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Doutorado em Biologia Parasitária

FEBRE AMARELA E MALÁRIA: INVESTIGAÇÃO DE DOIS SURTOS ZOONÓTICOS NO SUDESTE BRASILEIRO

FILIPE VIEIRA SANTOS DE ABREU

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Febre Amarela e Malária: Investigação de dois surtos zoonóticos no Sudeste brasileiro

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitária

Orientador: Prof. Dr. Ricardo Lourenço de Oliveira

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Ata da defesa de tese de doutorado em Biologia Parasitária de **Filipe Vieira Santos de Abreu**, sob orientação do Dr. Ricardo Lourenço de Oliveira. Ao vigésimo oitavo dia do mês de junho de dois mil e dezenove, realizou-se às quatorze horas, no Auditório Emmanuel Dias/FIOCRUZ, o exame da tese de doutorado intitulada: **"Febre Amarela e Malária: Investigação de dois surtos zoonóticos no sudeste brasileiro"**, no programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutor em Ciências - área de concentração: Ecologia e Epidemiologia, na linha de pesquisa: Epidemiologia de Doenças Infecciosas e Parasitárias e Hospedeiros.. A banca examinadora foi constituída pelos Professores: Dr. Fernando Dias de Ávila Pires - UFSC/SC (Presidente), Dr. Pedro Fernando da Costa Vasconcelos - IEC/PA, Drª. Joseli de Oliveira Ferreira - IOC/FIOCRUZ e como suplentes: Dr. Jeronimo Augusto Fonseca Alencar – IOC/FIOCRUZ e Dr. Josué da Costa Lima Junior – IOC/FIOCRUZ. Após arguir o candidato e considerando que o mesmo demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela aprovação da defesa da tese de doutorado. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutor em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Coordenador do Programa, Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 28 de junho de 2019.

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Aos brasileiros, continuamente espoliados pelos mais diversos parasitas.

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“*O mosquito fêmea não ferroa de-dia; está dormindo, com a tromba repleta de maldades (...) Disse que não era das frutas nem da água... Que era o mosquito que punha um bichinho amaldiçoado no sangue da gente*” (Guimarães Rosa, Sarapalha – 1946).

“*Nesta cidade todo mundo se acautela com a tal de febre amarela que não cansa de matar; E a dona Chica que anda atrás de mal conselho pinta o corpo de vermelho pro amarelo não pegar*” (Noel Rosa, Minha Viola – 1929).

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FEBRE AMARELA E MALÁRIA: INVESTIGAÇÃO DE DOIS SURTOS ZOONÓTICOS NO SUDESTE BRASILEIRO

RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Filipe Vieira Santos de Abreu

Tem-se observado expansão territorial da febre amarela silvestre da área endêmica (Amazônia e parte do Centro-oeste) para o sul e leste no Brasil. Paralelamente, a região serrana do Rio de Janeiro (RJ), onde a malária foi erradicada há décadas, registrava casos humanos autóctones de terçã benigna onde o caso índice não era identificado, sendo a hipótese de origem simiana do parasito aventada por pesquisadores. Entre 2015-2017, o RJ registrou surto de malária e a reemergência do vírus amarílico (YFV), este último se alastrando na Mata Atlântica, considerada indene por quase 80 anos. Registrou-se o maior surto silvestre do YFV no país. Estas situações sanitárias estimularam a realização deste estudo, onde buscamos esclarecer aspectos da transmissão desses agravos a partir de amostragens em primatas não-humanos (PNHs) e mosquitos. Após padronização das técnicas de captura, foi possível coletar e examinar, entre 2015 e 2019, 146 PNHs de seis espécies e 17.940 mosquitos de 89 espécies, em 44 municípios de cinco estados brasileiros sob influência da Mata Atlântica. A única espécie de PNH infectada com *Plasmodium sp.* foi *Alouatta guariba clamitans* (N=11), sendo cinco com *P. simium/vivax*, quatro com *P. brasilianum/malariae* e dois co-infectados. Polimorfismos de nucleotídeos únicos (SNPs) específicos de *P. simium* foram encontrados em todos os bugios infectados, e em todos os casos humanos autóctones fluminense, o que, aliado à sobreposição geográfica dos humanos e PNHs infectados, reforça a hipótese da transmissão zoonótica. Antes do surto de YFV, a região se mostrava receptiva, com detecção de vetores silvestres tradicionais *(Haemagogus* e *Sabethes*) em 82% dos municípios amostrados e PNHs susceptíveis em 100% deles. Após a reemergência do YFV, detectou-se o vírus em duas espécies de PNHs, *Callithrix jacchus* e *A. g. clamitans,* e em cinco espécies de mosquitos, *Hg. leucocelaenus*, *Hg. janthinomys*, *Sa. chloropterus*, *Aedes scapularis* e *Ae. taeniorhynchus*, os dois primeiros considerados os vetores primários devido às altas taxas de infecção e larga distribuição geográfica nos focos. Mosquitos urbanos ou periurbanos não estavam infectados, reforçando o caráter silvestre do surto. O vírus circulante pertence ao genótipo Sul Americano I*,* subclado 1E, e apresenta assinatura molecular representada por nove alterações de aminoácidos. O sequenciamento do genoma de 30 YFV obtidos de mosquitos, PNHs e humanos, revelou a circulação recente de duas sub-linhagens em Goiás, uma delas tendo chegado ao RJ e se dividindo em duas cadeias de transmissão – uma costeira e uma continental – separadas pela Serra do Mar. Demonstrou-se que o YFV é capaz de permanecer numa mesma área por três estações de transmissão consecutivas independentemente de nova introdução. Não houve evidência de reemergência da febre amarela urbana a partir do surto de caráter silvestre e nem de circulação silvestre do vírus ZIKV e de outros arbovírus no RJ. Em conjunto, nossos resultados alargam o conhecimento acerca da epidemiologia destas duas zoonoses na Mata Atlântica, confirmam o papel central dos bugios na epidemiologia destes dois agravos e contribuem com informações originais úteis na otimização de processos para vigilância e controle.

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ABSTRACT

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Filipe Vieira Santos de Abreu

A territorial expansion of wild yellow fever from the endemic area (Amazonia) toward the south and southeast in Brazil has been recently recorded. The mountainous areas of Rio de Janeiro state (RJ), where malaria was eradicated decades ago, have reported autochthonous human cases of benign tertian malaria. Their simian origin has been hypothesized as no index case was identified. Between 2015-2017, RJ registered a malaria outbreak as well as the re-emergence of yellow fever virus (YFV) transmission and spread in the Atlantic forest (AF), considered YFV-free for almost 80 years. Thus, Brazil recorded its most severe sylvatic YFV outbreak. These sanitary circumstances prompted the present study, where we aim to clarify transmission pieces concerning both diseases through sampling nonhuman primates (PNHs) and mosquitoes as well as conducting multidisciplinary analyzes of detected agents. After standardization of capture techniques, 146 PNHs of six species and 17,940 mosquitoes of 89 species were collected and examined between 2015 and 2019 in 44 municipalities of five Brazilian states under the influence of the AF. The only NHP species infected with *Plasmodium sp*. was *Alouatta guariba clamitans* (N = 11), five with *P. simium/vivax*, four with *P. brasilianum/malariae* and two co-infected. *P. simium* specific Single Nucleotide Polymorphisms were found in all infected howler monkeys, and in all RJ autochthonous human cases, which, together with the geographical overlap of humans and infected PNH, confirms the zoonotic transmission. Before the YFV outbreak, the region was receptive, with traditional sylvatic vectors (*Haemagogus* and *Sabethes*) detections in 82% of the sampled municipalities, and susceptible NHPs in 100% of them. After re-emergence of YFV, the virus was detected in two species of NHPs, *Callithrix jacchus* and *A. g. clamitans* and in five species of mosquitoes, *Hg. leucocelaenus*, *Hg. janthinomys*, *Sa. chloropterus*, *Ae. scapularis* and *Ae. taeniorhinchus*, the first two considered the primary vectors due to their high infection rate and wide geographic distribution in foci. Urban or peri-urban mosquitoes were not found infected, reinforcing the wild character of the YFV outbreak. The circulating virus belongs to the South American genotype I, subclade 1E, and presents a molecular signature represented by nine amino acid changes. Sequencing of 30 YFV genomes obtained from mosquitoes, PNHs and humans revealed the recent essentially simultaneous circulation of two sub-lineages in Goiás, one of them having arrived in RJ and dividing into two transmission chains, one coastal and one continental, separated by Serra do Mar. It was demonstrated that the YFV is able to remain in the same AF area at least for three consecutive transmission seasons without new introduction. There was no evidence of spillovers from the sylvatic outbreak to the urban ecosystem or from the urban to a wild cycle of ZIKA virus or any other arboviruses in RJ. Altogether, our results broaden the knowledge about the transmission features of these two zoonoses in the AF, confirm the central role of howler monkeys in the epidemiology of these two diseases and contribute with original and useful information for optimizing their surveillance and control.

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LISTA DE SIGLAS E ABREVIATURAS

AC	Antes de Cristo
CDC	Center for Disease Control and Prevention
CHIKV	Vírus Chikungunya
DENV	Vírus Dengue
DNA-mt	DNA mitocondrial
ES	Espírito Santo
FA	Febre amarela
GO	Goiás
Km	Quilômetro
MAYV	Vírus Mayaro
MG	Minas Gerais
ml	Mililitro
OMS	Organização Mundial de Saúde
PAHO	Pan American Health Organization
PCR	Polymerase Chain Reaction
PNH	Primata-não-humano
RJ	Rio de Janeiro
SC	Santa Catarina
SNPs	Single Nucleotide Polymorphisms
SP	São Paulo
WNV	Vírus do Oeste do Nilo
YFV	Vírus da Febre Amarela
ZIKV	Vírus Zika

1 INTRODUÇÃO

Ao longo das próximas seções discutiremos brevemente o conceito de zoonose e sua importância na história da humanidade. Posteriormente, daremos ênfase às zoonoses transmitidas por artrópodes, apresentando alguns exemplos relevantes e então focaremos naquelas que são transmitidas por mosquitos. Depois de breve exemplificação, será dada atenção especial às que têm Primatas-não-humanos (PNHs) como hospedeiros silvestres e, finalmente, apresentaremos o "estado da arte" das duas zoonoses que causaram surtos recentes no Sudeste do Brasil e nas quais nos debruçamos durante o doutoramento: febre amarela e malária de origem simiana.

1.1 O conceito de zoonose e sua relação com a sociedade

O termo zoonose existe desde o século XIX e foi originalmente cunhado para descrever doenças que acometiam animais não humanos (Ávila-Pires 2015, 2017). No entanto, em 1950, a Organização Mundial de Saúde (OMS) organizou a primeira reunião de especialistas para tratar do tema e convencionou-se uma definição mais próxima da ideia moderna, de que zoonoses seriam as doenças transmitidas naturalmente de animais vertebrados ao homem e vice-versa (WHO 1951). Na segunda reunião, realizada em 1958, o conceito foi levemente expandido e zoonose passou a ser entendida como doenças e infecções que são naturalmente transmitidas entre animais vertebrados e humanos (WHO 1959). Dessa forma, além da possibilidade de ocorrência de infecções cruzadas (*spillover* e *spillback*), o conceito passou a contemplar a ideia de que nem todo agente infeccioso causa doença e que, quando causam, as manifestações clínicas podem ser extremamente diferentes entre os indivíduos e as espécies, humana ou não-humana.

Apesar da conceitualização relativamente recente, o homem convive com as zoonoses há milhares de anos, as quais tiveram papel importante durante a construção da civilização moderna. Há, por exemplo, evidências artísticas, históricas e biológicas de infecções causadas por *Leishmania*, protozoário que acomete diversos mamíferos incluindo o homem, datadas de 1500-2500 anos AC, em múmias egípcias, e de 800 AC em múmias peruanas, como revisado por Steverding (2017). Mas é

provável que nenhuma zoonose tenha marcado tanto a história da humanidade quanto a peste. Tendo como agente a bactéria *Yersinia pestis* e transmitida, em sua forma zoonótica, de roedores a humanos através da picada de pulgas, a peste quase dizimou populações europeias e asiáticas nas três grandes pandemias registradas entre os séculos VI e XIX, causando profundas mudanças econômicas e culturais nas sociedades (Zietz and Dunkelberg 2004). Somente na última pandemia, em 1894, as pesquisas científicas conseguiram compreender os mecanismos de transmissão da peste e as relações existentes entre o conjunto de hospedeiros (roedores, sifonápteros e humanos) (Zietz and Dunkelberg 2004), o que resultou em maior entendimento e capacidade de contenção / prevenção de outros surtos zoonóticos que viriam a ocorrer posteriormente.

Hoje, sabe-se que a maioria dos agentes infecciosos que acometem o homem é de origem zoonótica. Dos 1.415 agentes que infectam humanos reconhecidos até 2001, 58% são zoonóticos. Além disso, estes agentes, principalmente os virais, apresentam maiores chances de causarem eventos emergentes ou reemergentes, quando comparados aos não zoonóticos (Taylor et al. 2001; Woolhouse and Gowtage-Sequeria 2005).

Apesar de frequente, o estabelecimento de uma infecção zoonótica a partir do *spillover* depende de uma série de fatores e gargalos, ligadas ao hospedeiro original (ex: a densidade e a distribuição da populações de hospedeiros, aliados à prevalência e intensidade de uma infecção); ao agente infeccioso (ex: sua virulência e modo de liberação/propagação a partir do hospedeiro - através de excreções, de tecidos ou por transmissão vetorial); e aspectos do novo hospedeiro (ex: a taxa de exposição, a competência imunológica e o comportamento) (Plowright et al. 2017). Com o passar dos anos, cresceu a noção da importância das relações ecológicas para ocorrência de transmissões zoonóticas. Hoje, é clara a ideia de que os diferentes padrões comportamentais, religiosos e climáticos, dentre outros, contribuíram para as diversas relações estabelecidas entre humanos, os animais e o ambiente que os cerca, favorecendo, em muitos momentos, a troca de organismos parasitas ou simbiontes entre ambos (Krause 1992; Thompson 2013). A percepção de que o parasitismo é uma relação ecológica moldada por diversos fatores biológicos, evolutivos e ambientais e que, portanto, a saúde de cada ser está integrada à totalidade da vida no planeta, levou ao surgimento da ideia de *One Health* (Cunningham et al. 2017; Waltner-Toews 2017).

One Health é definido como uma abordagem interdisciplinar e colaborativa que reconhece que o estado de saúde de humanos, de animais e a qualidade dos ecossistemas estão intimamente relacionados (Zinsstag 2012). Cresce a preocupação de *spillover* também a partir do homem para os animais domésticos e silvestres e os impactos ambientais destas transmissões (Thompson 2013; Jenkins et al. 2015). O conceito de *One Health* ganha força no atual contexto da globalização, onde todo o planeta está interligado através das tecnologias de comunicação e dos meios de transporte, favorecendo a ideia de interdependência. Apesar da emergência e reemergência de epidemias e de agentes infecciosos terem existido nos últimos milênios, como exemplificado anteriormente, hoje eles podem ser dispersos por todo o planeta em tempo recorde, o que faz crescer a preocupação com epidemias de dimensões globais (Jenkins et al. 2015). As emergências recentes de arbovírus como a Febre do oeste do Nilo (WNV), Chikungunya (CHIKV) e ZIKA (ZIKV) exemplificam o potencial de disseminação (de vetores e patógenos) no mundo globalizado (Weaver and Barrett 2004; Chevillon et al. 2008; Vasilakis et al. 2011).

1.2 Zoonoses transmitidas por artrópodes

Diversas zoonoses são transmitidas entre os hospedeiros vertebrados através de artrópodes vetores, principalmente aqueles adaptados à hematofagia. Em inglês, estas doenças são chamadas de *Vector-borne diseases* (Gubler 2009). Neste processo, parasitos presentes no sangue são ingeridos durante a alimentação dos artrópodes, podendo se reproduzir/replicar e/ou se desenvolver nos tecidos do hospedeiro invertebrado e são carreados até o momento da infecção de outros elos da cadeia de transmissão, como animais vertebrados por exemplo (Gubler 2009; Forrester et al. 2014). Para que isso ocorra, são necessárias adaptações dos parasitas às diferentes condições físicas (ex: temperatura, pH) e biológicas (ex: sistema imune, diversidade molecular e celular) encontradas nos dois (ou mais) hospedeiros, o que gera diversos gargalos e confere altos graus de especificidade à maioria das relações parasita-hospedeiro (Forrester et al. 2014; Plowright et al. 2017). Ainda assim, alguns parasitos conseguiram se adaptar a infectar com sucesso diferentes espécies de animais vertebrados e de artrópodes, o que os torna de especial interesse no estudo das zoonoses (Blitvich 2008; Vasilakis et al. 2011).

Dentre os artrópodes, há dois grupos de especial relevância na manutenção de ciclos zoonóticos, devido à evolução da hematofagia em algumas de suas espécies: a Ordem Acari, que compreende diversas espécies de carrapatos, e a Classe Hexapoda, onde insetos de diversas ordens se adaptaram à alimentação sanguínea. Os principais táxons de artrópodes e os principais parasitos transmitidos por eles foram listados por Gubler (2009). No presente trabalho, vamos apenas exemplificar agravos relevantes relacionados a estes dois grupos.

Os carrapatos podem transmitir um grande número de patógenos (diversos vírus, bactérias e protozoários) para os animais silvestres ou domésticos dos quais se alimentam, causando grande prejuízos econômicos e ecológicos (Jongejan and Uilenberg 2004). O homem pode servir de hospedeiro para muitas espécies de carrapatos, e eventualmente, infectar-se com parasitas, caracterizando-se a transmissão zoonótica (Dantas-Torres et al. 2012). Atualmente, a doença de Lyme, cujo agente é a bactéria *Borrelia burgdorferi,* transmitida através da picada de carrapatos ixodídeos, é a mais importante zoonose transmitida por carrapatos, com centenas de milhares de casos humanos notificados todos os anos no hemisfério norte (Steere et al. 2004; WHO 2017). No Brasil, foram notificados surtos de febre maculosa em humanos, causados por *Rickettsia rickettsii* e *Ri. parkeri,* agente transmitido pelo carrapato estrela (*Amblyomma cajennense* e *Amblyomma sculptum*) entre animais silvestres ou domésticos, podendo infectar acidentalmente o homem (Paddock et al. 2008; Krawczak et al. 2014; Martins et al. 2016; Ministério de Saúde 2018). Nestes surtos, o papel de reservatório exercido pelas capivaras pareceu ser importante, principalmente pelas grandes populações desses roedores em áreas urbanas ou suburbanas, resultado do desmatamento de seus habitats naturais, e das altas taxas de infestação por carrapatos em ambientes modificados (Krawczak et al. 2014), contexto que se aplica ao conceito de *One Health* (Dantas-Torres et al. 2012).

Dentre os hexápodes que apresentam importância médico-veterinária, destacam-se três ordens: Hemiptera, Siphonaptera e Diptera. A ordem Hemiptera, compreende diversas espécies de barbeiros capazes de transmitir o protozoário *Trypanosoma cruzi*, agente etiológico da doença de Chagas em humanos. Este parasito é especialmente interessante do ponto de vista científico, pois é capaz de se desenvolver em diversos grupos de vertebrados (Carnivora, Chiroptera, Rodentia, Cingulata, Didelphimorphia, Primates) e em diferentes espécies de barbeiros. O conceito de *One Health* é facilmente visualizado na epidemiologia desta zoonose. Sua

emergência e endemicidade estão diretamente ligados com a pobreza, que impacta a qualidade das moradias humanas tornando-as, muitas vezes, propícias à invasão e/ou estabelecimento dos insetos vetores, e hábitos socioculturais, como a caça de animais silvestres podem aumentar a exposição do homem aos hospedeiros não-humanos infectados (Thompson 2013).

A ordem Siphonaptera engloba centenas de espécies de pulgas e pelo menos algumas dezenas estão envolvidas na transmissão da peste no mundo (Gratz 1999). No Brasil a espécie *Xenopsylla cheopis*, introduzida a partir do Velho Mundo, alimenta-se predominantemente em roedores comumente encontrados nas proximidades de habitações humanas, como o *Rattus rattus* ou *R. novergicus*, que são hospedeiros potenciais da bactéria *Y. pestis* (Brasil et al. 1989) nos meios urbano e periurbano. As pulgas *X. cheopis* também podem se alimentar no homem e em animais domésticos, especialmente em caso de escassez dos roedores, como ocorre em períodos de epizootias de peste ou de campanhas de desratização. Estes eventos aumentam as chances dos humanos se infectarem, caracterizando a transmissão zoonótica (Ávila-Pires 1976; Brasil et al. 1989). Ciclos da *Y. pestis* também podem ser mantidos entre espécies de pulgas e de roedores silvestres, onde o homem se infectaria acidentalmente ao frequentar estes ambientes e ser picado por uma pulga susceptível infectada. Fatores sociais, como o crescimento das cidades e da produção de lixo, ecológicos, como a diversidade de roedores e de pulgas, e fisiológicos, como a capacidade das bactérias de lesar o aparelho digestório das pulgas, fazendo com que regurgitem mais bactérias em seus hospedeiros, têm contribuído para a manutenção desta zoonose ao longo da história (Ávila-Pires 1976; Hinnebusch and Erickson 2008; Tavares et al. 2012).

Finalmente, na ordem Diptera há duas famílias de maior importância zoonótica. Dentro da família Psychodidae estão os flebotomíneos, vetores de diversas espécies de *Leishmania* que podem infectar uma grande variedade de mamíferos, inclusive o homem (Campbell-Lendrum et al. 2001; Steverding 2017). Nos últimos anos, tem sido observada a expansão de áreas endêmicas de leishmanioses em direção aos grandes centros urbanos, influenciada por aspectos socioeconômicos e ambientais. Dentre eles, destacam-se: a) o desmatamento crescente das áreas contíguas às cidades pela especulação imobiliária, o que aproxima o humano do vetor (*Lutzomyia* sp.) e afasta os hospedeiros vertebrados naturais; b) a adaptação de vetores (particularmente a *Lu. longipalpis*) ao ambiente modificado pelo homem; c) a grande quantidade de animais

de estimação ou vadios pelas ruas, podendo servir de reservatórios aos parasitos (Lainson and Rangel 2005; Costa et al. 2013).

A outra família com grande importância na transmissão vetorial de zoonoses é a Culicidae, composta pelos insetos popularmente conhecidos como mosquitos. Por ser um dos temas centrais da presente tese, este grupo será tratado separadamente no tópico seguinte.

1.2.1 Zoonoses transmitidas por mosquitos

A noção de que os mosquitos seriam capazes de transmitir patógenos aos humanos ou animais é relativamente recente. O primeiro pesquisador a sugeri-lo academicamente foi Manson, em 1877, quando investigava o ciclo do parasito *Wuchereria bancrofti* (Manson 1878). Manson deu origem ao raciocínio que culminaria na descoberta do potencial dos mosquitos como importantes transmissores de agentes infecciosos, incluindo os causadores de doenças relevantes desde aquela época, como a malária e a febre amarela (Franco 1969; Ávila-Pires 2015).

Dentre as doenças transmitidas por mosquitos, a malária e as diversas arboviroses (do inglês *arthropod-borne virus*) apresentam grande importância na atualidade, pois têm causado epidemias emergentes e reemergentes de dimensões continentais ou globais (Weaver and Barrett 2004; Vasilakis et al. 2011). Parte delas tem caráter zoonótico, seja em sua origem ou durante os ciclos de expansão a partir dos locais de origem (Vasilakis et al. 2011; Gould et al. 2017). A epidemiologia do WNV, originário da África, e do vírus Mayaro (MAYV), da América, exemplificam a transmissão zoonótica.

O WNV é um membro do gênero *Flavivirus* – Família Flaviviridae, isolado pela primeira vez em Uganda, em 1937 (Smithburn et al. 1940; Blitvich 2008; Fall et al. 2017). Surtos pelo WNV já foram registrados nas américas, Europa, Ásia e África (WHO, disponível em https://www.who.int/en/news-room/fact-sheets/detail/west-nile-virus). Seu ciclo silvestre é mantido entre mosquitos do gênero *Culex* spp. e aves, que apresentam longos períodos de viremia, podendo carrear o vírus por grandes distâncias durante voos migratórios, o que parece ser o principal mecanismo de dispersão deste vírus (Work et al. 1955; Nasci et al. 2001). Ao contrário de outros *Flavivirus,* o WNV apresenta baixa especificidade de hospedeiros, tendo sido encontrado em diversas espécies de mosquitos e vertebrados. Os principais vertebrados afetados são as aves e os equídeos, mas animais de sangue frio como

répteis e anfíbios já foram encontrados infectados durante surtos (Klenk et al. 2004; Hayes et al. 2005; Jacobson et al. 2005; Martins et al. 2019). Até o momento especula-se que, com exceção das aves, os demais vertebrados – inclusive o homem – teriam baixa capacidade de amplificar o vírus a ponto de infectar os vetores e estabelecer um ciclo enzoótico (Bunning et al. 2002; Klenk and Komar 2003; Hayes et al. 2005). Estudos sorológicos realizados no Brasil encontraram alto percentual de equinos com anticorpos específicos para WNV no pantanal (Pauvolid-Corrêa et al. 2014, 2017) e, recentemente, o vírus foi isolado pela primeira vez no país, durante epizootias em equinos no Espírito Santo (Martins et al. 2019). O ciclo epidemiológico de WNV, afetando aves migratórias que se deslocam por todo o globo, mosquitos de diversas espécies e outros vertebrados (humanos, equinos, répteis) ilustra o conceito de *One Health*, fazendo com que as práticas de vigilância e controle desta arbovirose necessariamente partam de uma perspectiva holística e integrativa.

Já o MAYV, pertencente ao gênero *Alphavirus*, Família Togaviridae, foi isolado pela primeira vez, em 1954, em Trinidad e, posteriormente, no Brasil, no Rio Guamá, Pará (Anderson et al. 1957; Causey and Maroja 1957). O MAYV tem sido responsável por surtos de doença febril aguda e síndrome de artralgia na Região Amazônica e no Planalto Central do Brasil, bem como em outros países da América do Sul, como Peru, Bolívia e Venezuela (Figueiredo 2007; Azevedo et al. 2009; Mourão et al. 2011; Zuchi et al. 2014). Embora seu ciclo enzoótico ainda não esteja completamente elucidado, MAYV é predominantemente transmitido em meio silvestre por mosquitos do gênero *Haemagogus*, especialmente *Hg. janthinomys,* mas já foram encontrados mosquitos dos gêneros *Culex, Sabethes e Psorophora* infectados (Causey and Maroja 1957; Pereira Serra et al. 2016). Há evidências de infecção em PNHs, roedores, marsupiais e aves (Vasconcelos et al. 2001d; de Thoisy et al. 2004). Experimentalmente, *Ae. aegypti* mostrou moderada competência para transmitir MAYV e, por isso, há preocupação com o risco de surtos de transmissão urbana em cidades infestadas por *Ae. aegypti* e constante circulação de PNHs (ex: *Callithrix* sp.) (Weaver and Reisen 2010; Long et al. 2011; Esposito and Fonseca 2017). Recentemente a mídia brasileira repercutiu o encontro de humanos infectados com MAYV em Niterói, RJ (https://saude.estadao.com.br/noticias/geral,mayaro-parente-do-virus-chikungunya-ja-circula-no-sudeste-segundo-estudo-da-ufrj,70002831708).

Esse resumo do conhecimento sobre a história natural desses dois arbovírus ilustra a diversidades de espécies de mosquitos e, sobretudos, de vertebrados não humanos que podem fazer parte dos ciclos de transmissão.

1.2.2 Zoonoses transmitidas entre Primatas não humanos (PNHs), mosquitos e Humanos

O papel dos Primatas-não-humanos

A ordem Primates pode ser classificada em duas subordens, a Strepsirrhini, composta por Lêmures, Tárcios e outros primatas basais, e a Haplorrhini, dividida em Plathirhinii – macacos do Novo Mundo como os bugios e pregos (famílias Atelidae e Cebidae, respectivamente) e os saguis e micos (Família Callitrichidae), e Catarhinii – macacos do Velho Mundo como os Rhesus e Babuínos (Família Cercopithecidae) e os Gorilas, Chimpanzés e Humanos (Família Hominidae) (Goodman et al. 1998; Rylands and Mittermeier 2009).

Devido a semelhanças evolutivas, morfológicas e fisiológicas, humanos e PNHs podem compartilhar dezenas de parasitos, especialmente com os do Velho Mundo, devido a maior proximidade filogenética (Davies and Pedersen 2008). Há relatos bem documentados de adaptações e *spillovers* de importantes agentes infeciosos. Os exemplos mais relevantes são: o vírus da Imunodeficiência Humana (HIV), originado a partir de transmissões macaco-humano do vírus da Imunodeficiência Simiano (SIV) a partir de PNHs do Velho Mundo (Van Heuverswyn and Peeters 2007); e o vírus Ebola, capaz de infectar diversos animais, inclusive primatas humanos e não humanos (Le Guenno et al. 1995). Estima-se que ambos foram inicialmente contraídos a partir do manuseio de sangue e vísceras de PNHs, cuja caça para consumo de carne ainda é comum em regiões subsaarianas (Burgos-Rodriguez 2011; Mossoun et al. 2015). Existem muitos outros exemplos, mas, devido ao escopo do capítulo e do presente trabalho, serão discutidas apenas as zoonoses transmitidas através da picada de mosquitos.

Neste sentido, quatro arboviroses transmitidas por mosquitos que possuem, ou pelo menos originalmente possuíram PNHs como hospedeiros amplificadores, emergiram ou reemergiram nas últimas décadas, causando enorme dano sanitário: Chikungunya (CHIKV), quatro sorotipos do dengue (DENV), Zika (ZIKV) e febre amarela (YFV). Além de coincidentemente serem arboviroses e terem PNHs como hospedeiros amplificadores no ciclo silvestre original, estes arbovírus compartilham

quatro importantes características epidemiológicas: a) apresentam um ciclo silvestre onde circulam entre mosquitos primatofílicos e acrodendrófilos e PNHs; b) realizaram *spillover* para seres humanos; c) passaram a ser transmitidos de humano para humano em ciclos urbanos independentes do ciclo silvestre, através da picada de mosquitos antropofílicos, ex: *Ae. aegypti*; d) tiveram origem no Velho Mundo e se dispersaram por quase todo o globo através de rotas comerciais (CDC 1990; Vasilakis et al. 2011; Althouse et al. 2016; Gould et al. 2017). A figura 1 ilustra estas características e pontua algumas diferenças entre essas arboviroses.

Apesar da origem silvestre e dos outros aspectos ecológicos em comum, até agora apenas o YFV foi capaz de estabelecer um ciclo silvestre nas Américas. Por isso, há uma preocupação crescente com a possibilidade destes outros vírus, cuja história natural é semelhante ao YFV, estabelecerem também um ciclo silvestre no Novo Mundo, o que diminuiria drasticamente as chances de erradicação (CDC 1990; Althouse et al. 2016; Lourenço-de-Oliveira and Failloux 2017). Vale a pena, portanto, conhecer alguns aspectos da história natural e da disseminação do CHIKV, DENV e ZIKV além de seus limites originais, como descrevemos a seguir.

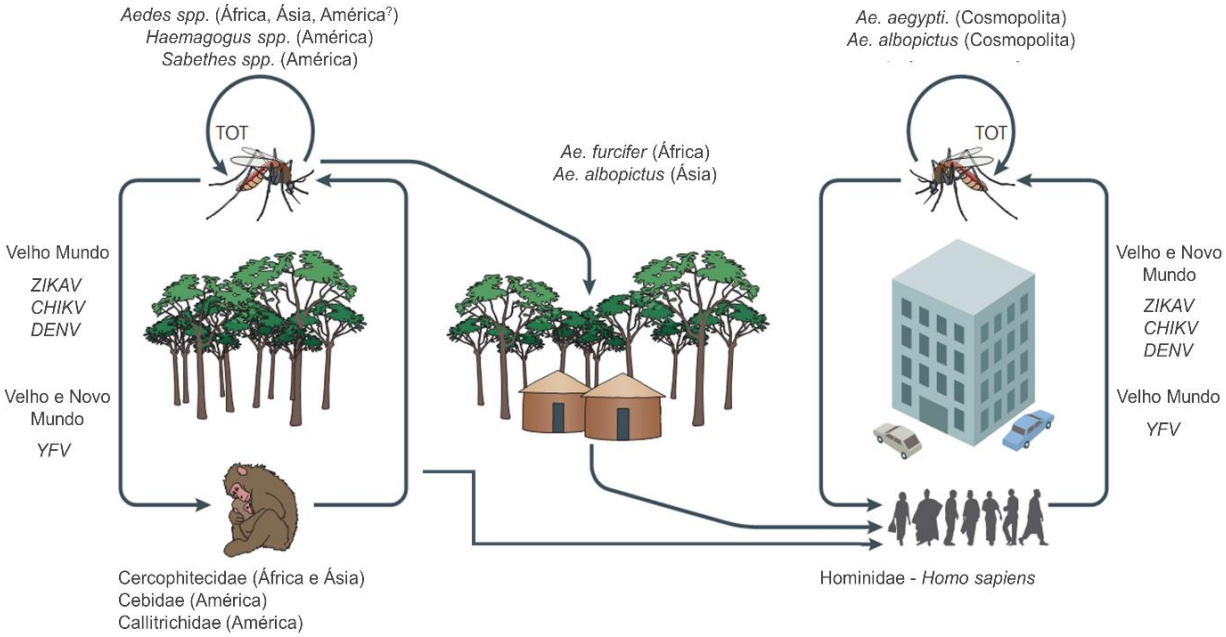

Figura 1: Desenho esquemático dos ciclos silvestre, rural e urbano (quando existentes) das quatro arboviroses epidêmicas mais importantes dos últimos anos - CHIKV, DENV, ZIKV e YFV, exemplificando os principais hospedeiros vertebrados e invertebrados no Velho e Novo Mundos. TOT (do inglês transovarian transmission) representa a possibilidade de transmissão entre vetores. (Adaptado de Vasilakis et al, 2011).

O CHIKV pertence ao gênero *Alphavirus,* família Togaviridae. Foi descrito durante epidemia em 1952-53 na África (Robinson 1955), único continente onde se reconhece a ocorrência do ciclo silvestre, mantido entre mosquitos *Aedes* spp*.* (ex*: Ae. taylori*, e *Ae. africanus*) (Weinbren et al. 1958; Diallo et al. 1999) e várias espécies de PNHs da família Cercopithecidae (Althouse et al. 2018). Humanos se infectam na floresta africana ou em suas proximidades e dispersam o vírus para ambientes modificados (Gould et al. 2017). No ciclo urbano, hoje encontrado em todos os continentes com exceção da Antártida, o vírus pode ser transmitido de humano para humano através da picada dos mosquitos antropofílico *Ae. aegypti* e *Ae. albopictus*. Após o primeiro diagnóstico de CHIKV no Brasil, em 2014, aventou-se a possibilidade que mosquitos silvestres pudessem se infectar em humanos virêmicos e transmiti-lo a PNHs. Preocupantemente, os mosquitos silvestres *Ae. terrens* e *Hg. leucocelaenus* oriundos de matas do Brasil, mostraram-se altamente competentes para disseminar e transmitir Chikungunya em infecções experimentais (Lourenço-de-Oliveira and Failloux 2017). Até agora, há poucas evidências de circulação de CHIKV entre os primatas neotropicais (Moreira-Soto et al. 2018).

A dengue é a arbovirose de maior importância na atualidade, constituindo um dos principais problemas mundiais de saúde pública (PAHO/WHO 2019). O agente é do gênero *Flavivirus,* família Flaviviridae*.* Atualmente são encontrados quatro sorotipos do vírus do dengue DENV-1.; DENV-2; DENV-3 e DENV-4. O DENV exibe ciclo silvestre – transmitido por espécies silvestres de mosquito do gênero *Aedes spp.* a PNHs da família Cercopithecidae tanto na África como no sudeste Asiático. O spillover de DENV para a espécie humana provavelmente ocorre no interior ou nas proximidades das florestas, onde mosquitos como *Ae. furcifer* e *Ae. albopictus* podem servir como vetor-ponte (Diallo et al. 2003). No entanto, a imensa maioria dos casos de dengue detectados em todo o globo terrestre estão em área urbana, onde o DENV é transmitido entre humanos através da picada dos vetores antropofílicos citados acima. Curiosamente, mesmo após séculos de transmissão na América, ainda não se comprovou o ciclo silvestre de DENV neste continente.

O ZIKV é do mesmo gênero que o DENV (*Flavivirus*) (Kuno and Chang 2007), tendo sido isolado, pela primeira vez, em 1947, a partir de macacos Rhesus utilizados como sentinelas em estudos de febre amarela silvestre na Floresta de Zika, em Uganda, na África (Dick et al. 1952). Em 1948, caracterizou-se o mosquito *Ae. africanus* como vetor transmissor do ZIKV, e posteriormente outras espécies de *Aedes*

spp. foram adicionados à lista de vetores silvestres (Dick et al. 1952; Haddow et al. 1964; Diallo et al. 2014). Dentre os vertebrados, PNHs dos gênero *Chlorocebus* e *Cercophitecus* seriam os amplificadores silvestres (Vasilakis and Weaver 2017). Portanto, assim como descrito para CHIKV e DENV, ZIKV tem um ciclo silvestre que atualmente é reconhecido apenas no Velho Mundo.

O primeiro isolado de ZIKV em humanos foi documentado na Nigéria, em 1954, ocasião em que outras infecções foram confirmados por dosagem de anticorpos neutralizantes (MacNamara 1954). No Brasil, os primeiros casos autóctones de Zika ocorreram em 2015, na região nordeste, nos estados da Bahia e Rio Grande do Norte e se espalharam por todo o Brasil e outros países americanos (Zanluca et al. 2015). O mosquito *Ae. aegypti* foi encontrado naturalmente infectado, tendo sido incriminado como o responsável pela transmissão (Ferreira-de-Brito et al. 2016). O surto teve consequências dramáticas como a associação com casos de microcefalia congênita, a comprovação de transmissão não vetorial, e a dimensão global da epidemia, levando a OMS a decretar emergência mundial (de Araújo et al. 2016). Cresceram também as preocupações com a possiblidade do ZIKV estabelecer um ciclo silvestre no Brasil, o que impediria sua erradicação tal como aconteceu com YFV (Althouse et al. 2016). Até o momento, há relatos de encontro de RNA de ZIKV em PNHs neotropicais, especialmente nas proximidades de residências onde casos humanos foram confirmados (Terzian et al. 2018; Favoretto et al. 2019). No entanto, até onde sabemos, não há trabalhos publicados que tenham investigado a presença de mosquitos silvestres naturalmente infectados com ZIKV no Brasil.

1.2.3 Zoonoses transmitidas entre PNHs – mosquitos – humanos que causaram surtos silvestres recentes no Brasil

A - Febre amarela.

A febre amarela é uma doença febril aguda cujo agente é o arbovírus protótipo do gênero *Flavivirus,* Família Flaviviridae, o YFV. Trata-se de um vírus composto por RNA de fita simples e polaridade positiva, com um genoma de aproximadamente 11 kilobases (kb) (Kaufmann and Rossmann 2011). A tradução do RNA gera uma poliproteína precursora, que dará origem a três proteínas estruturais (responsáveis pela formação do capsídeo, pré-membrana e envelope) e sete não estruturais (proteínas NS1, NS2a e NS2b, NS3, NS4a e NS4b, e NS5) (Kaufmann and Rossmann 2011). Até o momento foram descritos sete genótipos do YFV, sendo cinco na África

(*West Africa* 1 e 2, *East Africa*, *East/Central Africa* e Angola) e apenas dois nas Américas (Sul Americano I e II) (Bryant et al. 2007). Estudos filogeográficos apontam para a origem do YFV na África há cerca de 1500 anos (Bryant et al. 2007). Os genótipos americanos são monofiléticos e filogeneticamente próximos aos genótipos do oeste da África, dos quais divergiram há aproximadamente 470 anos. Após a chegada na América eles se divergiram entre si, há cerca de 300 anos, dando origem aos dois genótipos sulamericanos (Sul Americano I e II) citados acima. Estes dados temporais corroboram a hipótese previamente aventada de que o YFV teria surgido na África e chegado às Américas durante o tráfico negreiro, como será descrito a seguir (Bryant et al. 2007).

O genótipo Sul Americano I é o genótipo mais bem distribuído ao longo do continente americano e essencialmente o único encontrado nos isolados brasileiros, com exceção de uma detecção em Rondônia em 1983, que pertencia ao genótipo Sul Americano II. Este último é mais comum na região andina, sobretudo no Peru, de onde eventualmente se expande para países vizinhos como Bolívia e Equador por exemplo (Vasconcelos et al. 2004; Nunes et al. 2012). O genótipo Sul Americano I é dividido em seis subclados (Old Pará, 1A-1E) que estão relacionados com o período e região geográfica de circulação, sendo que as linhagens detectadas no Brasil a partir de 2004 pertencem ao subclado 1E (Vasconcelos et al. 2004; de Souza et al. 2010; Mir et al. 2017). Estudos recentes mostraram que este subclado provavelmente surgiu no norte da América do Sul, possivelmente na Venezuela, no final da década de 90 (de Souza et al. 2010; Mir et al. 2017).

Epidemias de febre amarela são reportadas, pelo menos, desde o século XVII nos países caribenhos da América Central. Estima-se que o YFV tenha chegado nas Américas através do tráfico de escravos e de mercadorias, hipótese recentemente comprovada por análises filogeográficas, como descrito anteriormente. Os navios europeus que faziam viagens entre a África e América traziam água armazenada em barris, que serviriam de criadouro para o mosquito africano *Aedes aegypt*i, além de humanos, muitas vezes infectados (Franco 1969). Isto contribuiu para o aparecimento de muitos surtos, em virtualmente todas as cidades portuárias americanas temperadas, tropicais e subtropicais, entre os séculos XVII e XIX, ceifando milhares de vidas. Entre 1685 e 1900, o Brasil registrou diversos surtos de febre amarela especialmente em grandes cidades litorâneas como Recife, Salvador e Rio de Janeiro, causando pânico entre os habitantes e sobretudo aos imigrantes, e prejudicando as

rotas comerciais, conforme descrito por Franco (1969). Durante todo este período, tanto o agente etiológico quanto o seu modo de transmissão eram desconhecidos, o que dificultava o controle e profilaxia. À época, acreditava-se que a infecção se dava através de “miasmas”, gases tóxicos exalados por plantas e animais em decomposição o que levou a diversas medidas de saneamento além de providências praticamente inócuas (Franco 1969).

A transmissão do YFV aos humanos através de insetos foi primeiramente aventada por Nott, em 1848, ao analisar os padrões epidêmicos das cidades localizadas próximas ao Rio Mississipi. O padrão de dispersão dos casos, que em determinados anos progredia de casa em casa mas em outros apresentava múltiplos focos afastados, levou-o a refutar a hipótese dos miasmas e a propor que tal padrão se encaixava melhor a uma transmissão realizada por insetos (Nott 1848). O médico cubano Carlos Finlay foi o primeiro a suspeitar, ainda em 1886, que um mosquito pudesse transferir patógenos do sangue de um hospedeiro doente para um saudável e também suspeitou, de maneira inédita, do papel do mosquito hoje conhecido como *Aedes aegypti* na transmissão da febre amarela urbana (Finlay 1886; Clements and Harbach 2017). No entanto, Finlay falhou nos seus experimentos de transmissão por desconhecer o período necessário para que o vírus se prolifere e invada os tecidos do mosquito, até chegar na glândula salivar – período de incubação extrínseco. Sua hipótese só foi cabalmente provada após uma sucessão de experimentos conduzidos pela comissão de febre amarela enviada dos Estados Unidos para estudar o problema em Cuba.

Tendo como apoio as proposições de Finlay, a comissão acabou por incriminar definitivamente o mosquito *Ae. aegypti* como o transmissor da febre amarela aos humanos em 1900, o que iniciou um vasto campo de combate e prevenção da doença (Reed et al. 1901; Clements and Harbach 2017). Àquela época, acreditava-se que a única espécie capaz de transmitir o YFV era o *Ae. aegypti*, mosquito exclusivamente urbano e de hábitos antropofílicos. Portanto, o controle da doença era baseado essencialmente no combate a este vetor. Diversas cidades e países, incluindo o Brasil, obtiveram sucesso na erradicação deste mosquito, o que fez com que se acreditasse que a erradicação global do YFV seria possível (Soper 1963). Paralelamente, a busca por um modelo animal para estudos do YFV lograva sucesso após a descoberta da susceptibilidade do macaco Rhesus ao vírus inoculado a partir de uma paciente africano, de nome Asibi (Stokes et al. 1928). O isolamento da cepa “Asibi” através de

sucessivas passagens de macaco a macaco ou macaco-mosquito-macaco foi um importante marco no estudo da febre amarela e no desenvolvimento da vacina (Strode 1951). Esforços sucessivos demonstraram que diversos gêneros de primatas neotropicais (Ex: *Cebus* / *Sapajus*, *Alouatta* e *Callithrix*) também eram susceptíveis ao YFV (Davis 1930a, 1930b). A partir dos avanços na compreensão do YFV, da possibilidade de eliminação do *Ae. aegypt*i urbano (Soper 1963) e do desenvolvimento de uma vacina eficaz, a possibilidade de erradicação da doença estaria avançando. No entanto, a partir da década de 1930 descobriu-se a possibilidade de transmissão do vírus sem a participação do *Ae. aegypti*, reduzindo as esperanças iniciais (Soper 1937).

A possibilidade de transmissão do YFV por mosquitos silvestres foi primeiramente aventada por Adolpho Lutz, em 1898 (Lutz 1930), e confirmada nos anos 1930, após investigação de surtos em regiões de Mata Atlântica durante a construção de estradas de ferro em São Paulo e no Vale do Canaã, no Espírito Santo, respectivamente (Lutz 1930, Soper et al. 1933). O dado que fomentou a hipótese da transmissão silvestre em ambas as áreas foi a impossibilidade de se detectar, mesmo após longos levantamentos entomológicos, a presença do mosquito urbano *Ae. aegypti* nas localidades com casos de febre amarela confirmados clínica e experimentalmente (Soper et al. 1933). A descoberta do ciclo silvestre mudou o paradigma de controle e epidemiologia desta doença e acabou com as esperanças ambiciosas de erradicação deste vírus no continente americano, uma vez que o ciclo seria constantemente mantido nas florestas entre mosquitos e primatas não humanos, infectando o homem eventualmente (Soper 1938). Os achados nos anos 1930, no Sudeste do Brasil, estimularam a realização de intensa pesquisa sobre a transmissão silvestre da febre amarela em outras partes do Brasil (ex: litoral baiano), e na África. Estações de estudos multidisciplinares foram implantadas em florestas africanas e brasileiras e, com o uso de plataformas nos dosséis florestais para captura de mosquitos, a instalação de animais-sentinela, a captura e exame do soro e vísceras de diversos vertebrados silvestres e rurais, e infecções experimentais de invertebrados e vertebrados, conseguiu-se caracterizar os principais vetores e hospedeiros silvestres do YFV nos dois continentes e isolar e caracterizar o vírus amarílico (Shannon et al. 1938; Soper 1938; Shannon 1939; Bugher et al. 1944; Causey et al. 1949; Kumm and Laemmert 1950), saga resumida por Strode em seu clássico livro "*Yellow Fever*" (Strode 1951). Já na década de 1930, como

consequência de tais esforços, os macacos *Alouatta* e *Cebus* / *Sapajus* e os mosquitos *Haemagogus* e *Sabethes* eram reconhecidos como os principais hospedeiros vertebrados e invertebrados, respectivamente, do vírus da febre amarela na América. Percebia-se que nem todo mosquito era capaz de adquirir, e nem todos que adquiriam eram capazes de transmitir o YFV (Davis and Shannon 1929, 1930; Whitman and Antunes 1937; Kumm and Cerqueira 1951). Diversos fatores (ecológicos, bioquímicos, genéticos, entre outros) influenciavam neste processo que hoje estão contemplados nos conceitos de capacidade e competência vetorial (Johnson et al. 2002; Vega-Rua et al. 2014).

A capacidade vetorial é um índice que leva em conta aspectos ligados ao vetor, ao parasito, ao hospedeiro vertebrado e ao ambiente que os cerca para quantificar a capacidade que uma espécie e/ou população de vetor tem para transmitir um patógeno em um determinado espaço e tempo. A fórmula para seu cálculo foi originalmente sugerida para a transmissão da malária e é expressa da seguinte forma:

$$CV = \frac{mbca^2P^n}{-\mathrm{Ln}\,(P)}$$

Onde **a** é o número de picadas por hospedeiro por dia; **b** é a probabilidade que um mosquito infectado tem de transmitir o parasito ao picar um hospedeiro susceptível; **c** é a probabilidade que um mosquito tem de se infectar com o parasito enquanto pica um hospedeiro infectado; **m** é o número de fêmeas de mosquito por pessoa em dada área, **n** é a duração do período extrínseco de incubação do parasito no inseto; **P** é a taxa de sobrevivência diária do inseto; logo, **– Ln (P)** representa a sobrevivência do vetor após o período de incubação extrínseco (Garrett-Jones 1964). Cada um destes fatores influencia na transmissão dos patógenos, como será exemplificado a frente.

A competência vetorial diz respeito à susceptibilidade dos mosquitos em se infectar, permitir a replicação, disseminação e transmissão de patógenos. Para isso é necessário que o patógeno seja capaz de sobreviver às condições fisiológicas do vetor e de atravessar as diversas barreiras enquanto se replica/multiplica/evolui no hospedeiro invertebrado (Lourenço-de-Oliveira et al. 2003; Lourenço-de-Oliveira and Failloux 2017).

Esses parâmetros são facilmente visualizados no ciclo silvestre da febre amarela, tomando o exemplo de transmissão por *Haemagogus*. O número de picadas (a) e o número de fêmeas do mosquito por hospedeiro (m) estão diretamente

relacionados à densidade dos mosquitos e sua preferência alimentar. A densidade populacional de *Haemagogus* é maior no período chuvoso, uma vez que este deposita seus ovos acima do nível da água em criadouros temporários como ocos de árvores, e parte dos ovos precisa de múltiplas imersões para eclodir (Alencar et al. 2008; Tátila-Ferreira et al. 2017b). Esse parâmetro ajuda a explicar a sazonalidade dos surtos de YFV, coincidentes com as estações quentes e chuvosas, que aceleram o desenvolvimento larval e aumentam a densidade populacional deste mosquito. Além disso, *Haemagogus* possuem hábitos acrodendrófilos e primatofílicos (Marcondes and Alencar 2010), o que, em caso de circulação viral aumenta as chances desta espécie adquirir o YFV e transmiti-lo a outro hospedeiro durante os repastos sanguíneos, uma vez que os primatas são os principais amplificadores vertebrados do vírus (Laemmert et al. 1946). Após uma alimentação sanguínea infectante, as fêmeas susceptíveis do mosquito permanecem infectadas pelo resto da vida. Quanto menor for o período de incubação extrínseco (n) (acelerado em maiores temperaturas como as registradas no verão) e quanto mais longevo for o mosquito (P) mais tempo ele permanecerá apto a transmitir o vírus através da picada. Além disso, os *Haemagogus* apresentam alta competência vetorial mesmo quando infectados com diferentes linhagens de YFV. Estudo recente mensurou taxas de transmissão (vírus viáveis encontrados na saliva após infecção experimental) de 36.6%, mostrando que o vírus foi capaz de atravessar todas as barreiras até a chegada à saliva (Couto-Lima et al. 2017).

Portanto, a capacidade e a competência vetorial podem variar enormemente entre as diferentes espécies de mosquitos, condições geográficas, estações do ano e linhagens de YFV. Na área endêmica por exemplo, recoberta pelo bioma amazônico, o vírus já foi isolado em diversas espécies de *Haemagogus*, tais como *Hg. albomaculatus*, *Hg. equinus* além de *Hg. janthinomys*, considerado vetor primário da doença. Mosquitos do gênero *Sabethes*, especialmente o *Sa. chloropterus,* também foram encontrados naturalmente infectados e são considerados os vetores secundários (Hervé et al. 1984; Vasconcelos et al. 2001c). Fora da região endêmica, o *Hg. janthinomys* é também considerado o vetor primário nas áreas de transição entre a Amazônia e o Cerrado e no Cerrado stricto senso onde também foram encontrados *Ae. scapularis*, *Sa. chloropterus*, *Sa. soperi* e *Sa*. *glaucodemus* naturalmente infectados (Dégallier et al. 1993; Vasconcelos et al. 1997, 2001a; Mascheretti et al. 2013). A depender da força de transmissão o vírus pode alcançar o extremo Sul do país, área subtropical recoberta pelo Bioma Pampas (Rio Grande do Sul), onde *Hg.*

janthinomys não é encontrado. Nesta região, o vírus foi isolado de *Hg. leucocelaenus*, considerado o vetor primário, e de *Ae. serratus* (Cardoso et al. 2010). Além disso, *Sa. albiprivus* foi encontrado naturalmente infectado em Missiones na Argentina, também na região dos Pampas (Goenaga et al. 2012). Intrigantemente, na região da Mata Atlântica costeira onde a Febre amarela silvestre foi descoberta, não havia registros de circulação do vírus desde a década de 1940. No entanto ensaios experimentais mostraram que populações de duas espécies coletadas na região (*Sa. albiprivus* e *Hg. leucocelaenus*) apresentaram alta competência vetorial (Couto-Lima et al. 2017).

A partir do reconhecimento da existência do ciclo silvestre, nos anos 1930, mantido por espécies de mosquito competentes, foi possível criar estratégias para se detectar os casos que apareciam em forma de surtos epidêmicos nos diversos estados do Brasil. Uma das estratégias foi o desenvolvimento do viscerótomo e do serviço de viscerotomia para coleta e remessa de fragmentos de fígado de pacientes falecidos após quadro febril agudo (Franco 1969; Costa et al. 2011). De fato, entre 1934 e 1967, foram feitos diversos esforços para identificar, mapear e controlar os surtos.

Nessas décadas iniciais de investigação de febre amarela silvestre, a confirmação diagnóstica era feita exclusivamente através de análises morfológicas das amostras de fígado dos pacientes que iam a óbito. Sabendo-se que apenas parte dos casos evolui para óbito, os números obtidos à época são enormemente subestimados. Mesmo assim, com base nos registros de FA silvestre disponíveis dos anos 1932 a 1967 (condensados no quadro 1) e de 1960 a 2015 (mostrados na figura 2), nota-se que o vírus circulou continuamente pelo país, causando surtos de maiores ou menores dimensões, taxa de mortalidade média variando de 30 a 40% (Franco 1969; Monath and Vasconcelos 2015).

Quadro 1: Casos de febre amarela silvestre entre 1932 e 1976, de acordo com a Unidade Federativa. Adaptado de Franco, 1969

Unidade da Federação	Nº de casos	Unidade da Federação	Nº de casos
Acre	14	Paraná	91
Amazonas	17	Piauí	1
Bahia	15	Rio Grande do Sul	7
Espírito Santo	281	Rio de Janeiro	65
Goiás	210	Rondônia	2
Maranhão	3	Roraima	5
Mato Grosso	115	Santa Catarina	40
Minas Gerais	417	São Paulo	329
Pará	63	**Total**	**1675**

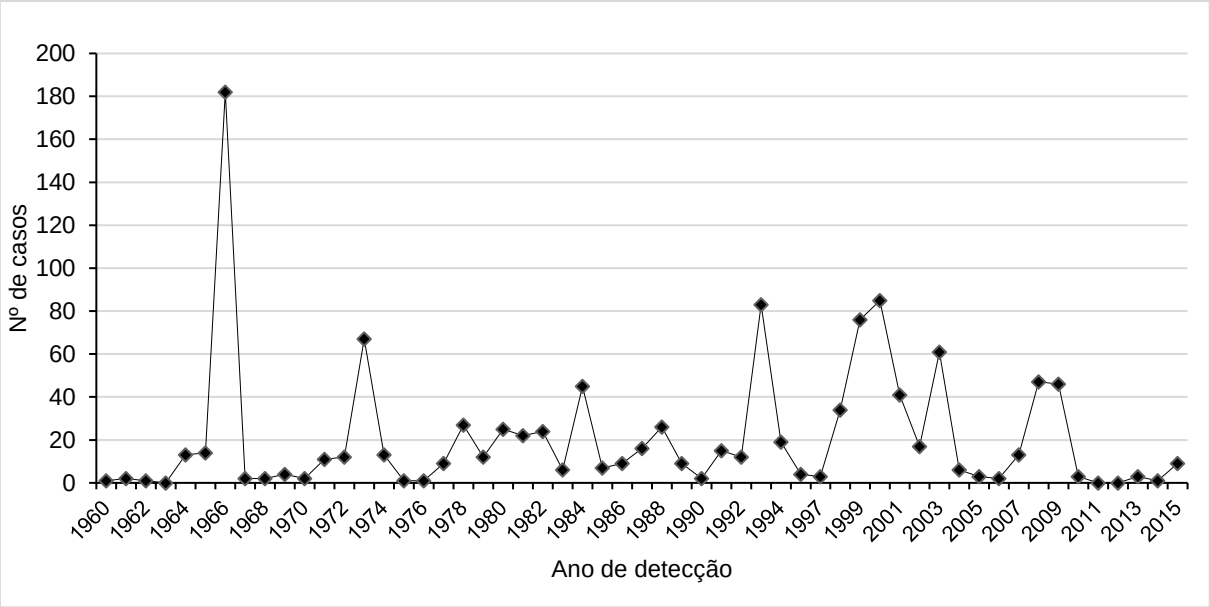

Figura 2: Número de casos de febre amarela silvestre no Brasil entre 1960 e 2015. Fonte OMS (http://ais.paho.org/phip/viz/ed_yellowfever.asp).

Com base na análise dos dados epidemiológicos históricos, postulou-se que os maiores surtos ocorreriam em intervalos periódicos de aproximadamente sete a dez anos, tempo necessário para a renovação de vertebrados susceptíveis, aliado a características ambientais favoráveis (Barrett 2003; Vasconcelos 2010). No entanto, o vírus circula de forma endêmica e, aparentemente, perene na região amazônica, onde se concentram as maiores florestas do país e, consequentemente o maior número de hospedeiros vertebrados e invertebrados (Vasconcelos 2003; Possas et al. 2018). Nesta região, o número de casos humanos geralmente é baixo, por se tratar de área de recomendação da vacina (onde a vacinação é obrigatória desde a infância) estabelecida pelo governo brasileiro (Romano et al. 2014). Quando encontra condições propícias (tais como clima quente e úmido e grande número de hospedeiros susceptíveis), o vírus se expande no território, dispersando-se provavelmente através das matas de galerias para outras regiões e biomas do país, carreados majoritariamente por mosquitos, mas também por PNHs e humanos, ocasionalmente (Vasconcelos et al. 2001a; Vasconcelos 2010). Entre 1950 e 1999, as expansões territoriais da febre amarela se limitaram ao centro-oeste brasileiro, onde predomina o bioma Cerrado (Vasconcelos et al. 2001a, 2004; Possas et al. 2018). Porém, nas duas últimas décadas, os ciclos de expansões do vírus aumentaram sua área de influência, ao se expandir em direção ao Sul, sudeste e/ou nordeste, podendo alcançar a Mata

Atlântica - outra floresta tropical úmida, como mostra a figura 3 (Vasconcelos et al. 2003; Ribeiro and Antunes 2009; de Souza et al. 2011; Mascheretti et al. 2013; Moreno et al. 2013; Romano et al. 2014).

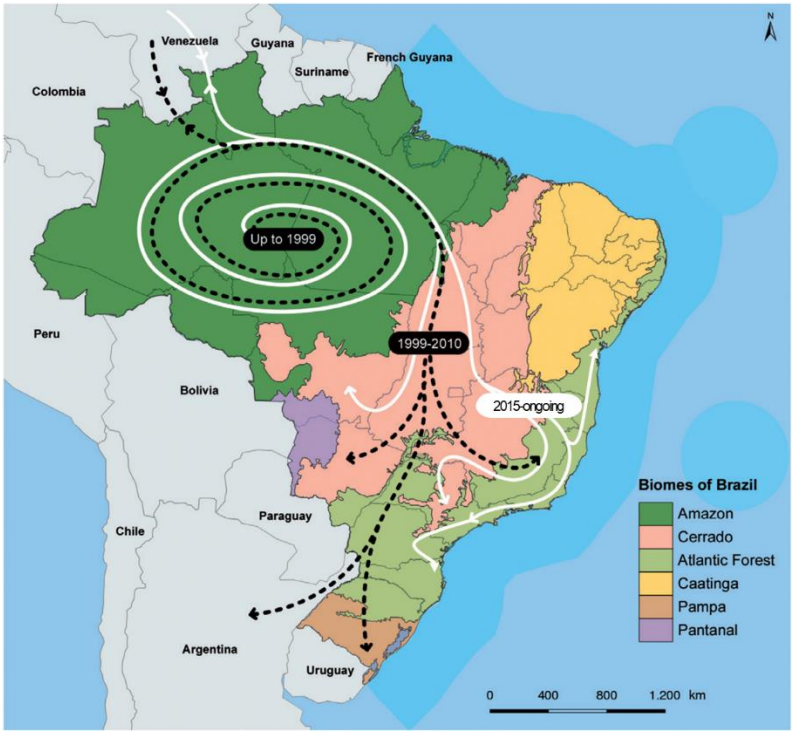

Figura 3: Esquema de dispersão do YFV a partir da floresta amazônica entre 1999-2010 (linha preta pontilhada) e de 2015 em diante, causando o surto atual (linha branca cheia). Adaptado de Possas et al. (2018).

Nestas ondas de expansão da febre amarela silvestre, o vírus geralmente se desloca de forma análoga a um incêndio, ou seja, avançando de um município afetado para os municípios vizinhos até então livres do vírus, e raramente retorna para áreas já afetadas, uma vez que a maioria dos hospedeiros vertebrados morreram ou ficaram imunizados através da exposição ao vírus circulante ou à vacina, no caso dos humanos (Bugher et al. 1944; Taylor and Da Cunha 1946). Em geral, é durante as expansões para as áreas não endêmicas que são registrados os maiores números de casos humanos, em função da menor cobertura vacinal. A procura pela vacina nestas áreas é baixa, seja por não vivenciarem a constante ameaça do vírus ou por se tratar de área cuja recomendação vacinal se iniciou apenas recentemente (Romano et al. 2014). De fato, o governo brasileiro tem aumentado as áreas de recomendação de vacinação em reposta às sucessivas expansões do vírus nas últimas duas décadas, como mostra a figura 4 (Romano et al. 2014). A vacinação universal não foi adotada em função do risco de efeitos adversos graves em decorrência da vacina, estimado

em aproximadamente 0.8 casos graves a cada um milhão de doses aplicadas. Portanto é necessário que que se faça análise do risco/benefício antes de preconizar a imunização em massa (Vasconcelos et al. 2001b; Struchiner et al. 2004; Romano et al. 2014).

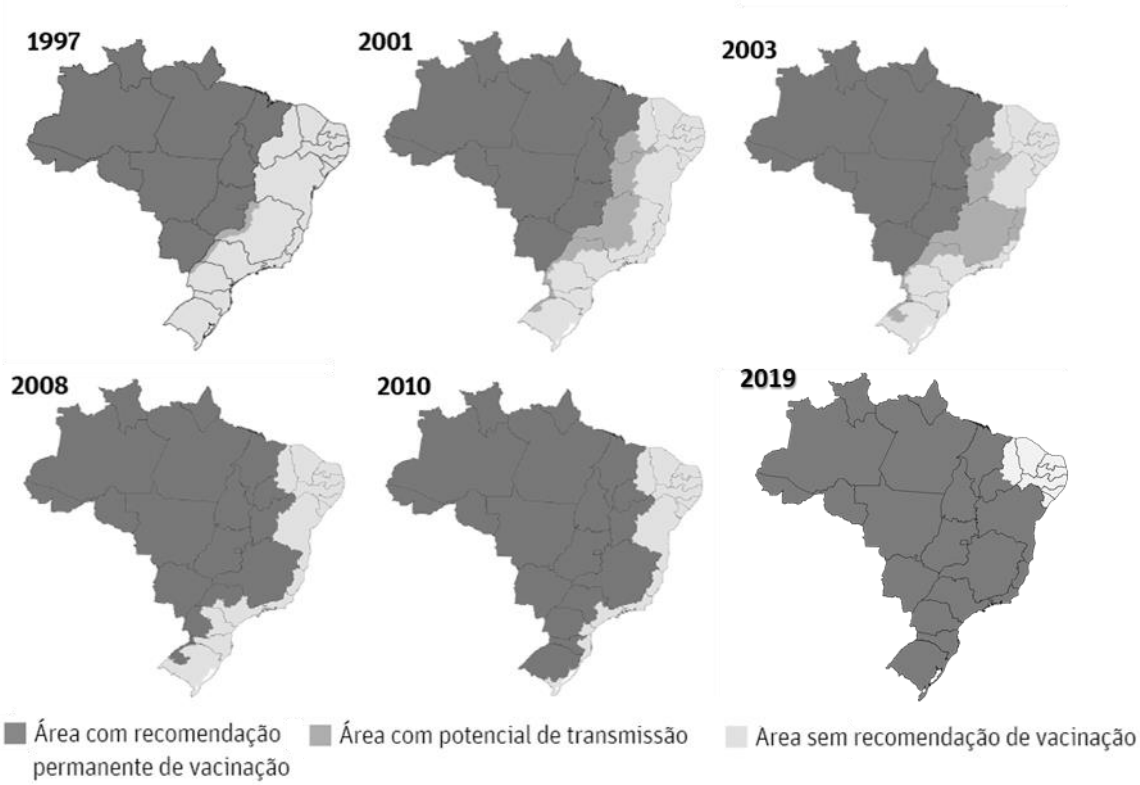

Figura 4: Expansão gradual das áreas de recomendação de vacina contra a febre amarela, preconizadas pelo ministério da saúde. Fonte: Adaptado a partir de Romano et al. 2014 (Romano et al. 2014). O mapa de 2019 foi obtido em https://www.saude.gov.br/o-ministro/920-saude-de-a-a-z/febre-amarela/10771-vacinacao-febre-amarela

Até o ano de 2016, toda a costa Brasileira desde o Piauí até o Rio Grande do Sul era considerada área indene e, portanto, sem recomendação da vacina (Fig. 4). A costa da região Sudeste é recoberta por remanescentes de Mata Atlântica que, depois da região amazônica, abriga a maior diversidade de primatas não humanos do Brasil (Chiarello 2003), fator que se correlaciona com o aumento do risco de reemergência de febre amarela (Hamrick et al. 2017; de Almeida et al. 2018). Além disso, é a região que concentra os maiores adensamentos populacionais do país. Após detecção da circulação do YFV no centro-oeste, entre 2014-2015, e um período de relativo silêncio, o vírus reemergiu no oeste de Minas Gerais e rapidamente se espalhou para a costa, tomando os estados do Espírito Santo e Rio de Janeiro, onde o vírus não era

detectado desde a transição entre os anos 30 e 40 (Possas et al. 2018). Condições ecoepidemiológicas propícias, tais como grande número de hospedeiros vertebrados susceptíveis (humanos não vacinados e diversas espécies de PNHs), presença de espécies de mosquitos reconhecidas tradicionalmente como vetoras, e despreparo da vigilância local para reconhecer e combater o agravo, contribuíram para a eclosão de um surto de febre amarela silvestre sem precedentes nos últimos 80 anos (Possas et al. 2018), como ilustra a figura 5, que consiste no mesmo gráfico mostrado anteriormente (Fig. 2), com o acréscimo dos casos confirmados no atual surto (2016 – 2018, dados 2019 ainda não estão disponíveis).

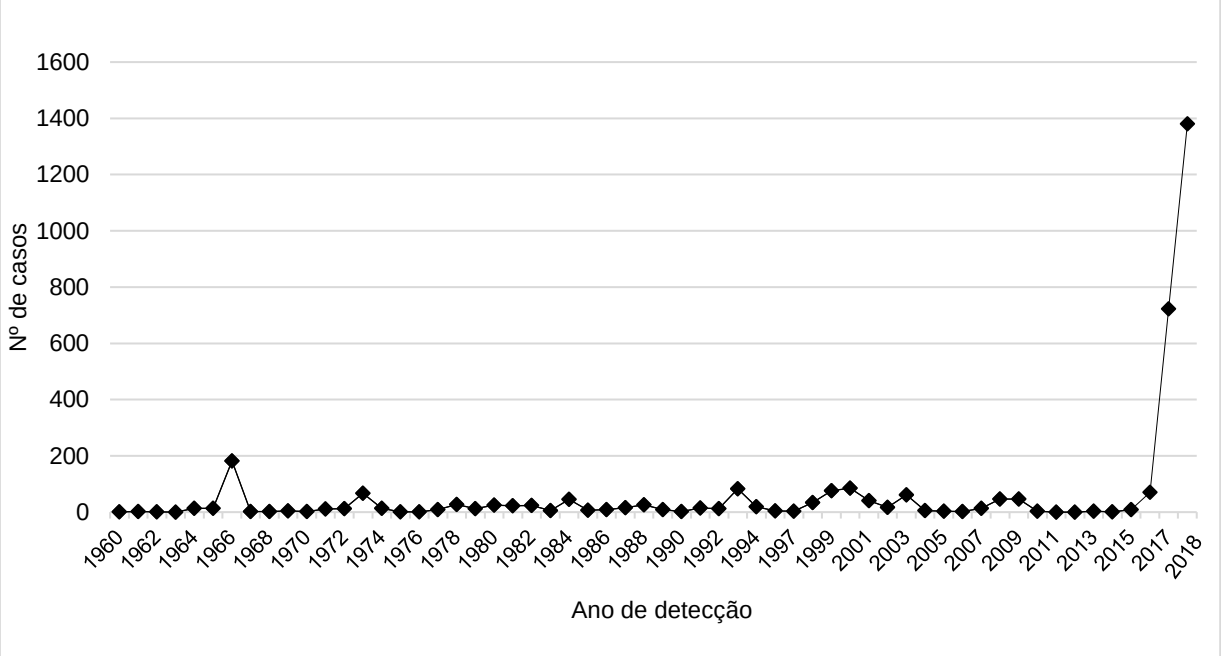

Figura 5: Número de casos de febre amarela silvestre no Brasil entre 1960 e 2018. Fonte OMS (http://ais.paho.org/phip/viz/ed_yellowfever.asp) e MS (http://portalms.saude.gov.br/saude-de-a-z/febre-amarela-sintomas-transmissao-e-prevencao).

Entre dezembro de 2016 e março de 2019, foram registrados 2.232 casos humanos, dos quais 754 evoluíram para óbito (Brasil 2017b, 2019b). No mesmo período, foram confirmadas 1.536 epizootias em PNHs, despertando preocupação com espécies ameaçadas de extinção e endêmicas do Sudeste (e.g. *Brachyteles arachnoides*, *Leontopithecus rosalia*) (Chiarello 2003; Bicca-marques et al. 2017; Brasil 2019a). O vírus “varreu” os estados do Sudeste e alcançou o nordeste (Bahia) e o Sul do país (primeiramente o Paraná e, mais recentemente, Santa Catarina) levando o Ministério da Saúde a ampliar a área de recomendação da vacina, como

ilustra a figura 4, mapa de 2019, e mostrando que a onda de transmissão continua a avançar (Brasil 2019a).

B - Malária

A malária, doença conhecida pelos quadros febris intermitentes, aliados à esplenomegalia, parece acompanhar a história do homem há muito tempo. Há registros datados de mais de 2.000 anos AC com descrições muito parecidas com os sintomas e sinais da malária, e provenientes de diferentes civilizações (Cox 2010). Tal como descrito para o YFV, até o final do século XIX não eram reconhecidos nem o agente etiológico, nem o modo de contaminação e a hipótese mais aceita na época era a dos miasmas exalados a partir de pântanos, o que dá nome à doença. A palavra malária é derivada do Italiano e significa “Mau ar” em alusão à ideia dos odores pestilentos exalados dos pântanos e brejos. Ao contrário da febre amarela, o agente etiológico foi descoberto antes do modo de transmissão.

O médico francês Alphonse Laveran investigava a doença enquanto trabalhava na Argélia, a partir de 1878, e conseguiu observar formas suspeitas em preparações de sangue fresco de pacientes. Ele conseguiu observar o processo de exflagelação e ficou especialmente interessado e convencido de que aquele ser vivo era o causador da doença. Em 1881, Laveran relatou os resultados, descrevendo o *Plasmodium* como agente etiológico da malária (Laveran 1881). A teoria de Laveran foi aceita após sua ratificação por cientistas proeminentes da época, como Louis Pasteur e Robert Roux, e aprimoramento das técnicas de coloração, que permitiram a melhor caracterização do parasito (Garnham 1966; Cox 2010). O modo de transmissão, no entanto, permanecia desconhecido. Como no caso do YFV, os estudos de Manson sobre a transmissão de filariose inspiraram as pesquisas sobre o possível papel dos mosquitos na transmissão dos parasitos causadores da malária. Os estudos realizados por Ross, em 1898, e quase paralelamente por Grassi e colaboradores, em 1900, finalmente comprovaram a transmissão via mosquito e incriminaram os “mosquitos das asas pintada”, gênero *Anopheles*, que se criam abundantemente em áreas alagadas e pantanosas, confirmando a suposição de que estas estariam associadas aos casos da doença (Grassi et al. 1898; Ross 1898; Cox 2010). Estudos posteriores confirmaram o papel do mosquito *Anopheles* como único vetor de malária humana, e identificaram as principais espécies envolvidas na transmissão ao redor do globo, inclusive no Brasil, como será mencionado a seguir (Deane 1986).

A malária humana é causada essencialmente por quatro espécies de protozoários do gênero *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*). É uma das doenças mais letais do mundo e, apesar dos esforços globais, estima-se que 219 milhões de pessoas foram infectadas e 435.000 morreram em consequência da doença apenas em 2017 (WHO 2018b). Mais de 90% dos casos, inclusive dos fatais, ocorreram no continente africano e foram causados por *P. falciparum,* transmitido por *Anopheles gambiae* ou outra espécie desse complexo. No Brasil, no mesmo, ano foram registrados cerca de 194.000 casos humanos, a maior parte concentrada na região amazônica e causada por *P. vivax*, tendo o *Anopheles darlingi* como principal vetor (WHO 2018b; Brasil 2019c).

Apesar da antiguidade das evidências da presença da malária no Velho Mundo, praticamente inexistem relatos de malária na América pré-colombiana e nem nas primeiras décadas pós colonização do Brasil. O primeiro relato de febres terçãs e quartãs em índios Tupinambás data de 1587 e estima-se que plasmódios humanos tenham chegado às Américas durante o tráfico de escravos, em africanos e/ou europeus mercantes infectados (Deane 1986). Nos séculos seguintes, a malária era comumente encontrada em áreas alagadas e grandes baixadas litorâneas. Os surtos aumentaram depois da abolição da escravatura, que contribuiu para a redução da mão de obra e consequente abandono da limpeza de margens de açudes e outras coleções de água de grandes fazendas, favorecendo o desenvolvimento das larvas de anofelinos do subgênero *Nyssorhynchus* nestes locais (Deane 1986; Oliveira-Ferreira et al. 2010; Griffing et al. 2015). Os primeiros grandes surtos só foram registrados no final do século XIX, quando ocorreu o ciclo da borracha na Amazônia levando muitos imigrantes oriundos de área livre de malária, a se estabelecerem na região, causando milhares de mortes.

Ao longo do século XX, a malária estava distribuída por todo o Brasil e diversas iniciativas visando ao controle e erradicação da doença foram desenvolvidas. As principais se baseavam em tratamento profiláticos para população e/ou trabalhadores de áreas afetadas através do quinino adicionado no sal ou em comprimidos, obras de engenharia sanitária para retificar rios e eliminar alagados, além de medidas de proteção individual como mosquiteiros nas redes/camas e telas nas janelas, reduzindo significativamente os casos de malária ao longo do século, apesar da descontinuidade dos programas por questões políticas ou sempre que o número de casos baixava. Dois acontecimentos do século XX são dignos de nota: o primeiro foi a invasão do *An.*

gambiae, principal vetor africano que exibe grande capacidade vetorial, tendo sido responsável por epidemias de grandes dimensões no nordeste entre as décadas de 1930 e 1940 (Oliveira-Ferreira et al. 2010; Griffing et al. 2015). A erradicação deste mosquito com apoio da fundação Rockfeller e do staff do serviço de febre amarela é um feito histórico no controle de mosquitos vetores. E em segundo, a nova onda migratória para Amazônia, iniciada em 1960 durante movimento do governo para povoar a região com a construção da estrada transamazônica e exploração vegetal e mineral. Hoje em dia, mais de 99% dos casos ocorrem na Amazônia, com predominância de *P. vivax*. Mas, tendo em vista o foco desta tese, focaremos a partir de agora na malária extra-amazônica, especialmente a que ocorre no Rio de Janeiro.

Devido às altas taxas de letalidade e a características epidemiológicas relacionadas à transmissão – entre elas o pensamento de que seria uma infecção transmitida apenas entre humanos, sem a participação de outros vertebrados – a OMS coordena um plano de redução de casos com vistas à erradicação da malária no mundo (WHO 2018a). No entanto, foi demostrado que há pelo menos uma espécie de *Plasmodium* de origem simiana capaz de infectar considerável número de humanos na natureza, o *P. knowlesi* (Singh et al. 2004; Cox-Singh and Singh 2008), originalmente descrito como exclusivo de PNHs do sudeste asiático. A comprovação de transmissão zoonótica levou a pesquisas mais detalhadas e, a partir de ferramentas moleculares mais modernas, demonstrou-se que as infecções por *P. knowlesi* são muito mais frequentes do que se imaginava, mas eram confundidas com *P. malariae* devido tanto a semelhanças morfológicas dos parasitos no sangue quanto clínicas (Chaudhury and Venkataramana 2017). A constatação da transmissão zoonótica na Ásia reduziu as pretensões de erradicação da doença e aumentou o interesse no esclarecimento da importância sanitária de transmissões do tipo PNH-Vetor-Humano ao redor do mundo (Ramasamy 2014), inclusive no Brasil, onde a malária humana de origem simiana já era tema de investigação desde os anos 1960 (Deane et al. 1966; de Arruda et al. 1989; Deane 1992).

O Brasil é o país que alberga a maior biodiversidade de primatas do globo, com 118 espécies descritas até o momento (Paglia et al. 2012). Apesar desta diversidade, apenas duas espécies de plasmódios simianos foram encontradas no Brasil (Deane 1992). A primeira, *P. brasilianum*, foi descrita em 1908 por Gonder e Berenberg-Gossler (apud Deane, 1992)(Deane 1992), a partir do exame do sangue de um uacari ou macaco inglês (*Cacajao calvus*), importado da Amazônia como atração de um circo

europeu. Esta espécie é praticamente indistinguível de *P. malariae*, parasita da malária quartã humana, apresentando apenas sutis diferenças morfológicas e moleculares (Guimarães et al. 2012; Lalremruata et al. 2015). Ainda assim, os ciclos esporogônico no mosquito e esquizogônico tecidual no vertebrado são idênticos. Por todas essas e outras razões, muitos autores acreditam que *P. brasilianum* e *P. malariae* seriam uma mesma espécie ou que a primeira se trataria de uma linhagem/cepa da segunda que teria se adaptado a PNHs do Novo Mundo (Coatney 1971; Deane 1992).

Plasmodium brasilianum possui ampla distribuição na natureza, tendo sido encontrada em pelo menos 11 gêneros das cinco famílias de PNHs neotropicais (Aotidae, Atellidae, Cebidae, Callitrichidae e Pitheciidae), em quatro das cinco regiões brasileiras (exceto no centro oeste) estendendo-se até a Costa Rica, na América Central (Deane 1992; Lourenço-de-Oliveira and Deane 1995; Figueiredo et al. 2015; Alvarenga et al. 2017; Fuentes-Ramírez et al. 2017). Desde o início dos anos 1960, já se sabe que *P. brasilianum* é capaz de infectar humanos experimentalmente, seja por inoculação de sangue de PNH infectados ou pela picada de anofelinos contendo esporozoítas na saliva (Coatney 1971). A suspeita da possibilidade de ocorrerem naturalmente infecções humanas por *P. brasilianum* passaram a ser consideradas. Porém, essa hipótese ganhou reforço quando Arruda et al (1989) descreveram elevada prevalência de anticorpos contra a proteína da superfície do esporozoíta de *P. brasilianum/malariae* em indivíduos que vivem em estreito contato com a floresta, na Amazônia, tais como indígenas, colonos rurais e garimpeiros. Os primeiros registros de infecção humana por *P. brasilianum* foram publicados em 2015 a partir da análise de sangue de índios Ianomâmis na Venezuela (Lalremruata et al. 2015).

A segunda espécie, *Plasmodium simium* foi descrita algumas décadas mais tarde, por Fonseca, em 1951, após a análise de preparações sanguíneas feitas a partir de um bugio (*Alouatta guariba clamitans*) capturado em Itapecerica-SP para estudos de febre amarela no ano de 1939 (Fonseca 1951). Assim como verificado entre *P. brasilianum* e *P. malariae*, *P. simium* é praticamente indistinguível de *P. vivax* (Fonseca 1951; Deane 1992), causador da malária terçã em humanos. No entanto, *P. simium* apresenta distribuição muito mais restrita, tendo sido encontrado apenas nos gêneros *Alouatta* e *Brachyteles* de vida livre e, mais recentemente, em *Cebus* e *Sapajus* de cativeiro, oriundos da Mata Atlântica do Sudeste e Sul do Brasil (Deane 1992; Duarte et al. 2013; de Alvarenga et al. 2015; Buery et al. 2017). A área coincide

com a região onde os casos de malária *vivax-like* têm sido reportados em humanos, levantado a suspeita de transmissão zoonótica.

De fato, desde 1966, após um guarda florestal que trabalhava capturando mosquitos em dossel da Mata Atlântica paulista ser infectado por *Plasmodium simium,* Deane e colaboradores alertaram sobre a possibilidade da malária humana autóctone nesse bioma poderia ter origem simiana (Deane et al. 1966). Com efeito, a malária humana foi erradicada há mais de 40 anos na maior parte das regiões Sul e Sudeste do Brasil, mas persistiu endêmica ou epidêmica em algumas áreas, como nos locais de grande influência da Mata Atlântica do litoral de Santa Catarina e São Paulo. Esta forma é chamada de malária das bromélias (Lutz 1903), pois é transmitida por mosquitos *Anopheles* do subgênero *Kerteszia* especialmente *An. cruzii* e *An. bellator* cujas larvas se criam na água acumulada nestas plantas, muito abundantes na região. *An. cruzii* é primatofílica e acrodendrófila, tendo sida encontrada naturalmente infectada e em simpatria com os casos humanos e simianos (Deane et al. 1984; Rezende et al. 2009; Duarte et al. 2013; Neves et al. 2013; Kirchgatter et al. 2014). Curiosamente, demonstrou-se que o comportamento de *An. cruzii* variava entre as regiões pesquisadas, tendo sido assinalado como estritamente acrodendrófilo em florestas de Santa Leopoldina (ES) e São Paulo (SP), mas eclético em Joinville (SC), onde era capturado em proporções semelhantes na copa e no solo (Deane et al. 1971, 1984). A ocorrência de focos de casos humanos coincidia exatamente com as regiões onde este vetor era abundantemente encontrado no solo. Além disso, em Santa Catarina *An. cruzii* era coletado também em ambiente peri e intradomiciliar (Rachou 1946). Portanto, as campanhas de combate à malária humana só obtiveram sucesso na redução dos casos quando mudaram o paradigma de controle, anteriormente focado na eliminação de grandes coleções de água, para controle e destruição de bromélias e de fragmentos florestais onde os *An. cruzii* se criavam, além da borrifação de casas com inseticidas de ação residual (Rachou 1952). Através do estudo do movimento vertical (entre a copa e o solo) deste mosquito, postulou-se que ele poderia transmitir plasmódios entre PNHs e humanos, sendo o responsável pela manutenção de focos isolados e enzoótico de malária na Mata Atlântica (Deane et al. 1971; Deane 1992).

Nas duas últimas décadas observou-se aumento expressivo de casos autóctones de malária causada por parasita identificado como *Plasmodium vivax* em regiões montanhosas da Mata Atlântica do Sudeste brasileiro, nos estados de São

Paulo, Espírito Santo e Rio de Janeiro (de Castro Duarte et al. 2008; Brasil et al. 2017; Buery et al. 2017). Estes casos apresentam aspectos clínicos e epidemiológicos semelhantes, tais como baixa parasitemia, pacientes com histórico recente de viagens para áreas montanhosas cobertas por densa floresta Atlântica, e ausência das recaídas esperados nas infecções por *P. vivax* (Curado et al. 2006; Cerutti et al. 2007; Brasil et al. 2017).

No Rio de Janeiro, pelo menos desde os anos 1990, são registrados casos humanos autóctones de malária de origem parasitária desconhecida. Entre 2006 e 2014 registraram-se 46 casos. Porém, estes números aumentaram drasticamente em 2015 e 2016: em apenas dois anos houve um surto com o registro de 49 casos comprovadamente autóctones, sem confirmação da origem dos parasitos (Fig. 6) (Pina-Costa et al. 2014; Brasil et al. 2017). Todos os casos estavam distribuídos ao longo da Serra do Mar, um complexo montanhoso coberto pelo mosaico florestal mais bem preservado da região (Pina-Costa et al. 2014).

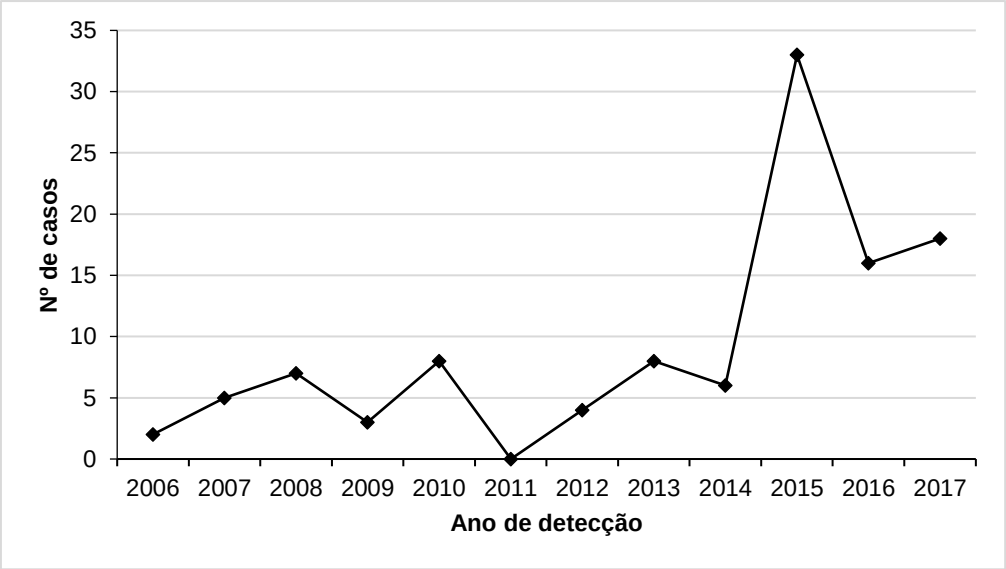

Figura 6: Número de casos autóctones de malária *tipo-vivax* em humanos entre 2006 e 2017 no Estado do Rio de Janeiro.

Apesar da hipótese de origem simiana e da rica fauna local de PNHs composta por seis gêneros (*Alouatta*, *Brachyteles*, *Callicebus* e *Sapajus* – Família Cebidae; e *Callithrix* e *Leontopithecus* – Família Callitrichidae) nenhum primata tinha sido encontrado infectado com *P. simium* no estado (Deane 1992). Durante quase 30 anos Leônidas Deane realizou, com auxílio de seus alunos e colaboradores, a mais extensa amostragem de PNHs brasileiros para pesquisa de *Plasmodium*. Dos 4585 macacos examinados, 148 estavam infectados com *P. simium*, sendo 146 *Alouatta spp.* e 02

Brachyteles sp., capturados em São Paulo, Espírito Santo, Santa Catarina e Rio Grande do Sul (Deane 1992; Lourenço-de-Oliveira and Deane 1995; Arruda 2010). No entanto, nenhum *Alouatta*, hospedeiro original e principal do *P. simium,* fora examinado no Rio de Janeiro devido às dificuldades de captura e à baixa densidade populacional desta espécie à época.

A determinação da origem do parasito é fundamental para a compreensão da epidemiologia e para o delineamento dos programas de erradicação. Por isso, desde 2015, iniciamos um trabalho de captura de PNHs no estado do Rio de Janeiro, com o objetivo de monitorar a frequência e a distribuição da infecção por plasmódios na região afim de colaborar com a elucidação da origem dos casos humanos fluminenses autóctones. As coletas foram concentradas geograficamente em torno desses casos e permitiram o encontro da infecção natural por *P. simium* em dois bugios pela primeira vez no Rio de Janeiro (Brasil et al. 2017). O sequenciamento completo do DNA (DNA-mt) mitocondrial dos plasmódios encontrado no sangue destes animais e no de casos humanos autóctone revelou elevada identidade entre eles e divergências moleculares entre eles e amostras de *P. vivax* de outra parte do Brasil e do mundo. Além disso, o DNA-mt dessas amostras simianas e os casos humanos fluminenses revelaram compartilhar a presença de *Single Nucleotide Polymorphisms* (SNPs), substituições de um nucleotídeo em região específica do genoma, cuja presença serviria como diagnóstico diferencial entre *P. vivax* e *P. simium* (Brasil et al. 2017; de Alvarenga et al. 2018). Esses achados não só confirmaram a origem simiana da malária humana autóctone fluminense, como permitiram que, doravante, se diferenciem os casos de origem simiana ou não nas malárias terçãs detectadas no Sul e Sudeste. Os artigos contendo estas informações estão apresentados nos anexos I e II deste documento.

No entanto, devido às dificuldades inerentes à captura de bugios, tais como a alimentação predominantemente folívora que torna o uso de armadilhas ineficaz, o habitat acrodendrófilo e o declive acentuado dos terrenos onde habitam que impedem a projeção de dardos anestésicos, o referido trabalho (Brasil et al. 2017) analisou apenas três PNHs, com distribuição geográfica restrita. Além disso, mesmo outros PNHs de vida livre como por exemplo saguis (*Callithrix sp*.) e macacos prego (*Sapajus sp.*) capturados para pesquisas ecológicas ou sanitárias (estudos de febre amarela ou *Trypanosoma cruzi*) não foram examinados em busca de plasmódios, devido à falta de colaboração entre grupos ou à baixa volemia encontrada nestes macacos

menores, o que gerou uma lacuna no entendimento da circulação de plasmódios no RJ.

1.3 Justificativa

Desde o início dos anos 2000, o perfil da febre amarela (FA) silvestre vem mudando no Brasil, influenciado pelos padrões de migração, expansão urbana, mudanças climáticas e comportamentais e deslocamento de pessoas não vacinadas. A contínua expansão da febre amarela silvestre da área tradicionalmente endêmica (Região Amazônica) para o sul e para o leste do Brasil, aproximando-se de áreas densamente povoadas e com baixa cobertura vacinal, como o Rio de Janeiro (RJ), despertou o interesse para a realização deste trabalho. Necessitava-se investigar a receptividade e vulnerabilidade do RJ à circulação do YFV a partir do exame de PNHs, mosquitos e das condições sócio geográficas que pudessem propiciar a entrada e a circulação do vírus da FA no RJ. Surpreendentemente, quando nosso estudo já estava avançando na realização das coletas, o YFV reemergiu na Mata Atlântica do Sudeste, área considerada indene por quase 80 anos causando um surto sem precedentes, impondo-se a necessidade de respostas à comunidade científica e leiga frente a novos questionamentos como: a origem do vírus, as espécies de mosquitos envolvidas (e as não envolvidas) na transmissão, as espécies de PNHs acometidas e principais hospedeiros, a caracterização molecular do vírus circulante e possibilidade de perenização do vírus na Mata Atlântica e do ressurgimento da transmissão urbana erradicada há décadas no país.

Desde os anos 1990, a região serrana fluminense, onde a malária também fora erradicada há décadas, tem registrado casos humanos autóctones de malária *tipo-vivax* de origem desconhecida. Um surto pouco antes daquela pelo YFV, em 2015 e 2016, contabilizou 49 casos, sem se confirmar a origem dos parasitos ou se detectarem casos índices importados de área endêmica. Nenhum PNH de vida livre tinha sido achado naturalmente infectado com plasmódio no RJ e bugios, gênero *Alouatta*, hospedeiro original e principal do *P. simium,* havia sido examinado no RJ, principalmente devido à grande dificuldade imposta na localização e captura destes animais. O exame desse e de outros PNHs, em especial das localidades onde houve registro de casos humanos, assim como a verificação de identidade entre os parasitos

infectando esses dois hospedeiros vertebrados, seriam fundamentais para a determinação da origem do parasito e, a partir disso, para o delineamento dos programas de erradicação desta doença. A ocorrência de um ciclo zoonótico poderia ser um grande empecilho no controle, sobretudo na região extra-amazônica. A captura e exame de PNHs de vida livre tornou-se indispensável também no esclarecimento desta malária autóctone de Mata Atlântica.

Se a captura de PNHs de vida livre já é difícil em alguns casos, a obtenção de suficiente amostras de sangue para várias investigações também era um obstáculo. O máximo aproveitamento de amostras sanguíneas de PNHs nos permitiu examiná-los para malária e YFV, ou mesmo para outros arbovírus da mesma família. Esse aproveitamento se justifica pelo fato de que, em 2015, também ocorreu a detecção das primeiras infecções pelo vírus ZIKV no Brasil. As características comuns entre ZIKV e YFV (ambos são *Flavivirus* de origem Africana, onde exibem um ciclo silvestre mantido entre PNHs e mosquitos) despertaram preocupação com a possibilidade de estabelecimento de um ciclo silvestre do ZIKV nas américas, tal qual o YFV, no início do século passado, reduzindo as chances de sua erradicação. A susceptibilidade de PNHs neotropicais infectados experimentalmente e o relato de infecções naturais destes animais nas proximidades dos surtos humanos, aumentaram tal preocupação. Não se sabe se o vírus já teria invadido o ambiente silvestre, se tem condições de se manter e se há mosquitos competentes para transmiti-lo neste nicho ecológico.

Em suma, as mudanças observadas na circulação da febre amarela silvestre que culminou no surto de dimensões catastróficas, o aumento de casos de malária tipo-*vivax* de origem desconhecida no Rio de Janeiro e o risco de “silvestrização” de arbovírus emergentes no país dilataram a necessidade de realização de grandes expedições de campo para avaliação dos hospedeiros silvestres (mosquitos e macacos) potenciais destes parasitos. Por isso, a partir de 2015 iniciamos uma sequência de expedições de campo nas matas do RJ e de suas fronteiras com o objetivo de encontrar, capturar e examinar uma amostragem robusta destes animais afim de reconhecer a fauna existente e os eventuais parasitos zoonóticos associados.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar aspectos epidemiológicos relativos aos surtos zoonóticos de febre amarela e malária que, recentemente, atingiram o estado do Rio de Janeiro.

2.2 Objetivos Específicos

I) Aprimorar abordagens e métodos de captura da fauna de PNHs de vida livre em Mata Atlântica, no estado do Rio de Janeiro (RJ) e suas fronteiras, com vistas a monitorar a circulação dos agentes zoonóticos nesses vertebrados;

II) Investigar a origem dos plasmódios responsáveis pelos casos de malária no Rio de Janeiro e confirmar o caráter zoonótico da malária autóctone de Mata Atlântica no estado.

II.a) Otimizar o uso de amostras sanguíneas de PNHs com a padronização de técnica molecular para a detecção de plasmódios em coágulos sanguíneos desses animais e de humanos;

II.b) Realizar inquérito da infecção plasmodial nos PNHs de vida livre;

II.c) Determinar, molecularmente, a identidade dos plasmódios detectados em PNHs com aqueles responsáveis por infecções humanas simpátricas

II.d) Determinar o reservatório animal da malária terçã benigna causada por *Plasmodium simium* no estado;

III) Investigar aspectos da transmissão do YFV no Rio de Janeiro e em outras áreas do Sudeste, especialmente naquelas limítrofes ao estado;

III.a) Investigar a fauna e a frequência espaço-temporal de mosquitos silvestres e as taxas de infecção natural pelo YFV antes e durante a transmissão recente em focos no Sudeste, visando a determinação dos vetores primários;

III.b) Investigar as prováveis rotas de disseminação do YFV antes e durante o surto no Sudeste;

III.c) Descrever a persistência da circulação do YFV no Sudeste durante três estações sazonais de transmissão independentemente de reintrodução do vírus de outra região;

III.d) Realizar inquérito virológico e sorológico em PNHs de vida livre visando a esclarecer aspectos epidemiológicos da transmissão epizoótica do YFV;

III.e) Investigar evidências de ressurgimento da transmissão urbana do YFV e de circulação de outros arbovírus, com ênfase no ZIKV, antes, durante e depois dos surtos de YFV e ZIKV, através da pesquisa de infecção natural em mosquitos e PNHs de vida livre e de inquérito sorológico em PNHs.

3 MATERIAL E MÉTODOS

As metodologias empregadas para o alcance do objetivo geral e de cada um dos objetivos específicos é detalhada nas respectivas seções de Material e Métodos dos artigos publicados em revistas científicas, produzidos ao longo do desenvolvimento desta tese.

O presente trabalho demandou a captura e análise de mosquitos e de PNHs essencialmente em fragmentos da Mata Atlântica do estado do Rio de Janeiro e de algumas áreas de seus estados fronteiriços, isto é: Minas Gerais, São Paulo e Espírito Santo. Após a eclosão do surto de febre amarela a área de coleta foi eventualmente ampliada visando a investigar municípios com suspeita de circulação recente do YFV e, a convite do Ministério da Saúde, participamos de esforços de coleta, por exemplo, em Salvador (BA). As capturas e métodos de exame foram amparados por licenças dos órgãos federais (Nº ICMBIO Sisbio 41837-3, 54707-6, 52472-2) e estaduais (L-012/2016, L-019/2018) e por autorização do comitê de ética institucional no uso de animais (CEUA Fiocruz/IOC – L004/2015, L037/2016), cujas cópias estão disponíveis no anexo V desta tese.

4 RESULTADOS

Os resultados serão apresentados em oito artigos científicos publicados, em que o candidato figura como primeiro autor, complementados por quatro artigos publicados em colaborações e reunidos nos Anexos I a IV.

Tendo em vista que o trabalho dependia do sucesso na captura de PNHs, o primeiro artigo (4.1) descreveu os principais desafios enfrentados nos terrenos de Mata Atlântica e a estratégia que desenvolvemos para superá-los, sobretudo para a captura de animais do gênero *Alouatta*, através da construção de uma rede de informações.

Outro gargalo enfrentado foi a dificuldade de obtenção de volume de sangue necessário para os testes moleculares e sorológicos visando a detecção tanto de plasmódios quanto de arbovírus, assim como de anticorpos anti-arbovírus numa mesma amostra. O desafio era maior, tanto menor fosse o PNH capturado devido à baixa volemia dos menores grupos como os saguis, por exemplo. Para contornar este problema, aventou-se a possibilidade de aproveitar o coágulo sanguíneo para extrair o DNA e diagnosticar eventuais infecções por *Plasmodium* ao invés do uso do sangue total, reservando-se o soro para os demais testes. Desta forma o segundo artigo (4.2) descreve de maneira inédita o uso de coágulos para detecção de duas espécies de Plasmódios em PNHs, e mesmo em humanos, mesmo com baixa parasitemia.

O terceiro artigo (4.3) descreve os resultados da maior amostragem de primatas de vida livre realizada no Rio de Janeiro para inquérito de *Plasmodium*. As taxas de infecções por *P. simium* e *P. brasilianum/malariae* são descobertas pela primeira vez no estado e discutidas em função da espécie de PNH, da distribuição no espaço e da sobreposição geográfica com casos humanos autóctones de malária terçã benigna provisoriamente identificadas como decorrentes da infecção pelo *P. vivax*. Os resultados, juntamente com os anexos I e II, apontam para a origem simiana das infecções humanas, a identidade do agente como sendo o *P. simium,* o caráter zoonótico da transmissão e os bugios como o único reservatório conhecido do parasito no RJ.

Em seguida, os artigos tratam de resultados sobre a investigação da febre amarela e de outros arbovírus. Assim, o quarto artigo (4.4) descreve os resultados da maior investigação entomológica em torno do surto de febre amarela silvestre no

Sudeste do Brasil. Quase 18 mil mosquitos foram capturados em 44 localidades e examinados, antes e depois do surto, permitindo-se descrever a abundância, riqueza, diversidade e frequência de mosquitos nos focos amarílicos e fora deles, assim como identificar os vetores primários e secundários da transmissão silvestre do YFV e suas respectivas taxas de infecção. Esse artigo e o anexo III também descrevem resultados de investigação entomológica e virológica quanto à ocorrência de transmissão do YFV por mosquitos domésticos e peri-domiciliares, como *Ae. aegypti* e *Ae. albopictus.*

No quinto artigo (4.5), juntamente com o anexo IV mostramos os achados moleculares, genéticos e filogenético do YFV, onde descrevemos a assinatura molecular correspondente a alterações nucleotídicas de aminoácidos existente no genoma do vírus que causou a epidemia no Sudeste, demonstramos que ela estava presente tanto nos mosquitos quanto nos humanos e macacos, discutimos as potenciais implicações destas alterações no genoma na estrutura do vírus, na transmissão vetorial e na gravidade do surto, e delineamos prováveis rotas de disseminação do YFV no RJ, uma ao sul ou porção costeira à Serra do Mar e outra ao norte ou na porção continental desta serra.

No sexto artigo (4.6), demostramos que duas linhagens do YFV do genótipo Sul Americano 1E circularam em Goiás antes e simultaneamente à epidemia no Sudeste, resultantes de introduções virais independentes na bacia do rio Araguaia. Apenas uma dessas linhagens parece ter gerado o surto na região Sudeste, exibindo a mesma assinatura molecular associada ao surto atual do YFV. Mostramos que essa linhagem seguiu dois caminhos de disseminação fora do GO, originando duas sub-linhagens principais do YFV: uma que parece ter se disseminado até nordeste de MG e, em sequência, atingido o ES e RJ, onde se dividiu nas duas cadeias descritas no artigo 4.5; e a outra se espalhou do sudoeste de MG para SP, seguindo a bacia do Paraná.

O sétimo artigo (4.7), a partir de análises moleculares e filogeográficas e genomas do YFV do RJ entre 2017 e 2019, demonstra, pela primeira vez, que o YFV é capaz de se manter numa mesma área de Mata Atlântica do Sudeste por pelo menos três estações sazonais de transmissão, sem necessidade de introdução de nova linhagem ou sub-linhagem viral vinda de áreas enzoóticas do Centro-Oeste ou Amazônia.

A procura da infecção natural por YFV, e também pelo ZIKV, em PNHs, assim como de outros 33 arbovírus em mosquitos silvestre, rurais e peri-urbanos, a

investigação da ocorrência de anticorpos contra YFV e ZIKV em PNHs capturados antes, durante e após surtos produzidos por estes dois vírus são descritas no último e oitavo manuscrito (4.8). A infeção por YFV foi detectada especialmente em bugios, mas também em saguis. Anticorpos neutralizantes anti-YFV não foram detectados nesses animais, mas sim em macacos-prego, animais aparentemente mais resistentes à infecção pelo YFV. Os resultados não mostraram que evidências de circulação do YFV no RJ antes do surto em curso, nem de transmissão urbana do YFV e nem de instalação de transmissão selvagem do ZIKV no Sudeste.

4.1 Artigo 1: Capture of *Alouatta guariba clamitans* for surveillance of sylvatic Yellow Fever and zoonotic Malaria: which is the best strategy in the tropical Atlantic Forest?

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NEW APPROACHES

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Capture of *Alouatta guariba clamitans* for the surveillance of sylvatic yellow fever and zoonotic malaria: Which is the best strategy in the tropical Atlantic Forest?

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Abstract

Howler monkey capture is an arduous and expensive task requiring trained and specialized professionals. We compared strategies and methods to most efficiently capture *Alouatta guariba clamitans* in remnants of the Atlantic Forest in Rio de Janeiro and its bordering states of Minas Gerais and São Paulo. We tested whether or not the success of expeditions in the forest with anesthetic darts, nets, and baited traps differed with and without the support of an information network, a contact chain built with key institutions and inhabitants to continuously monitor howler monkey presence. The influence of forest conditions (vegetation type and fragment size) upon darting success was also evaluated. We captured 24 free-living *A. guariba clamitans*. No howler monkey was caught with traps, probably due to the predominantly folivore feeding to high local plant diversity providing a great variety of food options. Captures based on an information network were significantly more efficient in terms of numbers of caught monkeys than without it. Captures with darts were considerably more efficient when performed in semideciduous forests and small forest fragments as opposed to ombrophilous forests or large woods. Although we walked great distances within the forest searching for howler monkeys, all but one animal were captured at the forest fringes. Hindrances to search and the darting method in the Atlantic Forest, for example, the steep terrain, high tree canopies, hunt pressure, and low *A. guariba clamitans* population density, were mitigated with the use of the information network in this monkey capture. Moreover, the information network enhanced the surveillance of zoonotic diseases, which howler monkeys and other nonhuman primates are reservoirs in Brazil, such as malaria and yellow fever.

KEYWORDS
anesthetic dart, howler monkey, information network, trapping

1 | INTRODUCTION

The capture of nonhuman primates (NHPs) has been performed for several scientific purposes, such as genetic, ecological, or sanitary aims. Traps are widely adopted for small and frugivorous neotropical primates capture (Watsa et al., 2015). However, the capture of wild howler monkeys is an arduous and expensive task requiring trained and specialized professionals. Howler monkey groups prefer the high forest canopies and feed predominantly on leaves, complicating access and reducing the efficacy of fruit-baited traps (Brasil, 2017a). Few authors have reported success in trapping howler monkeys such as Aguiar et al. (2007), capturing 70 *Alouatta caraya* with traps installed in the tree canopy. Therefore, despite the complexity and high risk of animal injury or even death, the most common technique for *Alouatta* capture is the anesthetic dart gun. With this method, 130 *Alouatta palliata* were captured and marked in La Pacifica, Costa Rica, between 1973 and 1975 (Scott, Júnior, & Malmgren, 1976), and 688 from 1973 to 1991 (Teaford & Glander, 1991), in a dry and deciduous forest environment within small woods surrounded by pastures (Clarke, Zucker, & Scott, 1986; Scott et al., 1976). There also have been reports of success with the same method in capturing howlers for ecological or epidemiological studies in Argentina, Costa Rica, Venezuela, and Panama, where 20–100 specimens were captured in a single month (Martínez, Kowalewski, Salomón, & Schijman, 2016; Milton, Lozier, & Lacey, 2009; Rumiz, 1990; Scott et al., 1976). In Brazil, the methodology was widely adopted during the 2008–2009 YFV outbreak in Rio Grande do Sul, where more than 270 animals were captured (Brasil, 2017a). Although the dart methodology is well established, little has been discussed on the importance of involving institutions and local inhabitants before and after the field expeditions. Approaches based on ethnoprimatology can provide clues and methods to take advantage of local resident knowledge and interaction with NHPs during the fieldwork (Jones-Engel, Engel, & Fuentes, 2011), contributing in the construction of an information network as a means of optimizing capture efforts.

NHPs can harbor several parasites infectious to man. In Brazil, since 1966, human autochthonous malaria in areas under the influence of the Atlantic forest has been suspected to have simian origin caused by *Plasmodium simium*, the original and primary host being howler monkeys, genus *Alouatta* (Deane, 1992). The confirmation of this zoonotic cycle has only been achieved very recently by molecular examination of local *Alouatta* (Brasil et al., 2017; Buery et al., 2017).

Sylvatic yellow fever is a viral zoonotic disease accidentally transmitted from viremic NHPs to man by arboreal primatophilic mosquitoes. Recently, the virus (YFV) has re-emerged in the Atlantic Forest, Southeast Region, causing numerous human cases and deaths besides having a huge impact on NHP biodiversity resulting from thousands of epizootic events (Bonaldo et al., 2017; Brasil, 2017b). Species of genus *Alouatta* are among the most susceptible to the YFV considering the number of dead animals or even population extinctions during epizooties (Araújo et al., 2011; Moreno et al., 2013; Moreno et al., 2015). The new affected area corresponds to the territory of three *Alouatta* taxa: *A. caraya*, *A. guariba guariba*, and mostly, *A. guariba clamitans*, the brown howler monkey, which has been the most affected since the beginning of this YFV expansion (Almeida et al., 2012) and the only *Alouattinae* found in Rio de Janeiro (RJ). Yellow fever surveillance includes active investigation, which consists of capturing free-living NHPs followed by the collection of blood samples for early detection of the virus and/or specific antibodies. This action aims to obtain evidence of viral circulation for the risk prediction of YFV transmission to human populations (Brasil, 2017a).

In 2015, we initiated a program for NHP capture focusing on howlers in response to the expansion of the YFV toward RJ and the increase in the number of autochthonous malaria cases in the state. Herein, we compare strategies and methods to most efficiently capture *A. guariba clamitans* in remnants of the RJ Atlantic Forest and its borders. Particularly, we wanted to test whether or not the capture success with darts or nets differs with or without the support of an information network composed of several kinds of agents, mostly from key institutions and residents in target areas. In addition, we wanted to find out the possible variation of dart capture efficiency in different forest types and fragment sizes. Finally, baited trap application on tree platforms for howler capture was evaluated in two different forest environments.

2 | DESCRIPTION

2.1 | Ethical statement

Our methods and protocols were previously approved by the Institutional Ethics Committee for Animal Experimentation (Protocol CEUA/IOC-029/2016, license L-037/2016) and by Brazilian Ministry of the Environment (SISBIO 41837-3 and 54707-4) and Rio de Janeiro's Environment agency (INEA 012/2016 and 019/2018). The research adhered to the American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates.

2.2 | Capture efforts

The following two main strategies were tested to capture *A. guariba clamitans* between 2015 and 2017: (a) Expeditions with active searches employing the use of anesthetic dart guns in the forest without any previous alert from an information network, and, alternatively, (b) the construction of an information network with key institution and resident contacts incorporating the use of anesthetic dart guns, canopy baited traps, or nets upon alerts raised in this information network.

Besides, the efficiency of capture with anesthetic darts was evaluated under influence of two different forest environments (semideciduous seasonal forests vs. ombrophilous forest) and two fragment size categories (<100 ha vs. >100 ha).

2.2.1 | Anesthetic dart capture during the expeditions without any previous alert from the information network

Seven long-term capture expeditions were orchestrated with an average duration of 13.5 days each. Six expeditions occurred in the regions of dense ombrophilous forest, covering 13 municipalities in 82 days, 63 of which were entirely devoted to the active search of howlers in the forest, the remaining days being spent journeying between forest fragments and inspection areas. One expedition was on the western slope, in the semideciduous seasonal forest, covering four municipalities in 13 days, 9 of which were totally devoted to howler search (Table 1). The howler search fieldwork was conducted by three to six trained investigators with a local guide entering the forest as quietly as possible. When howlers were encountered, anesthetic darts were targeted at the lower limbs of the largest animals in the group. The collection, biosafety, and anesthesia protocols complied with the rules and regulations of the Brazilian Ministry of Health (Brasil, 2017a). The darts contained a combination of ketamine (15 mg/Kg), midazolam (1 mg/Kg), and levomepromazine (1 mg/Kg), and alternatively, an association of tiletamine and zolazepam (4–5 mg/Kg). After anesthesia, a large nylon net was opened to catch the falling anesthetized animals. In some cases, the fall was induced by shaking the tree branch, supporting the animal with a rope. After sample collections and complete recovery of anesthetic effects, animals were released in the same place during the daytime. Only one monkey was injured by fracturing one femur during the fall while anesthetized, which was afterward treated and kept in a primate-breeding center (Centro de Primatologia do Rio de Janeiro—CPRJ).

2.2.2 | Information network-based captures—development and operation

The network was developed by establishing long-distance and personal contacts with three main types of agents/actions: (a) Residents, health agents, environmental guards, conservation unit managers, and local guides living in the target municipalities. To raise awareness about the importance of primate surveillance, we personally interviewed these agents in the field during the expeditions or short-term trips (1–3 days) before capture expeditions. We also organized lectures in local schools, community centers, and health departments aiming to amplify the network. (b) Patients affected by autochthonous malaria who usually have a secondary residence in the target area, introducing us to local residents, which were added to the groups amplifying the network. (c) We established collaborations with primate research groups working in several areas in the state of RJ.

Groups composed of all agents were created on WhatsApp mobile messenger application (WhatsApp Inc., Delaware Corporation, CA) for each location. Besides contact through WhatsApp, we made periodic phone calls and sent messages to key agents. By receiving information in real time, the howler monkey presence in specific sites, situation, and time was continuously monitored in each area even in our absence. Short-term capture trips to target areas were elaborated (Table 1) whenever our network agents efficiently informed the presence of healthy or sick howler monkeys in their locality.

2.2.3 | Installation of canopy traps in platforms

Two platforms were established with the main purpose of baiting and trapping whole groups of *A. guariba clamitans*, the traps being designed according to Aguiar et al. (2007). The selected trees for installation of both platforms were defined according to suggestions from the members of the information network (arrows in Figure 1). The trap front and back doors were kept open for 2 months to allow free circulation, enhanced by the continuously replenished baits. Afterward, the back door was sealed but the entrance remained open for the animals to access the bait, and about 8 kg of fresh fruits and vegetables was renewed every 3 days. The regular bait was banana, mango, guava, corn, and coconut. Eventually, other options such as peas and string beans were tested in an attempt to provide food closer to the predominantly folivorous *Alouatta* diet. Platform 1 was set at a 20-meter height in the Municipal Natural Park of Atalaia, a large forest fragment of approximately 1,183 hectares located at an altitude of 120 meter on the plains of the Macaé municipality,

TABLE 1 Number of howler monkeys captured according to strategies, capture methods, and forest characteristics

Strategies	Total days spent	Total captured howlers	Capture methodology	Vegetation type		Fragment size	
				SDF[a]	ODF[b]	Up to 100 ha	> 100 ha
Expeditions without the information network alert	95	9	9 (dart)	4 (9)	5 (63)	8 (29)	1 (43)
Expeditions with the Information network alert	23	15	3 (dart), 12 (net)	9 (11)	6 (11)	10 (12)	5 (10)
Platform baited trap	336	0	0 (trap)	0 (175)	0 (161)	0 (161)	0 (175)
Total	454	24	_	13 (195)	11 (235)	18 (202)	6 (228)

Note: The number of working days searching for monkeys in each kind of forest and forest fragment size in parenthesis.
[a]Semideciduous seasonal forest.
[b]Ombrophilous dense forest.

on the east side of Serra do Mar, 166 kilometers from the city of Rio de Janeiro, where our laboratory is located. The predominant vegetation is the dense ombrophilous lowland type (IBGE, 2012). Platform 2 was placed at a 12-meter height on a farm located on the western slope of Serra do Mar in the municipality of Sumidouro at an altitude of 1,080 meter and 147 kilometers from Rio de Janeiro. This forest consists of about 60 hectares of connected patches of predominantly semideciduous seasonal vegetation type in which the tree canopies were lower than those of the platform 1 area. According to reports of our information network, the high branches of few trees were the only passage for a group of howlers to cross an unpaved road that separates two forest fragments. We pruned these branches and linked the platform to the trees located on the opposite side of the road with ropes intending to force the animals to pass close to the trap entrance. Cameras with a motion infrared sensor were installed for 24-hr surveillance of the visiting animals in both platforms. Platform 1 was monitored for 23 weeks, from September 2015 to February 2016, the end of the dry and beginning of the wet seasons, respectively, while platform 2 was monitored for 25 weeks, between July and December 2016, essentially the dry season.

2.3 | Study area

The fieldwork was carried out between May 2015 and June 2017 in several locations in the Atlantic Forest, in RJ as well as frontier sites in the Southeastern states of Minas Gerais (MG) and São Paulo (SP; Figure 1). Due to deforestation, forests with ecological conditions to support larger NHPs have essentially been reduced to fragments on hilltops scattered in lowlands or mountain valleys and escarpments of "Serra do Mar" (Ribeiro, Metzger, Martensen, Ponzoni, & Hirota, 2009), which makes RJ different from other regions where howler monkeys have been captured. Serra do Mar is a mountain chain forming a large and long ecological corridor (CEPF, 2005). Its eastern slope, facing the ocean, has the largest continuous forest in the state and sustains primary or secondary dense ombrophilous forest predominant vegetation (IBGE, 2012). On the western slope, toward MG, the vegetation is replaced by semideciduous seasonal forest (IBGE, 2012) exhibiting high degrees of fragmentation (Figure 1). The choice of capture areas took into account scientific papers reporting howler occurrence, recent human malaria cases, and the existence of important river basins and denser forests, connecting states and wooded fragments, which may serve as a corridor for NHPs and YFV dispersal.

2.4 | Statistical analyses

The capture efficiency (CE) and its standard deviation (*SD*) were calculated by dividing the number of captured howlers by the capture effort (number of days spent in the capture) considering the sampled forest fragment. First, the CE was compared between the strategies with or without the use of an information network through a two-sided Kruskall-Wallis test, since this parameter was non-normally distributed (Shapiro-Wilk, $p < .05$). Subsequently, the CE of searches with anesthetic darts was contrasted between forest types (semideciduous seasonal and dense ombrophilous) and forest fragment sizes (smaller or larger than 100 ha) with the same statistical test. *p*-values were adjusted for multiple comparisons according to the Bonferroni criteria and were considered statistically significant when these values were equal to or less than .05. The response variables were not correlated according to

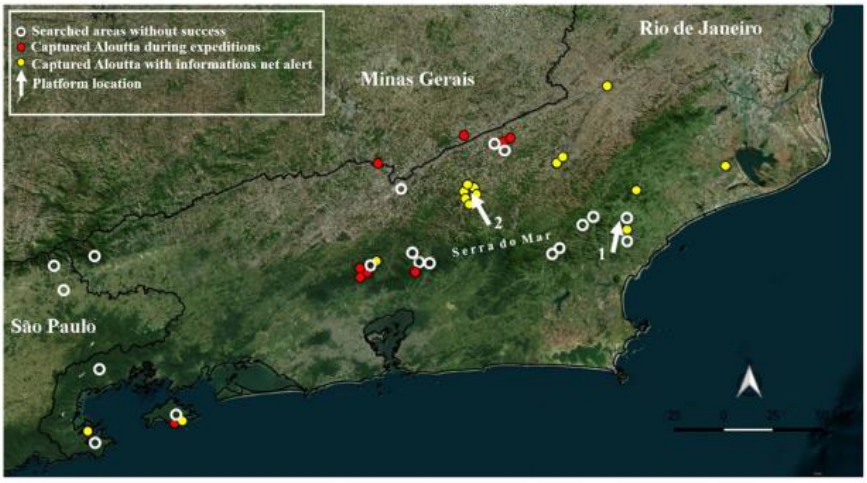

FIGURE 1 Satellite image of Rio de Janeiro showing sampled areas, relief, and vegetation. Red circles represent *Alouatta* captured with active searching during expeditions. Yellow circles represent *Alouatta* captured after our network alert. White transparent circles represent areas where we performed active search during expeditions but failed to capture *Alouatta*. White arrows indicate the locations of platforms 1 and 2

variation inflation factors (VIFs; i.e., VIF <5). All statistical analyses were performed with R version 3.0.2.

3 | EXAMPLES

Between 2015 and 2017, 24 wild *A. guariba clamitans* were captured and examined, nine during the active search in the forest without any previous alert from an information network, and 15 based on the information network (Table 1).

3.1 | Effects of the information network

The captures based on the information network alert were more efficient (Kruskal-Wallis, $p < .05$; Figure 2). On average, 0.92 howlers (*SD* =0.4) were captured per day during efforts based on alerts from the network versus 0.19 (*SD* =0.4) without the alert of the network information.

Of the 15 howlers captured based on the information network, six (40%) were reported by conservation unit managers, six (40%) by residents, two (13.3%) by other researchers, and one (6.6%) by the municipal health department manager. Six were captured in dense ombrophilous forests and nine in semideciduous. Concerning capture methods, 12 were captured with nets as they were either sick ($n = 8$) or easily attracted by fruits regularly offered by neighboring farmers ($n = 4$) and three with anesthetic darts in two field expeditions of 2 days each.

Importantly, during the YFV outbreak in Rio de Janeiro (2017–2018), the information network enabled us to find and collect material from 14 other sick, agonizing, or dead *A. guariba clamitans* besides two marmosets (genus *Callithrix*), but they were not included in the analysis to prevent bias in our statistical comparison of capture method efficiency.

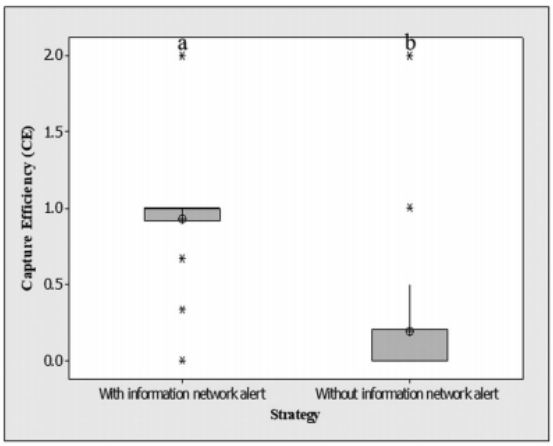

FIGURE 2 Boxplot of the capture efficiency (CE), of the two strategies tested: With and without the support of the Information Network. Different letters mean statistical difference (Kruskal-Wallis; $p < .05$)

3.2 | Effects of forest characteristics in dart capture efficiency

In total, 12 howlers were captured using darts, being seven in semidecidual and five in dense ombrophilous forest. The CE, when using anesthetic darts, was significantly higher in semideciduous seasonal forest (0.56 howler/day on average, *SD*: 0.67) compared to the ombrophilous forest (0.051 howler/day on average, *SD*: 0.1; Kruskall-Wallis, $p < .05$; Figure 3a).

Regarding fragment size, 11 howlers were captured in those considered small (up to 100 ha) and one in those termed large (>100 ha). The forest fragment size also influenced the capture success with darts. The CE (0.4 howler/day on average, *SD*: 0.5) in the 29 days of work in seven small forest fragments (0.5–100 ha) was significantly higher than those captured in 43 days of work in 17 large fragments (>100 ha, corresponding to 0.01 ± 0.03 howlers/day; Kruskall-Wallis, $p < .05$; Figure 3b).

Although we walked great distances and spent a lot of time in search of howler monkeys (up to 8 search hours per day), 11 of the 12 dart-captured animals were obtained less than 1 km from the forest fringe. Only one animal was captured deep inside the forest.

3.3 | Trapping evaluation

No howler monkey entered the traps or consumed the scattered fruits in proximity even after more than 23 weeks of the food supply in the two study areas. The target howler monkey groups were frequently detected using the highest arboreal branches above platform 1 and crossing the ropes installed close to platform 2 (Figure 4). After 4 weeks of baiting, a group of capuchin monkeys (*Sapajus nigritus*) began to access trap 1 (Figure 5a), of which we captured and examined two. The camera trap caught several rodents, marsupials, and birds consuming the bait and made the first record of a kinkajou *Potus flavus* in the Atalaia Park (Figure 5b).

After our survey, the traps were removed and the platforms were maintained for utility by other researchers, mostly for bird watching.

4 | COMPARISON AND CRITIQUE

This was the first time that different strategies for *A. guariba clamitans* capture were evaluated in different forest types and distinct fragment sizes of the Atlantic Forest. We showed that through an information network, it was possible to collect and examine more animals in less time. The number of animals we examined based on alerts from the information network was 66.6% higher than that obtained in active search without it. Both the capture time and the expended financial resources have significantly dropped with the network. In addition, during the YFV outbreak, the information network helped in reducing the time between finding monkeys and the laboratory diagnosis, contributed to rapid

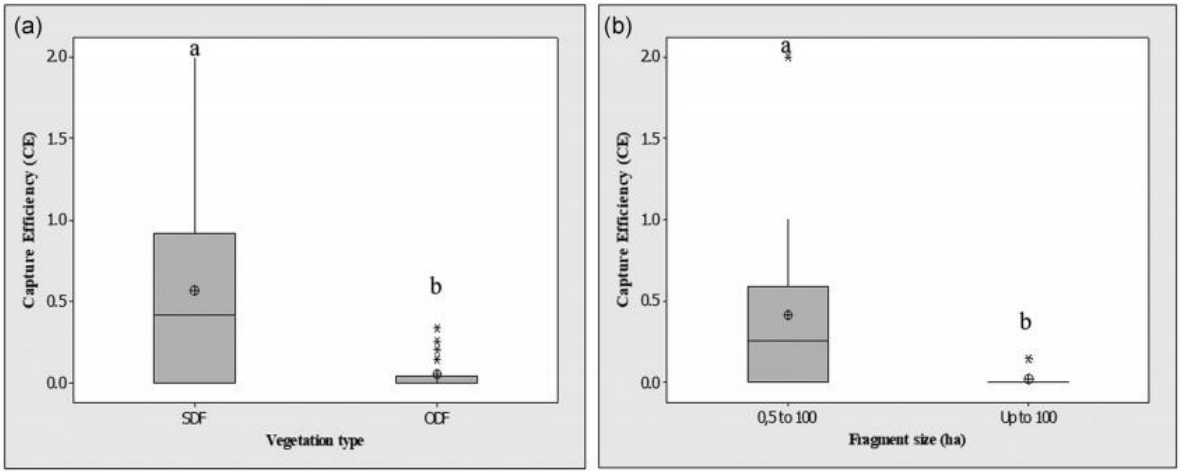

FIGURE 3 (a) Boxplot of the CE in two types of vegetation: Semideciduous seasonal and dense ombrophilous. (b) Boxplot of the CE in two categories of fragments sizes: Small (up to 100 ha) and large (more than 100 ha). Different letters mean statistical difference (Kruskall-Wallis; $p < .05$). CE, capture efficiency

notification in the state of Rio de Janeiro, minimizing the damage to the human population bordering the epizootics.

Although the elevated costs and high injury risks, the most popular technique for howler capture is the anesthetic dart, with which populations of *Alouatta* have been monitored for decades in different regions in Latin America (Martínez et al., 2016; Rumiz, 1990; Scott et al., 1976). These studies were conducted in highly fragmented or isolated patches of woods, in essentially flat terrains, generally covered with secondary semidecidual forests (Froehlich, Thorington, & Otis, 1981; Martínez et al., 2016; Milton et al., 2009; Rumiz, 1990; Scott et al., 1976).

However, we had lower capture success in Rio de Janeiro, especially when working in the dense ombrophilous forest type and in larger fragments (>100 ha). The dense ombrophilous forest in Rio de Janeiro has a canopy over 20 meters in height, composed of several intermediate tree limbs that reduce visibility and hamper efficient dart gun procedure. In addition, the steep terrain may provide lower vegetation sustenance, consequently, decreasing food availability, which results in lower primate densities (Assumpção, 1983; Pinto, Claudia, Costa, & Fonseca, 1993). In general, large forest fragments also present reduced monkey densities by favoring dispersal and movement. Also, this kind of forest may harbor large predators and competitors (González-Solís, Guix, Mateos, & Llorens, 2001). Besides, the hunting pressure reported by residents, park managers, and environmental guards during the interviews, may have contributed to the scared behavior of the howlers in Rio de Janeiro (Araújo, Souza, & Ruiz-Miranda, 2008; González-Solís et al., 2001; Pinto et al., 1993). Together, these factors may explain the capture difficulty, reinforcing the importance of other methodologies in this type of environment.

The great advantage of darting for yellow fever surveillance in NHPs is the mobility since it allows sampling different areas of

FIGURE 4 *A. guariba clamitans* crossing the road through the rope, very close to the baited trap in platform 2. It never entered or examined the platform or the baits. Panoramic image was assembled using the software Microsoft Image Composite Editor 2.0 (ICE)

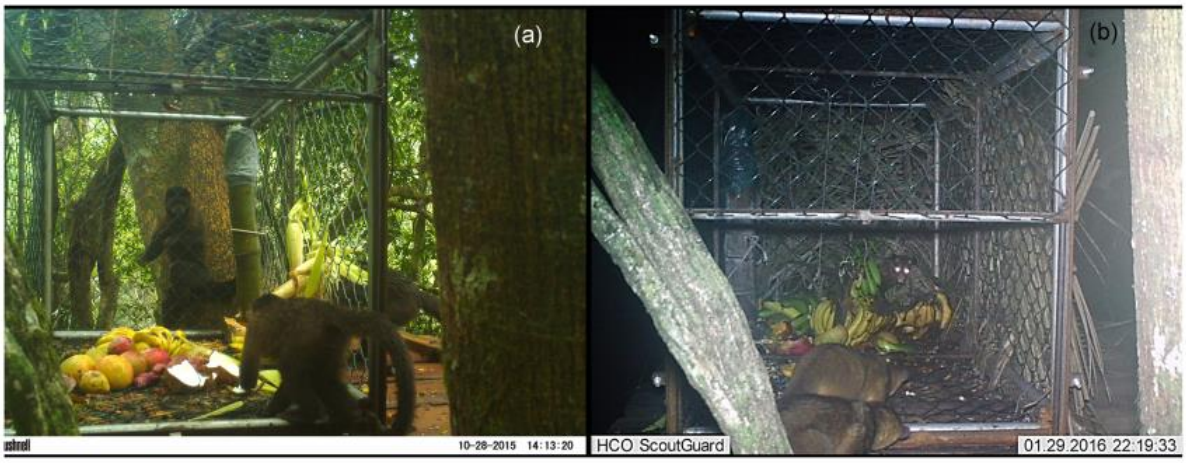

FIGURE 5 Records of animals consuming the bait inside the trap installed at platform 1. (a) Group of *Sapajus nigritus*. (b) First record of kinkajou (*Potus flavus*) in the Atalaia Park. It is possible to distinguish two kinkajou and an opossum

interest at any time (Brasil, 2017a). However, our results indicated that this application should be prioritized either in small and flat fragments of semideciduous secondary forest or on islands due to the confined area limiting animal dispersal, facilitating animal encounter. Due to the difficulties reported, we recommend monitoring based on the construction of an information network before captures, especially for the mountainous region (Serra do Mar) in the Brazilian southeastern states. As an initial approach, active search and howler capture with dart guns would be recommended at the fragment fringe as well as at sites with constant sightings of *Alouatta* previously reported by local inhabitants and the information network. Finally, net capture seems to be the simplest and cheapest technique in the case of diseased or baited animals reported by the information network.

Intriguingly, all but one *A. guariba clamitans* were caught less than 1 km from the forest fringe in Rio de Janeiro, which may suggest that long hikes in rough terrain deeper into the forest were inefficient. Probably the team movement within the forest might have startled the howler monkeys, inducing them to disperse or hide, especially under the hunter pressure reported by residents. The same was observed by Jones and Bush (1988) when capturing *Cercopithecus*. The only animal we captured deep inside the forest was encountered on a flat and well-marked trail in Ilha Grande State Park, Angra dos Reis, a protected and isolated island, which facilitated howler encounter (Oliveira, 2011).

The use of baited traps is inefficient for capturing howlers because they have a predominantly folivore feeding habits and can forage on dozens of tree species as well as consume sprouts, flowers, and fruits depending upon availability (Aguiar et al., 2007; Chiarello, 1994). Despite this, one study reports the success in capturing 70 *Alouatta caraya* in the Paraná river basin, Southern Brazil (Aguiar et al., 2007). In contrast, we failed to trap *A. guariba clamitans* in Rio de Janeiro. We attribute this failure to the following factors: (a) Higher plant and animal richness and diversity in RJ, providing more options to the howler monkeys and discouraging bait attraction. Moreover, baits were consumed more rapidly by birds and other mammals, differently from that reported in the isolated areas in Paraná (Aguiar et al., 2007). (b) Interspecific behavioral difference: The species trapped by Aguiar et al. (2007) was *Alouatta caraya*, commonly found in very disturbed secondary forests, presenting a more diverse diet than brown howlers when in sympatry (Agostini, Holzmann, & di Bitetti, 2010). The predominantly folivore feeding habits together with differences in population densities and group size could essentially explain the reduced attraction of *A. guariba clamitans* to the bait offered in our traps. Perhaps, a long-term program of baiting in special circumstances (very small forest fragments, low availability of preferred food, long dry season, and absence of competitors) may increase the chance of *A. guariba* clamitans allurement to traps but demands great physical effort and human resources.

Our results demonstrate that the most efficient strategy to enhance howler monkey capture in the Atlantic Forest is the use of an information network. Its creation requires an initial investment of time and we emphasize the importance of involving health and environmental agencies with the inclusion of reserve managers, environmental guards, and hiker associations in the information network as these agents deal daily with natural environments and are usually the first to come across sick or dead animals. Currently, technology is a helpful tool. Message exchange apps such as WhatsApp or Telegram facilitate communication, providing comprehensive and cost-effective interaction among the agents involved. Hence, it is an efficient and inexpensive method of monitoring the primate population, according to the researchers to take advantage of the best methods to capture howlers according to the species, and ecological aspects. Its application can be adopted for the collection of samples destined for ecological, evolutionary, and especially, epidemiological studies, with the

objective of understanding zoonotic diseases and predicting epidemics threatening man.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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REFERENCES

Agostini, I., Holzmann, I., & di Bitetti, M. S. (2010). Are howler monkey species ecologically equivalent? Trophic niche overlap in syntopic *Alouatta guariba* clamitans and *Alouatta caraya*. *American Journal of Primatology*, *72*, 173–186. https://doi.org/10.1002/ajp.20775

Aguiar, L. M., Ludwig, G., Svoboda, W. K., Teixeira, G. M., Hilst, C. L. S., Shiozawa, M. M., & Passos, F. C. (2007). Use of traps to capture black and gold howlers (*Alouatta caraya*) on the islands of the upper Paraná River, Southern Brazil. *American Journal of Primatology*, *69*, 241–247. https://doi.org/10.1002/ajp.20372

Almeida, M., Dos Santos, E., Da Cruz Cardoso, J., Da Fonseca, D. F., Noll, C. A., Silveira, V. R., & Brasil, R. A. (2012). Yellow fever outbreak affecting *Alouatta* populations in southern Brazil (Rio Grande do Sul State), 2008–2009. *American Journal of Primatology*, *74*, 68–76. https://doi.org/10.1002/ajp.21010

Araújo, F. A. A., Ramos, D. G., Santos, A. L., Passos, P. H., de, O., Elkhoury, A. N. S. M., … Romano, A. P. M. (2011). Epizootias em primatas não humanos durante reemergência do vírus da febre amarela no Brasil, 2007 a 2009. *Epidemiologia e Serviços de Saúde*, *20*, 527–536. https://doi.org/10.5123/S1679-49742011000400012

Araújo, R. M., Souza, M. Bde, & Ruiz-Miranda, C. R. (2008). Densidade e tamanho populacional de mamíferos cinegéticos em duas Unidades de Conservação do Estado do Rio de Janeiro, Brasil. *Iheringia. Série Zoologia*, *98*, 391–396. https://doi.org/10.1590/S0073-47212008000300014

Assumpção, C. T., de (1983). An ecological study of the primates of southeastern Brazil, with a reappraisal of Cebus paella races. University of Edinburgh (unpublished doctoral dissertation).

Bonaldo, M. C., Gómez, M. M., dos Santos, A. A., Abreu, F. V. S, Ferreira-de-Brito, A., Miranda, R. M, & Lourenço-de-Oliveira, R. (2017). Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. *Memórias do Instituto Oswaldo Cruz*, *112*, 447–451. https://doi.org/10.1590/0074-02760170134

Brasil. (2017a). *Guia De Vigilância De Epizootias Em Primatas Não Humanos E Entomologia Aplicada À Vigilância Da Febre Amarela. Segunda edición actualizada*. Retrieved from http://portalarquivos.saude.gov.br/images/pdf/2017/marco/24/Guia_Epizootias_Febre_Amarela_2a_ed_atualizada_2017.pdf

Brasil (2017b). Monitoramento Dos Casos E Óbitos De Febre Amarela No Brasil - Informe – No 43/2017. Retrieved from http://portalarquivos.saude.gov.br/images/pdf/2017/junho/02/COES-FEBRE-AMARELA---INFORME-43---Atualiza----o-em-31maio2017.pdf

Brasil, P., Zalis, M. G., de Pina-Costa, A., Siqueira, A. M., Júnior, C. B., Silva, S., & Daniel-Ribeiro, C. T. (2017). Outbreak of human malaria caused by Plasmodium simium in the Atlantic Forest in Rio de Janeiro: A molecular epidemiological investigation. *The Lancet Global Health*, *5*, e1038–e1046. https://doi.org/10.1016/S2214-109X(17)30333-9

Buery, J. C., Rodrigues, P. T., Natal, L., Salla, L. C., Loss, A. C., Vicente, C. R. … Cerutti, C. (2017). Mitochondrial genome of Plasmodium vivax/simium detected in an endemic region for malaria in the Atlantic Forest of Espírito Santo state, Brazil: Do mosquitoes, simians and humans harbour the same parasite? *Malaria Journal*, *16*(1), 1–10. https://doi.org/10.1186/s12936-017-2080-9

CEPF (2005). Atlantic Forest Hotspot: Brazil Briefing Book (1st.). Washington DC: Critical Ecosystem Partnership Fund. http://www.cepf.net/Documents/final.atlanticforest.brazil.briefingbook.pdf

Chiarello, A. G. (1994). Diet of the brown howler monkey *Alouatta fusca* in a semi-deciduous forest fragment of southeastern Brazil. *Primates*, *35*, 25–34. https://doi.org/10.1007/BF02381483

Clarke, M. R., Zucker, E. L., & Scott, N. J. (1986). Population trends of the mantled howler groups of La-Pacifica, Guanacaste, Costa-Rica. *American Journal of Primatology*, *11*, 79–88.

Deane, L. M. (1992). Simian malaria in Brazil. *Memórias do Instituto Oswaldo Cruz*, *87*, 1–20. https://doi.org/10.1590/S0074-02761992000700001

Froehlich, J. W., Thorington, R. W., & Otis, J. S. (1981). The demography of howler monkeys (*Alouatta palliata*) on Barro Colorado Island, Panamá. *International Journal of Primatology*, *2*, 207–236. https://doi.org/10.1007/BF02739331

González-Solís, J., Guix, J. C., Mateos, E., & Llorens, L. (2001). Population density of primates in a large fragments of the Brazilian Atlantic rainforest. *Biodiversity and Conservation*, *10*, 1267–1282.

IBGE (2012). De Geografia E Estatística – Ibge. Manual Técnico da Vegetação Brasileira (Vol. 1). Rio de Janeiro, Brasil. Retrieved from https://biblioteca.ibge.gov.br/visualizacao/livros/liv63011.pdf

Jones, W. T., & Bush, B. B. (1988). Darting and marking techniques for an arboreal forest monkey, *Cercopithecus ascanius*. *American Journal of Primatology*, *14*, 83–89. https://doi.org/10.1002/ajp.1350140108

Jones-Engel, L., Engel, G. A., & Fuentes, A. (2011). An ethnoprimatological approach to interactions between human and nonhuman primates, *Field and Laboratory Methods in Primatology: A Practical Guide, Second Edition* (pp. 21–32). Cambridge, UK: Cambridge University Press. https://doi.org/10.1017/CBO9780511921643.003

Martínez, M. F., Kowalewski, M. M., Salomón, O. D., & Schijman, A. G. (2016). Molecular characterization of trypanosomatid infections in wild howler monkeys (*Alouatta caraya*) in northeastern Argentina. *International Journal for Parasitology: Parasites and Wildlife*, *5*, 198–206. https://doi.org/10.1016/j.ijppaw.2016.05.001

Milton, K., Lozier, J. D., & Lacey, E. A. (2009). Genetic structure of an isolated population of mantled howler monkeys (*Alouatta palliata*) on Barro Colorado Island, Panama. *Conservation Genetics*, *10*, 347–358. https://doi.org/10.1007/s10592-008-9584-3

Moreno, E. S., Agostini, I., Holzmann, I., Di Bitetti, M. S., Oklander, L. I., Kowalewski, M. M., & Miller, P. (2015). Yellow fever impact on brown howler monkeys (*Alouatta guariba clamitans*) in Argentina: A

metamodelling approach based on population viability analysis and epidemiological dynamics. *Memorias do Instituto Oswaldo Cruz*, *110*, 865–876. https://doi.org/10.1590/0074-02760150075

Moreno, E. S., Spinola, R., Tengan, C. H., Brasil, R. A., Siciliano, M. M., Coimbra, T. L. M., & Suzuki, A. (2013). Epizootias de febre amarela em primatas não humanos no estado de São Paulo, Brasil, 2008–2009. *Revista do Instituto de Medicina Tropical de Sao Paulo*, *55*, 45–50. https://doi.org/10.1590/S0036-46652013000100008

Oliveira, A. B. M., de (2011). Comportamento de bugios (Alouatta clamitans Cabrera, 1940) na Ilha Grande, RJ. Universidade do Estado do Rio de Janeiro (unpublished master dissertation). Retrieved from http://arquivos.proderj.rj.gov.br/inea_imagens/downloads/pesquisas/PE_Ilha_Grande/Oliveira_2011.pdf

Pinto, L. P. S., Claudia, M. R., Costa, K. B. S., & da Fonseca, G. A. B (1993). Habitat, density and group size of primates in a Brazilian tropical forest. *Folia Primatologica*, *61*, 135–143. https://doi.org/10.1159/000156740

Ribeiro, M. C., Metzger, J. P., Martensen, A. C., Ponzoni, F. J., & Hirota, M. M. (2009). The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biological Conservation*, *142*, 1141–1153. https://doi.org/10.1016/J.BIOCON.2009.02.021

Rumiz, D. I. (1990). *Alouatta caraya*: Population density and demography. *American Journal of Primatology*, *21*, 279–294. https://doi.org/10.1002/ajp.1350210404

Scott, N. J., Júnior, A. F. S., & Malmgren, L. A. (1976). Capturing and marking howler monkeys for field behavioral studies. *Primates*, *17*, 527–533. https://doi.org/10.1007/BF02382910

Teaford, M. F., & Glander, K. E. (1991). Dental microwear in live, wild-trapped *Alouatta palliata* from Costa Rica. *American Journal of Physical Anthropology*, *319*, 313–319. https://doi.org/10.1002/ajpa.1330850310

Watsa, M., Erkenswick, G., Halloran, D., Kane, E. K., Poirier, A., Klonoski, K., & Zuniga, A. (2015). A field protocol for the capture and release of callitrichids. *Neotropical Primates*, *22*, 59–68.

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4.2 Artigo 2: Frozen blood clots can be used for the diagnosis of distinct *Plasmodium* species in human and non-human primates from brazilian Atlantic Forest

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Malaria Journal

METHODOLOGY Open Access

Frozen blood clots can be used for the diagnosis of distinct *Plasmodium* species in man and non-human primates from the Brazilian Atlantic Forest

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Abstract

Background: Zoonotic infections with epidemic potential, as non-human primate malaria and yellow fever (YF), can overlap geographically. Optimizing a small blood sample for diagnosis and surveillance is of great importance. Blood are routinely collected for YF diagnosis and blood clots usually discarded after serum obtention. Aiming to take sample advantage, the sensitivity of a PCR using extracted DNA from long-term frozen clots from human and non-human primates for detection of *Plasmodium* spp. in low parasitaemia conditions was assayed.

Results: Malaria diagnosis with DNA extracted from blood clots generated results in agreement with samples obtained with whole blood, including mixed *Plasmodium vivax/simium* and *Plasmodium malariae/brasilianum* infections.

Conclusion: Blood clots from human and non-human primates may be an important and low cost source of DNA for malaria surveillance in the Atlantic Forest.

Keywords: Blood clot, *Plasmodium*, *Alouatta guariba clamitans*, Non-human-primates, Yellow fever, Atlantic Forest

Background

Some zoonotic infections with epidemic potential may overlap geographically. Their surveillance and follow-up during epizooties and epidemics depends upon the screening for distinct infectious agents often in a single blood sampling from animal reservoirs. Frequently, reservoirs are low weight animals, and sometimes, hypovolaemia at the sampling moment prevents the collection of the ideal amount of blood. Therefore, optimizing the blood sample may be critical for diagnosis of different infections at the time.

Non-human primates (NHPs) are reservoirs of main zoonotic agents, such as *Plasmodium* spp. and yellow fever virus (YFV). For instance, in South America, the YFV coexists in the same areas where *Plasmodium* spp. from NHP origin has been recorded for decades [1–3]. Thus, simultaneous surveillance of these zoonotic infections in a single NHP blood sampling is very important.

Recently, outbreaks of human malaria and YF acquired in the sylvatic transmission cycle have been reported in the Atlantic Forest localities in Southeast Brazil, the states of Espírito Santo (ES) and Rio de Janeiro (RJ) were the most affected by both outbreaks [4–8]. Indeed, the most severe yellow fever outbreak in the last seven decades reemerged in the Atlantic Forest areas with 689 humans and 1394 NHPs confirmed deaths [7, 9]. In the same way, outbreaks caused by *Plasmodium simium*, a NHP parasite closely related to *Plasmodium vivax*, have

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been recently reported [1, 5, 10–14], as well as outbreaks by *Plasmodium brasilianum*, another New World NHP malaria parasite morphologically indistinguishable to *Plasmodium malariae* [1]. Conventional PCR protocols for malaria diagnosis are adopted worldwide [e.g. 15, 16] and also do not distinguish *P. vivax* and *P. malariae* from *P. simium* and *P. brasilianum*, respectively [5, 12, 17]. It has been postulated that *P. vivax* can only be differentiated from *P. simium* by two specific Single Nucleotide Polymorphisms (SNPs) in mitochondrial DNA from human or NHP red blood cells or whole blood [5, 6, 18]. *Plasmodium simium* parasitaemia is usually low to scanty both in NHP and human hosts. Concerning YFV, the Brazilian Ministry of Health preconizes the active surveillance of NHPs, which requires their capture with traps or anesthetic darts together with blood sampling for serological and molecular assays. Thus, blood is regularly collected in tubes without anticoagulant to obtain serum [19], normally the blood clot discarded after serum collection.

The main host of both *P. simium* and the YFV in Southeastern and Southern Brazil are howler monkeys, genus *Alouatta* [1, 19]. These animals hardly ever enter traps and their capture in the forest canopy is difficult and expensive. Due to the difficulties to obtain blood samples from wild howler monkeys and other neotropical NHPs, it is important to optimize such samples. Therefore, the methodology proposed by Lundblom et al. [20] was improved and assayed for the detection of *Plasmodium* spp. DNA in blood clots from man and NHPs taking into account the low parasitemia conditions frequently associated with these malaria infections.

Methods

Non-human primate samples

During a YFV surveillance in 2016, two *Alouatta guariba clamitans* (M1 and M3) and one *Callithrix* hybrid (M2) (Table 1) were captured in the Atlantic Forest areas of Rio de Janeiro state, Brazil, under the technical guidelines of sampling and security measures [19]. A total of 5 and 3 mL of venal blood was, respectively, collected from femoral puncture of M1/M3 and M2 and then equally split into two tubes, one without anticoagulant and the other containing EDTA. Following centrifugation (2000*g* × 10 min), sera, clot, plasma and whole blood samples were stored at −80 °C until DNA extraction. Whole blood and clot samples were not depleted of leucocytes and platelets.

Human samples

Five mL blood samples collected in January 2017 from *P. vivax* patients (H1 and H2) infected in the Atlantic Forest areas of Rio de Janeiro state, Brazil and from a clinically healthy donor (H3) were used. Both whole blood and blood clots were obtained, processed and stored as described for NHP samples.

Parasitological diagnosis

Giemsa-stained thick and thin blood films of human and NHP samples were examined prior to PCR assays. Samples H1, H2, M1 and M3 were positive, displaying 208, 368, 300 and 40 malaria parasites/μL, respectively. The remaining two samples, a human (H3) and one NHP (M2) were negative (Table 1).

Table 1 PCR results from whole blood and blood clots according to malaria species and estimated parasitaemia according to microscopy examination (thick and thin blood film)

Code	Species	Parasitaemia (parasites/μL)	PCR[a]: whole blood	PCR[a]: 1st blood clot DNA extraction[b]	PCR[a]: 2nd blood clot DNA extraction[b]	PCR[a]: 3rd blood clot DNA extraction[b]
H1	*Homo sapiens*	208	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. vivax/P. simium*
H2	*Homo sapiens*	368	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. vivax/P. simium*	*Plasmodium; P. vivax/P. simium*
H3	*Homo sapiens*	0	–	–	–	–
M1	*Alouatta g. clamitans*	300	*Plasmodium; P. vivax/P. simium* and *P. malariae/P. brasilianum*	*Plasmodium; P. vivax/P. simium* and *P. malariae/P. brasilianum*	*Plasmodium; P. vivax/P. simium and P. malariae/P. brasilianum*	*Plasmodium; P. vivax/P. simium and P. malariae/P. brasilianum*
M2	*Callithrix* hybrid	0	–	–	–	–
M3	*Alouatta g. clamitans*	40	*P. malariae/P. brasilianum*	*P. malariae/P. brasilianum*	*P. malariae/P. brasilianum*	*P. malariae/P. brasilianum*

[a] Gene targets were cysteine proteinase for *P. vivax*, ssrRNA for *P. malariae* and 18S rRNA for genus *Plasmodium*; [b] DNA extractions from clots disrupted by high-speed shaking (6000 rpm/40 s) in lysis buffer, without glass beads; –: negative result

DNA extraction and PCR

After thawing, 600–800 µL of lysis buffer (AL lysis buffer provided in QIamp® DNA mini kit, Qiagen) were added to 300–400 µL (2:1) of each blood clot in tubes containing or not glass beads, a high-speed shaking (6000 rpm/40 s; Bertin Precellys 24) was performed for clot disruption. Afterwards, this DNA was extracted with the QIAamp® DNA mini kit, according to manufacturer's instructions, except for the elution step volume that was made in 50 instead of 100 µL. The unprocessed whole blood DNA was extracted from around 600 µL with both the same kit and elution procedures. In order to check DNA extraction efficiency, 3 sets of extractions were realized with the same blood clot sample, as already described. The presence of gDNA was evaluated after electrophoresis (110 A/1 h) on ethidium bromide agarose gel under UV light. To check PCR assay precision, these samples were tested in three replicates (intra-assays) on each of three different days (between assays).

For the conventional PCR assays, all DNA samples were tested for 18 s rRNA *Plasmodium* genus-specific gene [21], and than for cysteine proteinase *P. vivax* [22] and ssrRNA *P. malariae* genes [15]. The sensitivity threshold of the genus [21] and *P. vivax* assay [22] specific protocols optimized and routinely used are 0.5 and 0.019 parasites/µL respectively, while that of the *P. malariae* PCR is of 1 parasite/µL according to the authors [15]. PCR products were visualized under UV light after electrophoresis on 2% agarose gels. DNA from patients infected by *P. vivax* or *P. malariae* was the positive control in each round of amplification. Non-infected human and NHP DNA as well as blank samples (no DNA) were the negative controls.

Sensitivity comparison assay

In order to compare the sensitivity of PCR based on blood clots we used similar quantities of blood cells from whole blood and blood clot samples from another *A. g. clamitans* (M3) naturally infected with *P. malariae* (parasitaemia of 120 parasites/µL). Blood clots are composed of red and white blood cells, platelets and fibrin, which together roughly correspond to almost 50% of all human and NHP blood volume. Thus, aiming to equalize the amount of blood cells we used 300 µL and 600 µL of clot and whole blood, respectively. Whole blood and disrupted blood clot samples were diluted (1:2, 1:4, 1:10; 1:100; 1:1000; 1:10,000, 1:100,000) (Table 2) and DNA extractions from each dilution were processed for *P. malariae* PCR amplification, as described above [15]. In addition, we performed PCRs assays changing the last hold cycle of the first and second rounds to 58°–5 min instead of 58°–2 min and 72°–10 min instead of 72°–5 min as well as the number of cycles in the second round changed to 35 instead of 30.

Results

The PCR results with DNA extracted from clots were in agreement with those of the whole blood (Table 1). Concerning extraction without beads, there were small residual fragments, but they were dissolved in subsequent incubations, the gDNA strongly detectable under UV light. Thus, the DNA clot PCR results reported herein were from disruptions performed without glass beads. H1 and H2 samples were PCR positive for genus *Plasmodium* and *P. vivax* and negative for *P. malariae,* whereas M1 (*A. g. clamitans*) was positive for genus, *P. vivax* and *P. malariae*. Despite the low parasitaemia, samples were consistently intra-assay (repeatability) and between assay (reproducibility) positive on the 3 different days (Table 1). Inversely, clot disruption with glass beads, besides requiring centrifugation steps, generated a weak and indistinct gDNA band under UV light, PCR reactions negative regardless of the PCR target.

Concerning sensitivity, *P. malariae* was detected in blood clot and whole blood of M3 till the dilution of 1:10 and 1:100 that corresponds to 4 and 0.4 parasites/µL, respectively. By increasing the number of cycles of the PCR, the limit of detection (LoD) increased to 0.4 parasites/µL for blood clot samples and to 0.04 parasites/µL for whole blood samples (Table 2).

Table 2 PCR results from different *Plasmodium malariae* concentrations of blood clot and whole blood samples from an infected *Alouatta g. clamitans* (M3) monkey

M3 *Alouatta g. clamitans*		Dilutions							
Conditions	**Sample**	**1**	**1:2**	**1:4**	**1:10**	**1:100**	**1:1000**	**1:10,000**	**1:1,000,000**
Regular PCR[a] conditions	Blood clot[b]	+	+	+	+	−	−	−	−
	Whole blood	+	+	+	+	+	−	−	−
Improved PCR[a] conditions	Blood clot[b]	+	+	+	+	+	−	−	−
	Whole blood	+	+	+	+	+	+	−	−

[a] The gene target was ssrRNA; [b] DNA extractions from clots disrupted by high-speed shaking (6000 rpm/40 s) in lysis buffer, without glass beads; + and −: positive and negative result, respectively

Discussion

Human and non-human primate blood clots could be important sources of DNA that can be advantageous for *Plasmodium* spp. detection even in low parasitaemias. This is the first report of blood clots for the detection of *Plasmodium* DNA in NHPs as well as an unprecedented description of *P. vivax/simium* and *P. malariae/brasilianum* detection in clot samples even with low parasitaemias. In fact, blood clot disruption with high speed shaking was only reported for the detection of *P. falciparum* in larger volume samples of human blood clots with no information of a precision (repeatability and reproducibility) parameter [20]. It was also demonstrated that the LoD from the clot was smaller than that of whole blood under standard PCR conditions. However, when the number of cycles was changed, the sensitivity increased for both clot and whole blood samples. Thus, increasing the number of cycles can compensate a possible loss of sensitivity for blood clots with very low parasitaemia. Therefore, the use of often-discarded blood clots allows the investigation of malaria parasites in the same blood sampling from which serum was extracted for simultaneous surveillance of YFV as well as other arboviruses in NHPs.

Interestingly, the high-speed shaking technique without the use of beads exhibited best blood clot disruption performance. This corresponds to an advantage by reducing risk of contaminations due to handling as described for other disruption techniques [23–25]. In addition to malaria diagnosis, PCR templates from NHP blood can be used for epidemiological, ecological and evolutionary purposes. In fact, NHP trypanosomes infecting man, such as *Trypanosoma cruzi*, can be even more sensitively detected in blood clots when compared to whole blood and leukocyte samples [26] or serum in the case of detecting fungi causing aspergillosis in man [27].

Furthermore, human blood clots stored at −20 °C from 1 to 2.5 years were still useful for the investigation of single-nucleotide polymorphisms [28]. Indeed, PCR results with blood clots were essentially equivalent to whole blood (Tables 1, 2), as demonstrated in this paper with blood clots stored at −80 °C for around 2 years, revealing the maintenance quality of the DNA template. Consequently, blood clot storage may be also a stable source of primate genetic samples for several phylogeny, dispersion and gene flow studies as whole blood tissues, hair or even feces, which have already been adopted as DNA sources by several researchers [29–33] (non-exhaustive list). Along with increasing deforestation, studies of habitat fragmentation effects, especially in small populations of endangered NHPs, are necessary to support management strategies [34], and clotted blood could also be a stable genetic bank for several populations.

Finally in view of the results reported here, we suggest that the Brazilian Ministry of Health surveillance protocols recommend NHP blood clot collection storage and examination. This would indeed correspond to a low-cost initiative that could optimize the results of NHP capture and safety as well as contribute to knowledge expansion in different fields of primatology and epidemiology for the protection of man against zoonotic diseases.

Conclusion

In conclusion, blood clots can be a valuable source of genetic information, optimizing costs, handling time and reducing the amount of blood collected from each subject. Thereby, the creation of a blood clot bank could aid the resolution of several ecological, evolutionary and epidemiological issues.

Authors' contributions

FVSA, LRG and ARLM carried out blood clot DNA extractions and PCR assays; DST, ES and FVSA captured and collected NHP samples; APC, CBJ and PB provided human samples; MFFC and ARLM diagnosed by PCR malaria patients; MFFC and RLO conceived the study; RLO and FVSA examined PNH microscopic slides; CTDR, MFFC and RLO raised grants for funding the work; FVSA, MFFC and RLO drafted and finalized the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The protocols for handling and blood collection of NHP were approved by the Institutional Ethics Committee of Animal use at IOC (CEUA licenses LW-34/2014 and L037/2016, respectively). Wild NHP captures were in agreement with the Brazilian environmental authorities: SISBIO-MMA licenses 54707-137362-2 and 52472-1, and INEA license 012/2016012/2016. Protocol for human blood collection was approved by INI-Fiocruz Ethical Board (#0062.0.009.000-11) and by Ethical Research Committees of Fiocruz (# 32839013.6.00005248). All participants provided informed written consent.

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References

1. Deane LM. Simian malaria in Brazil. Mem Inst Oswaldo Cruz. 1992;87(suppl 3):1–20.
2. Barrett ADT. Epidemiology and ecology of yellow fever virus. Adv Virus Res. 2003;61:291–315.
3. Gonzalez JP, Prugnolle F, Leroy E. Men, primates, and germs: an ongoing affair. Berlin: Springer; 2012. p. 337–53.
4. Bonaldo MC, Gómez MM, dos Santos AA, de Abreu FVS, Ferreira-de-Brito A, de Miranda RM, et al. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. Mem Inst Oswaldo Cruz. 2017;112:447–51.
5. Brasil P, Zalis MG, de Pina-Costa A, Siqueira AM, Júnior CB, Silva S, et al. Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. Lancet Glob Health. 2017;5:e1038–46.
6. Buery JC, Rodrigues PT, Natal L, Salla LC, Loss AC, Vicente CR, et al. Mitochondrial genome of *Plasmodium vivax*/simium detected in an endemic region for malaria in the Atlantic Forest of Espírito Santo state, Brazil: do mosquitoes, simians and humans harbour the same parasite? Malar J. 2017;16:437.
7. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Monitoramento Dos Casos E Óbitos De Febre Amarela No Brasil—Informe—No. 43/2017. 2017:1–7. http://portalarquivos2.saude.gov.br/images/pdf/2017/junho/02/COES-FEBRE-AMARELA---INFORME-43---Atualiza----o-em-31maio2017.pdf. Accessed 15 Dec 2017.
8. Possas C, Martins RM, de Oliveira RL, Homma A, Possas C, Martins RM, et al. Urgent call for action: avoiding spread and re-urbanisation of yellow fever in Brazil. Mem Inst Oswaldo Cruz. 2017;113:1–2.
9. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Monitoramento do Período Sazonal da Febre Amarela. 2018. http://portalarquivos2.saude.gov.br/images/pdf/2018/fevereiro/21/Informe-n14-FA-20fev18-c.pdf. Accessed 3 Mar 2018.
10. Deane LM, Deane MP, Ferreira Neto J. Studies on transmission of simian malaria and on a natural infection of man with *Plasmodium simium* in Brazil. Bull World Health Organ. 1966;35:805–8.
11. Cerutti C, Boulos M, Coutinho AF, Hatab MCL, Falqueto A, Rezende HR, et al. Epidemiologic aspects of the malaria transmission cycle in an area of very low incidence in Brazil. Malar J. 2007;6:33.
12. de Pina-Costa A, Brasil P, Santi SM, de Araujo MP, Suárez-Mutis MC, Santelli ACF, et al. Malaria in Brazil: what happens outside the Amazonian endemic region. Mem Inst Oswaldo Cruz. 2014;109:618–33.
13. Costa DC, da Cunha VP, de Assis GMP, Souza Junior JC, de Hirano ZMB, de Arruda ME, et al. *Plasmodium simium*/*Plasmodium vivax* infections in southern brown howler monkeys from the Atlantic Forest. Mem Inst Oswaldo Cruz. 2014;109:641–53.
14. de Alvarenga D, de Pina-Costa A, de Sousa T, Pissinatti A, Zalis MG, Suaréz-Mutis MC, et al. Simian malaria in the Brazilian Atlantic Forest: first description of natural infection of capuchin monkeys (Cebinae subfamily) by *Plasmodium simium*. Malar J. 2015;14:81.
15. Snounou G, Viriyakosol S, Zhu Xin Ping, Jarra W, Pinheiro L, do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993;61:315–20.
16. Rubio JM, Benito A, Roche J, Berzosa PJ, García ML, Micó M, et al. Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of *Plasmodium vivax* infection in Equatorial Guinea. Am J Trop Med Hyg. 1999;60:183–7.
17. Fuentes-Ramírez A, Jiménez-Soto M, Castro R, Romero-Zuñiga JJ, Dolz G. Molecular detection of *Plasmodium malariae*/*Plasmodium brasilianum* in non-human primates in captivity in costa rica. PLoS ONE. 2017;12:e0170704.
18. de Alvarenga DAM, Culleton R, de Pina-Costa A, Rodrigues DF, Bianco C, Silva S, et al. An assay for the identification of *Plasmodium simium* infection for diagnosis of zoonotic malaria in the Brazilian Atlantic Forest. Sci Rep. 2018;8:86.
19. Ministério Da Saúde. Guia De Vigilância De Epizootias Em Primatas Não Humanos E Entomologia Aplicada À Vigilância Da Febre Amarela. 2017. http://portalarquivos.saude.gov.br/images/pdf/2017/marco/24/Guia_Epizootias_Febre_Amarela_2a_ed_atualizada_2017.pdf. Accessed 15 Dec 2017.
20. Lundblom K, Macharia A, Lebbad M, Mohammed A, Färnert A. High-speed shaking of frozen blood clots for extraction of human and malaria parasite DNA. Malar J. 2011;10:229.
21. Gama BE, Silva-Pires FES, Lopes MNR, Cardoso MAB, Britto C, Torres KL, et al. Real-time PCR versus conventional PCR for malaria parasite detection in low-grade parasitemia. Exp Parasitol. 2007;116:427–32.
22. Torres KL, Figueiredo DV, Zalis MG, Daniel-Ribeiro CT, Alecrim W, de Ferreira-da-Cruz M. F. Standardization of a very specific and sensitive single PCR for detection of *Plasmodium vivax* in low parasitized individuals and its usefulness for screening blood donors. Parasitol Res. 2006;98:519–24.
23. Garg UC, Hanson NQ, Tsai MY, Eckfeldt JH. Simple and rapid method for extraction of DNA from fresh and cryopreserved clotted human blood. Clin Chem. 1996;42:642–7.
24. Salazar LA, Hirata MH, Cavalli SA, Machado MO, Hirata RD. Optimized procedure for DNA isolation from fresh and cryopreserved clotted human blood useful in clinical molecular testing. Clin Chem. 1998;44:1748–50.
25. Se Fum Wong S, Kuei JJ, Prasad N, Agonafer E, Mendoza GA, Pemberton TJ, et al. A simple method for DNA isolation from clotted blood extricated rapidly from serum separator tubes. Clin Chem. 2007;53:522–4.
26. Fitzwater S, Calderon M, Lafuente C, Galdos-Cardenas G, Ferrufino L, Verastegui M, et al. Polymerase chain reaction for chronic *Trypanosoma cruzi* infection yields higher sensitivity in blood clot than buffy coat or whole blood specimens. Am J Trop Med Hyg. 2008;79:768–70.
27. McCulloch E, Ramage G, Jones B, Warn P, Kirkpatrick WR, Patterson TF, et al. Don't throw your blood clots away: use of blood clot may improve sensitivity of PCR diagnosis in invasive aspergillosis. J Clin Pathol. 2009;62:539–41.
28. Bank S, Nexø BA, Andersen V, Vogel U, Andersen PS. High-Quality and -quantity DNA extraction from frozen archival blood clots for genotyping of single-nucleotide polymorphisms. Genet Test Mol Biomarkers. 2013;17:501–3.
29. Nascimento FF, Bonvicino CR, da Silva FCD, Schneider MPC, Seuánez HN. Cytochrome b polymorphisms and population structure of two species of *Alouatta* (Primates). Cytogenet Genome Res. 2005;108:106–11.
30. Ruiz-Garcia M, Escobar-Armel P, Alvarez D, Mudry M, Ascunce M, Gutierrez-Espeleta G, et al. Genetic variability in four *Alouatta* species measured by means of nine DNA microsatellite markers: genetic structure and recent bottlenecks. Folia Primatol. 2007;78:73–87.
31. Oklander LI, Kowalewski MM, Corach D. Genetic consequences of habitat fragmentation in Black-and-Gold Howler (*Alouatta caraya*) populations from Northern Argentina. Int J Primatol. 2010;31:813–32.
32. Van Belle S, Estrada A, Strier KB, Di Fiore A. Genetic structure and kinship patterns in a population of Black Howler Monkeys, *Alouatta pigra*, at Palenque National Park, Mexico. Am J Primatol. 2012;74:948–57.
33. Bonvicino CR, Viana MC. Genetic diversity of *Alouatta* (Primates) from Brazilian Atlantic Forest. J Primatol. 2015;4:2–6.
34. Knapp LA. Primates in fragments. Molecular genetic tools for evaluating the consequences of habitat fragmentation. New York: Springer; 2013. p. 389–98.

4.3 Artigo 3: Howler monkeys are the reservoir of malaria parasites causing zoonotic infections in the Atlantic forest of Rio de Janeiro

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RESEARCH ARTICLE

Howler monkeys are the reservoir of malarial parasites causing zoonotic infections in the Atlantic forest of Rio de Janeiro

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Abstract

Background

Although malaria cases have substantially decreased in Southeast Brazil, a significant increase in the number of *Plasmodium vivax*-like autochthonous human cases has been reported in remote areas of the Atlantic Forest in the past few decades in Rio de Janeiro (RJ) state, including an outbreak during 2015–2016. The singular clinical and epidemiological aspects in several human cases, and collectively with molecular and genetic data, revealed that they were due to the non-human primate (NHP) parasite *Plasmodium simium;* however, the understanding of the autochthonous malarial epidemiology in Southeast Brazil can only be acquired by assessing the circulation of NHP *Plasmodium* in the foci and determining its hosts.

Methodology

A large sampling effort was carried out in the Atlantic forest of RJ and its bordering states (Minas Gerais, São Paulo, Espírito Santo) for collecting and examining free-living NHPs. Blood and/or viscera were analyzed for *Plasmodium* infections via molecular and microscopic techniques.

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Competing interests: The authors have declared that no competing interests exist.

Principal findings

In total, 146 NHPs of six species, from 30 counties in four states, were tested, of which majority were collected from RJ. Howler monkeys (*Alouatta clamitans)* were the only species found infected. In RJ, 26% of these monkeys tested positive, of which 17% were found to be infected with *P. simium*. Importantly, specific single nucleotide polymorphisms–the only available genetic markers that differentiate *P. simium* from *P. vivax*–were detected in all *P. simium* infected *A. clamitans* despite their geographical origin of malarial foci. Interestingly, 71% of *P. simium* infected NHPs were from the coastal slope of a mountain chain (Serra do Mar), where majority of the human cases were found. *Plasmodium brasilianum/malariae* was initially detected in 14% and 25% free-living howler monkeys in RJ and in the Espírito Santo (ES) state, respectively. Moreover, the malarial pigment was detected in the spleen fragments of 50% of a subsample comprising dead howler monkeys in both RJ and ES. All NHPs were negative for *Plasmodium falciparum*.

Conclusions/Significance

Our data indicate that howler monkeys act as the main reservoir for the Atlantic forest human malarial parasites in RJ and other sites in Southeast Brazil and reinforce its zoonotic characteristics.

Author summary

The present work comprises an unprecedented capture effort and large-scale field survey of *Plasmodium* species in non-human primates (NHPs) in RJ, a state recording three-decade history of autochthonous human cases of benign tertian malaria lacking epidemiological clarification of their origin. This is the first study to describe the infection rates by *Plasmodium* spp. in free-living NHPs in RJ, thereby matching the spatial distribution of *P. simium* in NHP with that of the local human cases of benign tertian malaria occurring due to this parasite. This study confirmed howler monkeys as the only reservoir of this zoonotic malarial parasite in RJ and reported that specific single nucleotide polymorphisms were present in all *P. simium* infected howler monkeys, despite their geographical origin of malarial foci. Moreover, this is the first study to record *P. brasilianum/malariae* in free-living NHPs from RJ, and to illustrate their widespread distribution in this state. Collectively, these findings help us in evaluating the simian malaria prevalence in the Atlantic Forests and in assessing the zoonotic characteristics of autochthonous human malaria in Rio de Janeiro, thus providing assistance in shaping surveillance and control.

Introduction

In Brazil, more than 99% of malarial infections are acquired from the Amazon, and few isolated cases are occasionally found and recorded in regions outside the Amazon [1]. Malaria transmission was considered eradicated from South and Southeast regions of Brazil approximately 40 years ago [1]; however, in the last three decades, a significant increase in autochthonous malarial cases by *Plasmodium vivax*-like parasites in the Atlantic Forest areas in Southeast Brazil, where no index case that could have introduced the parasite from a malaria

endemic region, has been reported [1,2]. These cases present similar parasitological, clinical, and epidemiological characteristics, such as low parasitemia, no *P. vivax* expected relapses, and recent visits to dense rain forest areas, where the bromeliad-inhabiting *Anopheles* mosquitoes belonging to the subgenus *Kertezsia*, specially *An. cruzii*, are found [2–4]. *An. cruzii* is the main vector of "bromeliad malaria", which is endemic in South and Southeast Brazil, and is the only known natural vector of simian malaria in the country [5]. This particular epidemiological context revived the hypothesis raised by Deane et al. in the 1960s regarding the existence of human malaria cases of simian origin in Brazil. Specifically, these authors reported a human natural infection attributed to the Neotropical primate parasite *P. simium* Fonseca [6] in São Paulo (SP), Southeast region [7]. The patient presented a benign tertian malaria after being exposed to the mosquito bites during a tree-canopy entomological survey in a forest densely populated by *An. cruzii*. The description of vertical movement of *An. cruzii* between the canopy and ground level in the Atlantic rain forest of Southern Brazil reinforced Deane's hypothesis that part of the transmission of bromeliad malaria in Southern and Southeastern Brazil would be of zoonotic character, with monkeys being the parasite reservoir [5–8].

Two species of *Plasmodium* have been described in the Neotropical non-human primates (NHP): *P. brasilianum* Gonder e Berenberg-Gossler (1908) and *P. simium*, almost indistinguishable from the human malaria parasites *P. malariae* and *P. vivax*, respectively [5,6,9]. Besides subtle morphological variations [2,5], molecular markers such as microsatellites and single nucleotide polymorphisms (SNPs) were the only differences so far described between *P. malariae* and *P. brasilianum*, and *P. vivax* and *P. simium* [2,10,11]. *P. brasilianum* is widely distributed compared to *P. simium*, with its presence from México to Southern Brazil, thereby infecting at least 11 genera of the five families of Neotropical primates (Aotidae, Atelidae, Callitrichidae, Cebidae, and Phiteciidae) [5,12–15]. In contrast, *P. simium* has been found essentially in species belonging to two genera (*Alouatta sp*. and *Brachyteles sp*., family Atelidae) [5], from the Atlantic forest of South and Southeast Brazil.

To completely understand the epidemiology of recent autochthonous malaria in Southeast Brazil, it is necessary to confirm the circulation of NHP *Plasmodium sp*. in the transmission foci as well as to determine the parasite reservoirs. Studies on the prevalence of *P. simium* and *P. brasilianum* infections in NHPs and their potential reservoirs in Southeast Brazilian states were conducted during 1960–1990s. Almost 800 NHPs were sampled and their blood slides were examined by microscopy, which is a less sensitive technique compared to the molecular assays, thus recording a variation in the *Plasmodium* infection from 10.9% in the states of Espírito Santo to 56.5% in SP [5]. During these surveys, only free-living lion-tamarins (Callitrichidae) were examined within RJ and all were negative to malaria parasites [16]. However, RJ recorded 110 autochthonous human cases of benign tertian malaria between 2005 and 2018, with an outbreak in 2015–2016 of 49 cases [1,2]. Interestingly, all these human infections were acquired at the RJ sites located along Serra do Mar, an extensive mountain chain covered by the best-preserved rain forest mosaic in Southeast Brazil. This biome harbors a rich NHP fauna comprising species of six genera (*Alouatta*, *Brachyteles*, *Callicebus*, *Callithrix*, *Leontophitecus*, and *Sapajus*) [17], with *An. cruzii* being the most common anopheline mosquito [18]. Consequently, the hypothesis of simian origin in these RJ malarial cases has been raised [5,7]. In response, multidisciplinary malaria studies including clinical, epidemiological, parasitological, and molecular approaches have been conducted in RJ [2,4,19]. More recently, the molecular studies of parasites infecting humans and three howler monkeys clearly demonstrated that they shared the same *P. simium* parasite [2]; however, to date, scarce number of wild NHPs of few species from only three out of numerous autochthonous malaria foci in RJ and surroundings could be examined [2]. This study presents the largest sampling effort ever carried out in the Atlantic forest of RJ and its borders for capturing and examining free-living NHPs in order

to describe the geographical distribution and frequency of simian malaria, as well as to determine the local animal reservoirs and confirm the identity of the parasite infecting humans and NHPs in the autochthonous malaria foci.

Material and methods

Study area

The work was carried out between May 2015 and January 2019, with a total of 120 days of fieldwork at 44 sites in 30 counties of the Atlantic Forest biome, mainly in RJ but also in its bordering areas including the states of Minas Gerais (MG), ES, and SP. In this survey, we included forest fragments from lowlands areas to mountain valleys and escarpments of mountain chains such as Serra do Mar, which divides the state territory into two sides, one facing the ocean (hereinafter called the coastal slope) and other the continent (continental slope) [20,21]. The choice of capture areas considered the local existence of NHPs, recent human malaria cases as well as alerts from the information network built with key institutions to continuously monitor the presence of howler monkeys, as previously described [21].

Capture and sample collection

The expeditions included up to 10-day surveys in the forests, conducted by 2–6 trained people in the target areas to search NHPs. The capture method was selected according to the NHP species, behavior, and size [22]. Briefly, Tomahawk model traps baited with banana were used for *Callithrix*, *Leontopithecus*, and *Sapajus* genera [22,23]. The traps were opened early during daytime and were inspected every hour until 3:30 pm, when they were closed. The captured animals were anesthetized with ketamine (15 mg/kg) + xilazine (0.5 mg/kg). Anesthetic darts containing ketamine (15 mg/kg), midazolam (1 mg/kg), and levomepromazine (1 mg/kg), or alternatively, a combination of tiletamine and zolazepam (4–5 mg/kg) were used for the *Alouatta* and *Brachyteles* genera, as well as for one titi monkey (*Callicebus*) [21,24]. Sick animals reported by the information network during the 2017–2018 yellow fever epizooties [25] were captured with nets [21]. A sample of 3–6 mL of blood was collected from the anesthetized or recently dead animals. Thick and thin blood films were immediately prepared, and the remaining blood was allowed to coagulate. After collections and the complete recovery from anesthesia (2–3 h), they were released to their habitats during the daytime from where they were captured. Liver samples were obtained from dead animals, which recently died due to yellow fever or any other disease. Liver and blood samples were stored at −80˚C until DNA extraction. Importantly, only one monkey was injured during the fall post anesthesia, which was then treated and kept in a primate-breeding center (Centro de Primatologia do Rio de Janeiro—CPRJ) [21].

Malaria diagnosis

Giemsa's solution stained thick and thin blood films were examined under a microscope with a 100× oil-immersion objective by two trained and independent microscopists. DNA was extracted from the blood clots as previously described [26] and from the liver samples [27] using the QIAGEN DNeasy mini kit according to manufacturer's instructions. Molecular diagnosis was made via conventional PCR. All DNA samples were tested in triplicate for 18S rRNA *Plasmodium* genus-specific gene [28,29], and then for cysteine proteinase *P. vivax* and ssrRNA *P. malariae* and *P. falciparum* genes, as previously described [29,30]. The sensitivity thresholds of the protocols used were 0.5, 0.019, 1.0, and 1.0 parasite per μL for the *Plasmodium* genus, *P vivax*, *P. malariae*, and *P. falciparum* assays, respectively [28–30]. Moreover, an internal

control (betaglobin primers) was used to assess eventual enzyme inhibitors that could generate false negative results and all the samples generated betaglobin amplicons.

P. vivax-positive samples were subjected to *P. simium* differential diagnosis based on a mitochondrial SNP, the only genetic marker available to differentiate them [2,10]. The molecular diagnosis was performed via nested-PCR of *coxI* gene fragment and subsequent enzymatic digestion, using primers and a previously described protocol, which detected 3.12 parasites/μL [10]. All the PCR products were visualized under UV light after electrophoresis on 2% agarose gels.

Histopathological analysis

Spleen fragments of a subsample comprising 16 howler monkeys (12 from RJ and 4 from ES), presumably found dead due to yellow fever, were fixed in Carson's formalin-Millonig [31] and further processed according to the standard histological techniques for paraffin embedding. Sections (5-μm-thick) from each block were stained with hematoxylin–eosin [32] or Lennert Giemsa [33] and analyzed for malarial pigments under an AxioHome microscope equipped with an HRc Axiocam digital camera (Carl Zeiss, Germany).

Ethical issues

The collection methods, biosafety, and anesthesia protocols adhered to the Brazilian law (11.794 of July 8, 2008) on the use of animals in scientific research, and complied with the rules and regulations of Brazilian Ministry of Health [22], having been previously approved by the institutional Ethics Committee for Animal Experimentation of Instituto Oswaldo Cruz (protocol CEUA/IOC-004/2015, license L-037/2016) and by Brazilian Ministry of the Environment (SISBIO 41837–3 and 54707–4) and Rio de Janeiro's Environment agency (INEA 012/2016 and 019/2018). The research also adhered to the American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates.

Results

In total, we examined 146 animals belonging to six species from 30 counties in four Brazilian states, with majority of animals being from RJ (S1 Table and Fig 1); of these, 130 animals were screened by microscopy and PCR using blood samples, seven by microscopy and PCR from blood samples and histopathology of spleen fragments, and nine by PCR of viscera and histopathology of spleen fragments.

Despite their geographical origin, the only NHP species found to be infected with *Plasmodium* was the howler monkey *Alouatta guariba clamitans* (Table 1). The PCR method was more sensitive compared to the microscopic examination of blood films, which eventually failed to detect *Plasmodium* in two infected howler monkeys, one harboring *P. simium* and another *P. brasilianum/malariae* (Table 1). Nevertheless, the results suggest that infected howler monkeys generally exhibit detectable parasitemia during microscopic examination of blood slides, for both *Plasmodium* species. The parasitemia ranging from 15–300 parasites/μL (median = 40 p/μL). Trophozoites were the most commonly visualized blood forms; however, schizonts and gametocytes were also detected (Fig 2). In addition, by using PCR, we were able to detect both *Plasmodium* species in four animals from liver, spleen and blood samples, which showed concordant diagnostic results.

Only 12 NHPs were examined from the bordering states of RJ, being two howler monkeys from MG and four from ES and none of these were infected by *P. simium*. One of the four examined *A. clamitans* from ES was PCR positive for *P. brasilianum/malariae* (25%; Table 1).

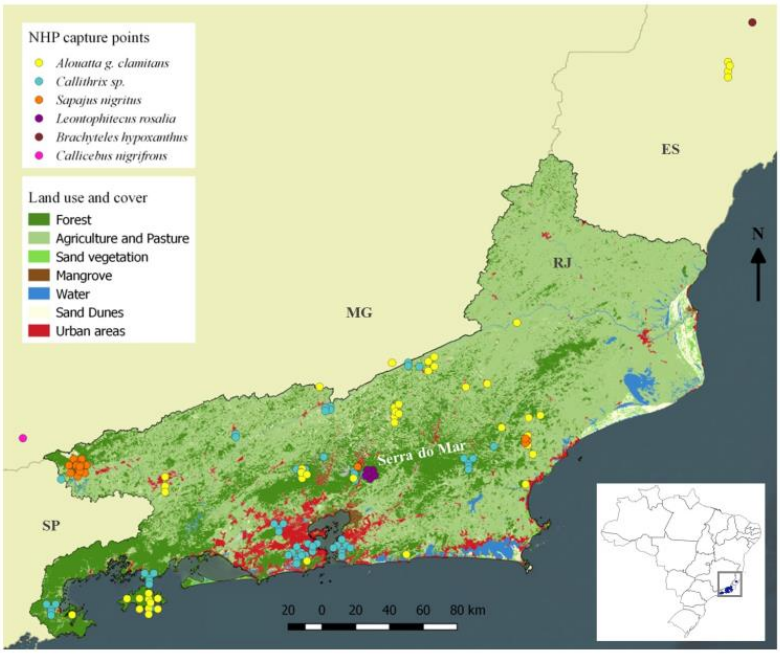

Fig 1. Map presenting collection points of non-human primates in Rio de Janeiro and bordering states in Brazil. Each circle represents one examined NHP. The figure was prepared using free software QGIS 2.18.

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Regarding RJ, 11 (26.1%) howler monkeys were infected with malarial parasites during sampling and, among these, seven (16.7%) were infected by *P. simium*, the causative agent of the autochthonous human malaria in this state (Tables 1 and 2). Importantly, the unique specific *P. simium* SNPs used to distinguish *P. simium* from *P. vivax* were detected in 100% of these tertian malarial parasite infected howler monkeys. Moreover, most of these animals originated from the coastal slope of Serra do Mar, where counties face the ocean and are influenced by its humidity, where several human cases have been recorded in the last decade (Table 2 and Fig 3). Six *A. g. clamitans* from RJ were infected by the quartan-malarial parasite *P.*

Table 1. *Alouatta g. clamitans* captured and examined per state, with the infection rate for each *Plasmodium* species and detection method for the present and previous infections. Number (%).

State	N	Total with *Plasmodium*	*P. simium*	*P. brasilianum* / *malariae*	*P. simium* and *P. brasilianum*/ *malariae*	*P. falciparum*	Diagnosis		Malarial Pigment[$]	
							Blood slides + PCR	Only PCR	N	Positive
RJ	42	11 (26.1)	5 (11.9)	4 (9.5)	2 (4.7)	-	7	4*	12	6 (50)
ES	4	1 (25.0)	-	1 (25.0)	-	-	NR	1	4	2 (50)
MG	2	-	-	-	-	-	-	-	-	-
TOT.	**48**	**12 (25.0)**	**5 (10.4)**	**5 (10.4)**	**2 (4.1)**	**-**	**7**	**5**	**16**	**8 (50)**

*Two harboring *P. simium* and two with *P. malariae/brasilianum*. NR: not realized, as they were found dead during a yellow fever outbreak.

[$]Search in spleen tissues in a subsample comprising dead animals.

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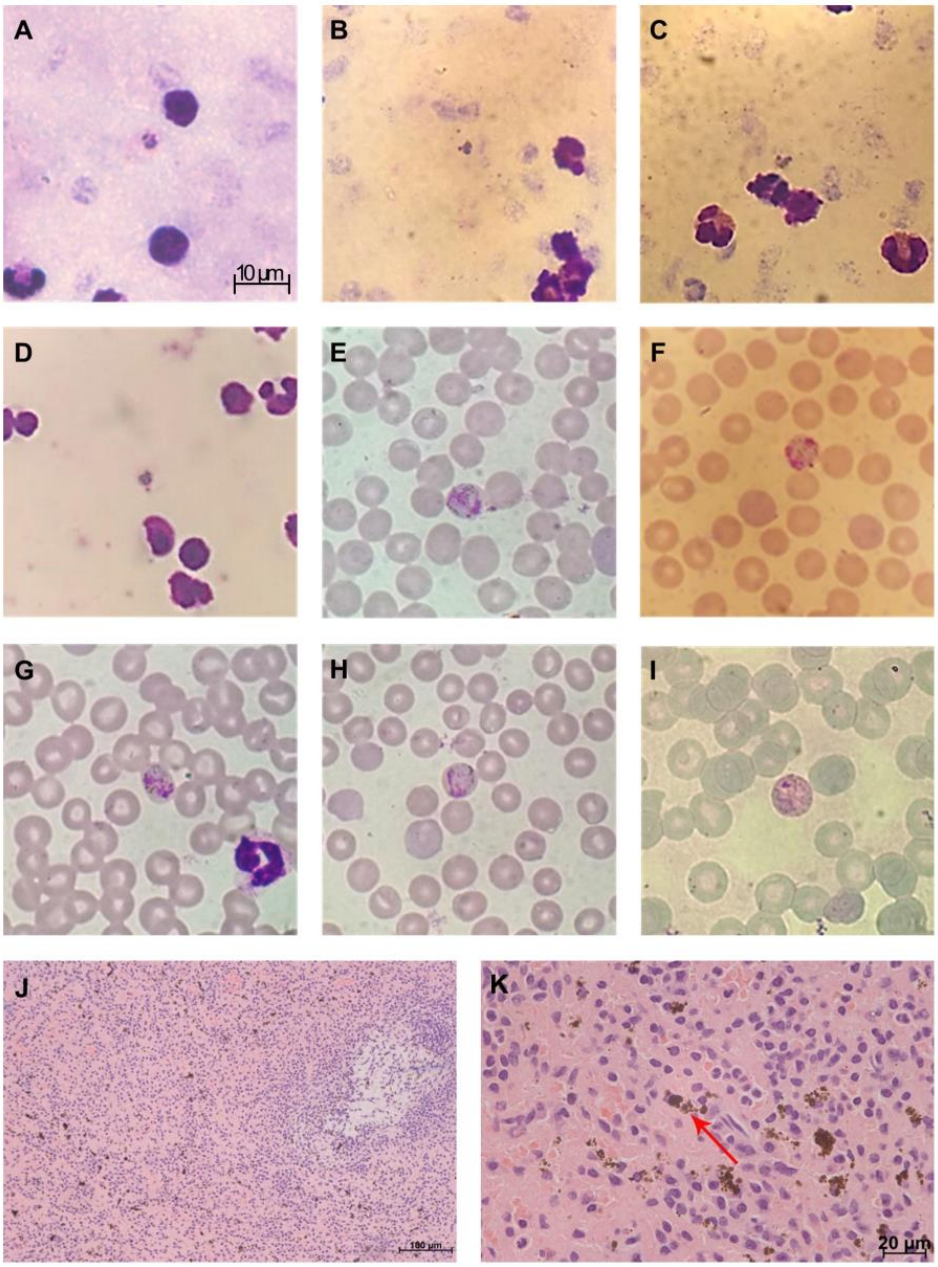

Fig 2. Giemsa's solution-stained thick (A-D) and thin (E-I) blood samples, and histopathological analysis of hematoxylin-eosin-stained spleen fragments of howler monkeys that were naturally infected with *Plasmodium* in Rio de Janeiro state, Brazil, presenting (J) hypertrophy of red pulp with malarial pigments and white pulp atrophy and (K) details of malarial pigments in the red pulp.

https://doi.org/10.1371/journal.pntd.0007906.g002

Table 2. ***Plasmodium*-positive howler monkeys, based on their plasmodial species, county, year, slope of capture, and occurrence of autochthonous human cases of benign tertian malaria, recorded in the respective county and the year of detection in Rio de Janeiro.**

Plasmodium infections in NHP					Previous NHP *Plasmodium* infection[$]	Human "*vivax*-like" cases	
Serra do Mar Slope	County	NP (%)	Parasitemia (p/mm^3)	*Plasmodium* species		N	Year
Coastal	Miguel Pereira	2 (50)	40 300	*P. brasilianum* *P. simium* + *P. brasilianum*	0 of 1	10	2015–2017
	Macaé	1 (16.6)	25	*P. simium*	3 of 3	12	2011, 2013, 2015–2017
	Petrópolis	1 (100)	0	*P. simium*	NA	3	2015–2016
	Angra dos Reis	4 (40)	0 NR 250 NR	*P. brasilianum* *P. brasilianum* *P. simium* *P. simium*	1 of 4	3	2015, 2017
Continental	Teresópolis*	1 (33.3)	40	*P. brasilianum*	NA	0	_
	Sumidouro	2 (100)	15	*P. simium*	NA	0	_
			240	*P. simium* + *P. brasilianum*	NA	0	_

NP = number of *Plasmodium* positive howler monkeys.

[$]Eight howler monkeys were found dead due to yellow fever virus (YFV), with *Plasmodium*-negative results (PCR and/or blood slides) in three counties where *Plasmodium*-positive howler monkeys were found. Histological preparations of spleen fragments revealed malarial pigment in four (50%) of these PCR-negative animals, suggesting previous infections.

*The *P. brasilianum* infection was found in the district of Água Quente, in the continental side of Teresópolis. NA = viscera non available.

https://doi.org/10.1371/journal.pntd.0007906.t002

brasilianum/malariae (14.3%), of which two were co-infected with *P. simium* (Tables 1 and 2). All samples were negative for *P. falciparum* parasites.

Previous malaria infections could be investigated by searching the malarial pigment in a subsample of 16 dead howler monkeys. Accordingly, the malarial pigment (Fig 2) was detected in the spleen fragments of five out of 13 animals with negative PCR at the time of death and, as expected, in three animals with positive PCR (Table 1). Interestingly, this pigment was found in the spleen samples of 50% (eight out of 16) of dead howler monkeys in both RJ (six of 12) and ES (two of four), indicating that malarial parasite is frequent in monkeys from both states.

Howler monkeys were examined from 15 counties in RJ, of which 6 counties presented records of autochthonous human malaria in the last years. Present infections by *P. simium* were diagnosed in howlers from four (66.6%) of the surveyed counties reporting human cases of benign tertian malaria in the state, and in a neighboring county (Sumidouro) where the human cases were never detected (Fig 3). Interestingly, in Macaé, where the highest number of human cases was recorded, all dead howler monkeys had malarial pigments in their spleen, suggesting that simian malaria is highly enzootic in that county (Table 2).

Discussion

The present study demonstrates an unprecedented capture effort and large-scale field survey of plasmodial species in NHPs in RJ, a state recording a three-decade history of autochthonous human cases of benign tertian malaria [1,2]. To our knowledge, this is the first study to describe the NHP infection rates by *Plasmodium*, and match the spatial distribution of *P. simium* in NHP with the local human cases previously recorded; howler monkeys were the only confirmed reservoirs of this zoonotic malaria in the state, and the presence of specific SNPs was demonstrated in all *P. simium* infected howler monkeys, despite their geographical

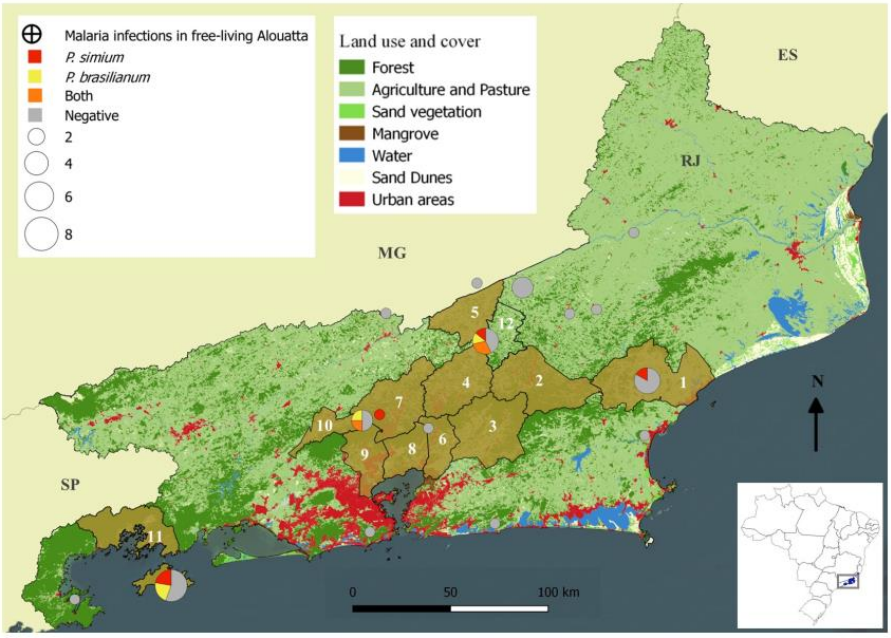

Fig 3. Map presenting the distribution, number, and *Plasmodium* infections of the examined *Alouatta g. clamitans* in Rio de Janeiro. Brown shaded areas represent the counties with registered autochthonous malaria in humans: 1. Macaé, 2. Nova Friburgo, 3. Cachoeira de Macacu, 4. Teresópolis, 5. Sapucaia, 6. Guapimirim, 7. Petrópolis, 8. Magé, 9. Duque de Caxias 10. Miguel Pereira, 11. Angra dos Reis. Number 12 represents Sumidouro, where human cases were not detected. The figure was prepared using free software QGIS 2.18.

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origin. Although *P. brasilianum/malariae* has already been found in captive NHPs from RJ [13], this is the first study to demonstrate that this parasite species was located in free-living NHPs, thereby contributing to its widespread distribution and zoonotic potential in the state.

P. simium and *P. vivax* have similar morphologies [5], immune response [34], and several genetic targets [35,36], for example, PCR based on 18S SSU rRNA, largely used for malaria diagnosis in humans [30], was unable to distinguish these parasites [10,13]. The only genetic markers that can differentiate *P. simium* from *P. vivax* are the SNPs (3535 T>C and 3869 A>G) in the 6 Kb region of the mitochondrial genome [2]. Excluding the taxonomic issue and focusing on public health impacts, our results confirm that, to date, the *P. simium* specific SNP is carried by all parasites isolated from NHPs in the Atlantic Forest, which is in accordance with the study by Alvarenga et al. (2018) [10]. Furthermore, *P. simium* was detected in howler monkeys captured in five out of 11 counties recently reporting autochthonous human malaria cases in RJ (Fig 2). Despite numerous efforts, we failed in capturing howler monkeys in four malarial foci due to some local hindrances such as the hunting pressure that scared the monkeys, steep terrains, and low *A. g. clamitans* population densities [21,37]. Nevertheless, the strong geographical overlap of howler monkey and human infections by parasites displaying specific *P. simium* SNPs in five of six malarial foci, strengthens the importance of howler monkeys as the main reservoir of benign tertian human malaria over the zoonotic transmission areas in Southeast Brazil [4,5,38,39]. Howler monkeys have also been by far the NHPs most commonly parasitized with both *P. simium* and *P. brasilianum* in Southern and Southeastern

Brazil [5,40]. Besides their susceptibility to *Plasmodium* infection [5], their acrodendrophilic behavior, huge bodies, and the slower movement through the tree canopies (compared to the smaller monkeys) [41] may make them more prone to mosquito biting vectors. Importantly, the detection of malarial pigment in spleen fragments of eight (50%) of the subsample consisting of 16 howler monkeys from RJ and ES, some of which were *Plasmodium*-negative by PCR at the time of death, suggest that simian malaria is very frequent in this species. Indeed, in some areas with the highest human cases (e.g., Macaé), four out of six (66.6%) howler monkeys were exposed to *Plasmodium* sp., when considering those with present and past malarial infections. This finding may also suggest that spontaneous healing from malarial infections may occur in the howler monkeys found in nature, as observed in the *P. simium* experimental infections in certain Neotropical NHPs [42] and in a human natural infection [7].

The frequency of *P. simium* infection in free-living howler monkeys in RJ (16.6%) was higher than that previously found in the bordering state of SP (5.8%) [39]; however, it was lower than that found in the entire South and Southeast Brazilian regions (26.3–35%) [5,43]. No previous data are available for comparison in RJ, as this was the first time that howler monkeys were captured in the state in a systematized manner. *A. g. clamitans* was also the only free-living NHP from RJ in which blood forms by microscopy and plasmodial DNA were detected. Recently, DNA but not blood forms of *P. simium* was detected in captive *Cebus* and *Sapajus* from the Southeast [27]. The parasite could be unable to establish the erythrocytic cycle or erythrocytic infection could be transitory, due to unspecificity during cell invasion or host immune competence. Thus, their role as a reservoir for zoonotic malaria in the region remains unclear [27]. *P. brasilianum* DNA was detected in captive capuchin, titi, howler, and owl monkeys, as well as in tamarins and marmosets [13,27], most of these being exotic species, that are not found in RJ. All these NHPs were confined in a breeding institution CPRJ located in a well-known simian malarial enzootic transmission area in RJ. Therefore, it was suspected that the local ecological conditions favored the accidental infection of these captive NHPs by parasites carried from infected free-living howler monkeys. Moreover, one free living specimen was infected by *P. simium* near CPRJ [27]; however, no evidence is available confirming if the parasite DNA found in the blood of these captive animals implies that they really undergo *Plasmodium* infections or only bear a transient parasitemia. Nevertheless, it is important to continuously monitor their potential role as a zoonotic *Plasmodium* reservoir, besides howler monkeys.

Although *P. brasilianum* has been found in several NHP genera [5,44–48] around other Brazilian regions, the previous studies conducted in Brazilian Atlantic forest and Cerrado biomes did not find any capuchin (56 examined) or marmosets (out of 44) infected with *Plasmodium* [39]. Similarly, more than 270 marmosets and lion-tamarins from the Southeast region were *Plasmodium* negative [5]. Splenectomized capuchins remained uninfected when injected with *P. simium* infected blood, whereas splenectomized marmoset endured low parasitemia [42]. Thus, the epidemiological role of other NHPs besides howler monkeys in the zoonotic transmission of malaria in Southeast Brazil, including the RJ state, if any, is presumably negligible.

P. brasilianum/malariae was found in a frequency similar to that of *P. simium*, six (14.3%) versus seven (16.7%), respectively–in howler monkeys from RJ, and mixed infections were recorded in two (4.6%) animals. *P. brasilianum/malariae* was the only malarial parasite detected in *A. clamitans* from ES (Table 1). Besides, the geographical co-occurrence of these parasites seems to be frequent in RJ, as it was revealed in three out of five counties, wherein howler monkeys were detected with the malarial parasites (Fig 2). Interestingly, despite this coincident distribution and similar frequency of *P. brasilianum/malariae* and *P. simium* in RJ, autochthonous human cases in this state have been diagnosed microscopically and/or

molecularly as benign tertian malaria due to *P. vivax* for decades [1,5,7,38,49,50]. In particular, *P. simium* was only identified by molecular tests and DNAmt sequencing as the causative agent in the 2015–2016 malaria outbreak in RJ, wherein the patients were essentially nonresidents of foci [2]. Nevertheless, six human asymptomatic infection by *P. malariae* were detected by PCR in residents of Guapimirim in RJ, in 2011 [19], and a subsample of reactive local individuals for any plasmodial species revealed antibodies against erythrocytic antigens of *P. malariae* in 30.9%. The hypothesis of infection of NHP origin due to *P. brasilianum* was proposed because no index case of introduced or imported human case of *P. malariae* was identified in Guapimirim, and because the cases had close contact with the Atlantic forest [19]. Similar situations have been reported in neighboring states, covered by the Atlantic forest, such as SP and ES [3,4,11,51–54]. Noteworthy, *P. brasilianum* is a widespread and common simian malarial parasite in the Amazon [5,14] that is experimentally found to infect humans, either by inoculation of parasitized monkey blood or by the bite of infected mosquitoes [9]. High prevalence of antibodies against sporozoites antigens and erythrocytic forms of *P. brasilianum/malariae* in people living or frequently working in the Amazon forests (e.g., Indians, miners, settlers) of Brazil, French Guiana, and Venezuela suggested infection of this simian quartan malaria parasite in humans [55–57]. Infections by *P. brasilianum/malariae* in humans would be, therefore, expected to occur in RJ and other southeast states where *P. simium* has been described. In particular, the natural vector of both parasites is the same (*An. cruzii*) [5]. However, it remains unclear why malaria cases due to *P. brasilianum/malariae* have not been consistently reported in the state. Further, strengthening of malaria surveillance either in residents or visitors of the Atlantic forest to evaluate the zoonotic potential of *P. brasilianum/malariae* in South and Southeast Brazil is recommended [1].

Noteworthy, most of the *P. simium* and *P. brasilianum/malariae*-infected howler monkeys (eight of 11;73%) were from the forest coastal slope of Serra do Mar, where all autochthonous human malaria cases have been acquired [2,19]. At least two main premises may explain this geographical association: from the entomological and climatic view point, the higher relative humidity in the costal slope [58,59] may increase *An. cruzii* survival rates, supporting the sporogonic cycle of the *Plasmodium*. Sea moisture also favors the density of epiphyte shade bromeliads, the larval habitat of *An. cruzii*, and generates higher rainfall indexes [58,59], which in turn increases the amount of water accumulated in the vector larval habitats, positively influencing the mosquito density. Greater longevity and density directly influence the vector capacity of the mosquito to transmit *Plasmodium* [60,61]. Vector competence is governed by genetics of vector population, and therefore, influences *Plasmodium* transmission dynamics [61,62]. Indeed, Deane (1992) has emphasized that environmental conditions highly influence the presence and densities of Neotropical NHP hosts, bromeliads, and *An. cruzii*, and consequently, define the occurrence or absence of simian malaria in nearby sites. Moreover, two genetic lineages of *An. cruzii* with partial reproductive isolation have been recently described in Serra do Mar, one curiously occurring in the coastal region and another in the continental slopes [63]. Compared to the continental side, coastal slopes of Serra do Mar comprise a higher number of sites where people from major cities choose to reside in country houses in the forest and include ecotourism areas such as waterfalls and natural parks attracting many visitors (personal observation). As elaborated, the autochthonous human cases in the Atlantic forest in RJ have been reported mainly in the nonresidents [1,2,19]. Collectively, the environmental, entomological, ecological, and epidemiological characteristics seem to indicate that the costal slope of Serra do Mar is the riskiest place to acquire malaria of NHP origin. Protective measures such as the use of repellents and long clothes should be encouraged specifically for those who live or practice ecotourism in this slope.

During the present study, a YFV outbreak erupted in the Southeast Brazil, a region without records of this virus presence for almost 80 years [25,64]. Hundreds of epizootics of NHPs were reported, causing a significant impact on the population size of howler monkeys, an extremely susceptible host to YFV [22,65–70]. Considering the role of the howler monkeys as a reservoir of *Plasmodium* infective to humans, it is plausible to suppose that dynamics of zoonotic transmission of malaria will undergo short or mid-term changes in RJ and bordering states affected by the YFV epizooties. In this context, we postulate that the rapid decrease of *Alouatta* populations would also reduce the source of plasmodial infection to *An. cruzii*, which would further hamper the circulation of *Plasmodium sp*. in the Atlantic forest. Despite the short duration since the 2016–2018 YFV epizootics, records from the Brazilian Ministry of Health surveillance program seem to confirm this scenario. In fact, there was an abrupt drop in the human malaria case records between 2018 and 2019 (only one autochthonous case) [71], which was contradictory with the numbers reported between 2006 and 2014, when 4.7 cases were registered per year, on an average [2]. If the reduction of autochthonous malarial cases in the Atlantic Forest is a consequence of the *Alouatta* deaths, leading to plasmodial sources reduction, the role of howler monkeys for the occurrence of malaria in the Atlantic Forest would be reinforced.

Previous sampling efforts on examining free-living NHPs in the Southeastern Atlantic Forest over the last 30 years revealed limited geographical coverage, with samplings essentially limited to the wildlife rescues or carried out in areas close to cities, or were based on few individuals [4,38,39]. As a result, our data contribute in understanding the simian malarial parasite distribution and frequency as well as the zoonotic characteristics of autochthonous human malaria in RJ, which in turn provides assistance in shaping surveillance and control. The evidence of the NHP origin of parasites infecting humans and the widespread occurrence of anophelines vectors in the Southeast region increased the concern of the reemergence of endemic or epidemic autochthonous transmission in the region independent of the enzootic cycle [2]. However, it is not clear whether the parasitemic humans infected by the bite of *An. cruzii* carrying esporozoites of *P. simium* acquired from howler monkeys could be a source of infection to *An. cruzii* or any other malarial vector present in the region. It is known that *P. simium* infected humans usually display scanty to null parasitemia, and can be cured spontaneously in few days without treatment and any relapse; moreover, the molecular detection of parasites during treatment follow-ups has been described [2,5]. Besides, all autochthonous human cases of benign tertian malaria detected for decades in Southeast have reported recent contact with the *P. simium* enzootic forest, and no secondary transmission directly derived from a human infected in the zoonotic cycle has ever been detected outside the sylvatic foci. These epidemiological and parasitological profiles appear to indicate that humans are not a source of *P. simium* infection for mosquitoes. Thus, determining vector competence of *An. cruzii* and other traditional human malaria vector occurring in the Southeast region (e.g., *An. darlingi*, *An. Aquasalis*, and *An. albitarsis*) for transmitting *P. simium* and *P. brasilianum* between humans and from NHPs and humans and vice-versa is imperative.

Supporting information

S1 Table. Number of examined NHPs, by species, habitat, and capture method.
(DOCX)

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References

1. Pina-Costa A de, Brasil P, Santi SM Di, Araujo MP de, Suárez-Mutis MC, Santelli ACF e S, et al. Malaria in Brazil: what happens outside the Amazonian endemic region. Mem Inst Oswaldo Cruz. 2014; 109: 618–633. https://doi.org/10.1590/0074-0276140228 PMID: 25185003
2. Brasil P, Zalis MG, de Pina-Costa A, Siqueira AM, Júnior CB, Silva S, et al. Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. Lancet Glob Heal. 2017;5. https://doi.org/10.1016/S2214-109X(17)30333-9
3. Curado I, dos Santos Malafronte R, de Castro Duarte AMR, Kirchgatter K, Branquinho MS, Bianchi Galati EA. Malaria epidemiology in low-endemicity areas of the Atlantic Forest in the Vale do Ribeira, São Paulo, Brazil. Acta Trop. 2006; 100: 54–62. https://doi.org/10.1016/j.actatropica.2006.09.010 PMID: 17126279
4. Cerutti C, Boulos M, Coutinho AF, Hatab M do CL, Falqueto A, Rezende HR, et al. Epidemiologic aspects of the malaria transmission cycle in an area of very low incidence in Brazil. Malar J. 2007; 6: 33. https://doi.org/10.1186/1475-2875-6-33 PMID: 17371598
5. Deane LM. Simian malaria in Brazil. Mem Inst Oswaldo Cruz. 1992; 87: 1–20. https://doi.org/10.1590/S0074-02761992000700001 PMID: 1343676
6. Fonseca F. Plasmódio de primata do Brasil. Mem Inst Oswaldo Cruz. 1951. pp. 543–553. https://doi.org/10.1590/s0074-02761951000100008 PMID: 14890537
7. Deane LM, Deane MP, Ferreira Neto J. Studies on transmission of simian malaria and on a natural infection of man with *Plasmodium simium* in Brazil. Bull World Health Organ. 1966; 35: 805–8. PMID: 5297817
8. Deane LM, Ferreira Neto JA, Lima MM, Deane LM, Ferreira Neto JA, Lima MM. The vertical dispersión of Anopheles (Kerteszia) cruzi in a forest in southern Brazil suggests that human cases of malaria of simian origin might be expected. Mem Inst Oswaldo Cruz. 1984; 79: 461–463. https://doi.org/10.1590/S0074-02761984000400011 PMID: 6533421
9. Coatney GR. The Simian Malaria: Zoonoses, Anthroponoses, or Both? Am J Trop Med Hyg. 1971; 20: 795–803. https://doi.org/10.4269/ajtmh.1971.20.795 PMID: 5002245
10. de Alvarenga DAM, Culleton R, de Pina-Costa A, Rodrigues DF, Bianco C, Silva S, et al. An assay for the identification of Plasmodium simium infection for diagnosis of zoonotic malaria in the Brazilian Atlantic Forest. Sci Rep. 2018; 8: 86. https://doi.org/10.1038/s41598-017-18216-x PMID: 29311638
11. Guimarães LO, Bajay MM, Wunderlich G, Bueno MG, Röhe F, Catão-Dias JL, et al. The genetic diversity of *Plasmodium malariae* and *Plasmodium brasilianum* from human, simian and mosquito hosts in Brazil. Acta Trop. 2012; 124: 27–32. https://doi.org/10.1016/j.actatropica.2012.05.016 PMID: 22705349
12. Rylands AB, Mittermeier RA, Silva JS. Neotropical primates: taxonomy and recently described species and subspecies. Int Zoo Yearb. 2012; 46: 11–24. https://doi.org/10.1111/j.1748-1090.2011.00152.x
13. Alvarenga DAM, Pina-Costa A, Bianco C, Moreira SB, Brasil P, Pissinatti A, et al. New potential Plasmodium brasilianum hosts: tamarin and marmoset monkeys (family Callitrichidae). Malar J. 2017; 16: 71. https://doi.org/10.1186/s12936-017-1724-0 PMID: 28187764
14. Lourenço-de-Oliveira R, Deane LM. Simian malaria at two sites in the Brazilian Amazon. I—The infection rates of Plasmodium brasilianum in non-human primates. Memórias do Instituto Oswaldo Cruz. 1995. pp. 331–339. https://doi.org/10.1590/s0074-02761995000300004 PMID: 8544737
15. Eyles DE. The Species of Simian Malaria: Taxonomy, Morphology, Life Cycle, and Geographical Distribution of the Monkey Species. J Parasitol. 1963; 49: 866–87. https://doi.org/10.2307/3275712 PMID: 14084190
16. Lourenço-de-Oliveira R, Ziccardi M. Natural infection of Golden lion tamarin, Leontopithecus rosalia, with Trypanosoma cruzi, in the state of Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 1990; 85: 15.
17. Paglia AP, Rylands AB, Herrmann G, Aguiar LMS, Chiarello AG, Leite YLR, et al. Lista anotada dos mamiferos do Brasil, segunda edicao. 2nd ed. Arlington: Conservation International; 2012.
18. Guimarães AE, Arlé M, Machado RNM. Mosquitos no Parque Nacional da Serra dos Órgãos, Estado do Rio de Janeiro, Brasil. II. Distribuição vertical. Mem Inst Oswaldo Cruz. 1985; 80: 171–85.
19. Miguel RB, Albuquerque HG, Sanchez MCA, Coura JR, Santos S da S, Silva S da, et al. Asymptomatic Plasmodium infection in a residual malaria transmission area in the Atlantic Forest region: Implications for elimination. Rev Soc Bras Med Trop. 2019; 52: 1–9. https://doi.org/10.1590/0037-8682-0537-2018 PMID: 30942262
20. Ribeiro MC, Metzger JP, Martensen AC, Ponzoni FJ, Hirota MM. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. Biol Conserv. 2009; 142: 1141–1153. https://doi.org/10.1016/J.BIOCON.2009.02.021

21. Abreu FVS, dos Santos E, Gomes MQ, Vargas WP, Oliveira Passos PH, Nunes e Silva C, et al. Capture of *Alouatta guariba clamitans* for the surveillance of sylvatic yellow fever and zoonotic malaria: Which is the best strategy in the tropical Atlantic Forest? Am J Primatol. 2019; 81: e23000. https://doi.org/10.1002/ajp.23000 PMID: 31192493

22. Brasil. Guia de vigilância de epizootias em primatas não humanos e entomologia aplicada à vigilância da febre amarela. 2nd ed. Brasília: Ministério da Saúde; 2017.

23. Santos Sales I dos, Ruiz-Miranda CR, Santos C de P. Helminths found in marmosets (Callithrix penicillata and Callithrix jacchus) introduced to the region of occurrence of golden lion tamarins (Leontopithecus rosalia) in Brazil. Vet Parasitol. 2010; 171: 123–129. https://doi.org/10.1016/j.vetpar.2010.02.044 PMID: 20378249

24. Scott NJ, Scott AF, Malmgren LA. Capturing and marking howler monkeys for field behavioral studies. Primates. 1976; 17: 527–533. https://doi.org/10.1007/BF02382910

25. Possas C, Lourenço-de-oliveira R, Tauil PL, Pinheiro FDP, Pissinatti A, Venâncio R, et al. Yellow fever outbreak in Brazil: the puzzle of rapid viral spread and challenges for immunisation. 2018; 113: 1–12. https://doi.org/10.1590/0074-02760180278

26. de Abreu FVS, Gomes LR, Mello ARL, Bianco-Júnior C, de Pina-Costa A, dos Santos E, et al. Frozen blood clots can be used for the diagnosis of distinct *Plasmodium* species in man and non-human primates from the Brazilian Atlantic Forest. Malar J. 2018; 17: 338. https://doi.org/10.1186/s12936-018-2485-0 PMID: 30249260

27. de Alvarenga D, de Pina-Costa A, de Sousa T, Pissinatti A, Zalis MG, Suaréz-Mutis MC, et al. Simian malaria in the Brazilian Atlantic forest: first description of natural infection of capuchin monkeys (Cebinae subfamily) by Plasmodium simium. Malar J. 2015; 14: 81. https://doi.org/10.1186/s12936-015-0606-6 PMID: 25889933

28. Gama BE, Silva-Pires F do ES, Lopes MNR, Cardoso MAB, Britto C, Torres KL, et al. Real-time PCR versus conventional PCR for malaria parasite detection in low-grade parasitemia. Exp Parasitol. 2007; 116: 427–432. https://doi.org/10.1016/j.exppara.2007.02.011 PMID: 17433301

29. Torres KL, Figueiredo D V., Zalis MG, Daniel-Ribeiro CT, Alecrim W, Ferreira-da-Cruz M de F. Standardization of a very specific and sensitive single PCR for detection of *Plasmodium vivax* in low parasitized individuals and its usefulness for screening blood donors. Parasitol Res. 2006; 98: 519–524. https://doi.org/10.1007/s00436-005-0085-8 PMID: 16416292

30. Snounou G, Viriyakosol S, Zhu Xin Ping, Jarra W, Pinheiro L, do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993; 61: 315–320. https://doi.org/10.1016/0166-6851(93)90077-b PMID: 8264734

31. Carson FL, Martin JH, Lynn JA. Formalin Fixation for Electron Microscopy: A Re-evaluation. Am J Clin Pathol. 1973; 59: 365–373. https://doi.org/10.1093/ajcp/59.3.365 PMID: 4119901

32. Mayer P. Die Caprellidae der Siboga-Expedition. Leiden, Buchhandlung und druckerei vormals E.J. Brill; 1903.

33. Lennert K. Methodologic Prerequisites for a Differential Diagnosis of Lymphomas. Malignant Lymphomas Other than Hodgkin's Disease. Berlin, Heidelberg: Springer Berlin Heidelberg; 1978. pp. 73–82. https://doi.org/10.1007/978-3-642-81092-3_2

34. Barnwell J. Antigens of *Plasmodium vivax* blood stage parasites identifiedby monoclonal antibodies. Mem Inst Oswaldo Cruz. 1986; 81: 56–61.

35. Tazi L, Ayala FJ. Unresolved direction of host transfer of *Plasmodium vivax* v. *P. simium* and *P. malariae* v. *P. brasilianum*. Infect Genet Evol. 2011; 11: 209–221. https://doi.org/10.1016/j.meegid.2010.08.007 PMID: 20849978

36. Escalante AA, Barrio E, Ayala FJ. Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. Mol Biol Evol. 1995; 12: 616–626. https://doi.org/10.1093/oxfordjournals.molbev.a040241 PMID: 7659017

37. Araújo RM, Souza MB, Ruiz-Miranda CR. Densidade e tamanho populacional de mamíferos cinegéticos em duas Unidades de Conservação do Estado do Rio de Janeiro, Brasil. Iheringia Série Zool. 2008; 98: 391–396.

38. Buery JC, Rodrigues PT, Natal L, Salla LC, Loss AC, Vicente CR, et al. Mitochondrial genome of Plasmodium vivax/simium detected in an endemic region for malaria in the Atlantic Forest of Espírito Santo state, Brazil: do mosquitoes, simians and humans harbour the same parasite? Malar J. 2017; 16: 437. https://doi.org/10.1186/s12936-017-2080-9 PMID: 29084553

39. de Castro Duarte AMR, Malafronte R dos S, Cerutti C, Curado I, de Paiva BR, Maeda AY, et al. Natural Plasmodium infections in Brazilian wild monkeys: Reservoirs for human infections? Acta Trop. 2008; 107: 179–185. https://doi.org/10.1016/j.actatropica.2008.05.020 PMID: 18620330

40. Yamasaki T, Duarte AMRC, Curado I, Summa MEL, Neves DVDA, Wunderlich G, et al. Detection of etiological agents of malaria in howler monkeys from Atlantic Forests, rescued in regions of São Paulo city, Brazil. J Med Primatol. 2011; 40: 392–400. https://doi.org/10.1111/j.1600-0684.2011.00498.x PMID: 21933192

41. Kowalewski MM, Garber PA, Cortés-Ortiz L, Urbani B, Youlatos D. Howler monkeys: behavior, ecology, and conservation. New York, NY: Springer; 2014. https://doi.org/10.1007/978-1-4939-1960-4

42. Deane LM. Studies on simian malaria in Brazil. Bull World Health Organ. 1964; 31: 752–3. PMID: 14278011

43. Costa DC, Cunha VP da, Assis GMP de, Souza Junior JC de, Hirano ZMB, Arruda ME de, et al. *Plasmodium simium*/*Plasmodium vivax* infections in southern brown howler monkeys from the Atlantic Forest. Mem Inst Oswaldo Cruz. 2014; 109: 641–653. https://doi.org/10.1590/0074-0276130578 PMID: 25099335

44. Lourenço-de-Oliveira R, Luz SLB. Simian malaria at two sites in the Brazilian Amazon—II. Vertical distribution and frequency of anopheline species inside and outside the forest. Mem Inst Oswaldo Cruz. 1996; 91: 687–694. https://doi.org/10.1590/s0074-02761996000600005 PMID: 9283645

45. Figueiredo MAP, Di Santi SM, Manrique WG, André MR, Machado RZ. Identification of Plasmodium spp. in Neotropical primates of Maranhense Amazon in Northeast Brazil. PLoS One. 2017; 12: 1–14. https://doi.org/10.1371/journal.pone.0182905 PMID: 28796820

46. Bueno MG, Catão-Dias JL, de Oliveira Laroque P, Arruda Vasconcellos S, Ferreira Neto JS, Gennari SM, et al. Infectious Diseases in Free-Ranging Blonde Capuchins, Sapajus flavius, in Brazil. Int J Primatol. 2017; 38: 1017–1031. https://doi.org/10.1007/s10764-017-9994-5

47. Araújo MS, Messias MR, Figueiró MR, Gil LHS, Probst CM, Vidal NM, et al. Natural Plasmodium infection in monkeys in the state of Rondônia (Brazilian Western Amazon). Malar J. 2013; 12: 1–8. https://doi.org/10.1186/1475-2875-12-1

48. Bueno MG, Rohe F, Kirchgatter K, Di Santi SMF, Guimarães LO, Witte CL, et al. Survey of Plasmodium spp. in free-ranging neotropical primates from the brazilian Amazon region impacted by anthropogenic actions. Ecohealth. 2013; 10: 48–53. https://doi.org/10.1007/s10393-012-0809-z PMID: 23404035

49. Gomes ADC, De Paula MB, Duarte AMRDC, Lima MA, Malafronte RDS, Mucci LF, et al. Epidemiological and ecological aspects related to malaria in the area of influence of the lake at Porto Primavera dam, in western São Paulo State, Brazil. Rev Inst Med Trop Sao Paulo. 2008; 50: 287–295. https://doi.org/10.1590/s0036-46652008000500008 PMID: 18949348

50. Brasil P, Costa A, Longo C, Silva S, Ferreira-da-Cruz M, Daniel-Ribeiro CT. Malaria, a difficult diagnosis in a febrile patient with sub-microscopic parasitaemia and polyclonal lymphocyte activation outside the endemic region, in Brazil. Malar J. 2013; 12: 402. https://doi.org/10.1186/1475-2875-12-402 PMID: 24200365

51. de Alencar FEC, Malafronte R dos S, Cerutti Junior C, Natal Fernandes L, Buery JC, Fux B, et al. Assessment of asymptomatic *Plasmodium* spp. infection by detection of parasite DNA in residents of an extra-Amazonian region of Brazil. Malar J. 2018; 17: 113. https://doi.org/10.1186/s12936-018-2263-z PMID: 29540186

52. de Alencar FEC, Malafronte R dos S, Cerutti C, Natal Fernandes L, Buery JC, Fux B, et al. Reassessment of asymptomatic carriers of Plasmodium spp. in an endemic area with a very low incidence of malaria in extra-Amazonian Brazil. Malar J. 2017; 16: 452. https://doi.org/10.1186/s12936-017-2103-6 PMID: 29121963

53. Lima GFMC, Arroyo Sanchez MC, Levi JE, Fujimori M, Da Cruz Caramelo L, Sanchez AR, et al. Asymptomatic infections in blood donors harbouring *Plasmodium*: an invisible risk detected by molecular and serological tools. Blood Transfus. 2018; 16: 17–25. https://doi.org/10.2450/2016.0118-16 PMID: 27893347

54. Hristov AD, Sanchez MCA, Ferreira JJB, Lima GFM de C, Inoue J, Costa-Nascimento M de J, et al. Malaria in pregnant women living in areas of low transmission on the southeast Brazilian Coast: molecular diagnosis and humoural immunity profile. Mem Inst Oswaldo Cruz. 2014; 109: 1014–1020. https://doi.org/10.1590/0074-0276140229 PMID: 25494466

55. de Arruda M, Nardin EH, Nussenzweig RS, Cochrane AH. Sero-epidemiological studies of malaria in Indian tribes and monkeys of the Amazon Basin of Brazil. Am J Trop Med Hyg. 1989; 41: 379–85. https://doi.org/10.4269/ajtmh.1989.41.379 PMID: 2508499

56. Volney B, Pouliquen J-F, De Thoisy B, Fandeur T. A sero-epidemiological study of malaria in human and monkey populations in French Guiana. Acta Trop. 2002; 82: 11–23. https://doi.org/10.1016/s0001-706x(02)00036-0 PMID: 11904099

57. Lalremruata A, Magris M, Vivas-Martínez S, Koehler M, Esen M, Kempaiah P, et al. Natural infection of Plasmodium brasilianum in humans: Man and monkey share quartan malaria parasites in the

Venezuelan Amazon. EBioMedicine. 2015; 2: 1186–1192. https://doi.org/10.1016/j.ebiom.2015.07.033 PMID: 26501116

58. Brito TT, Oliveira-Júnior JF, Lyra GB, Gois G, Zeri M. Multivariate analysis applied to monthly rainfall over Rio de Janeiro state, Brazil. Meteorol Atmos Phys. 2017; 129: 469–478. https://doi.org/10.1007/s00703-016-0481-x

59. Machado RL, Ceddia MB, Carvalho DF de, Cruz ES da, Francelino MR. Spatial variability of maximum annual daily rain under different return periods at the Rio de Janeiro state, Brazil. Bragantia. 2010; 69: 77–84. https://doi.org/10.1590/S0006-87052010000500009

60. Coutinho JO, Rachou R. Data on the biology and the malaria vector capacity of anophelines of subgroup Kerteszia under natural conditions. Rev Bras Malariol Doencas Trop. 1966; 18: 557–80. PMID: 5998435

61. Cohuet A, Harris C, Robert V, Fontenille D. Evolutionary forces on Anopheles: what makes a malaria vector? Trends Parasitol. 2010; 26: 130–136. https://doi.org/10.1016/j.pt.2009.12.001 PMID: 20056485

62. Beerntsen BT, James AA, Christensen BM. Genetics of mosquito vector competence. Microbiol Mol Biol Rev. 2000; 64: 115–37. https://doi.org/10.1128/mmbr.64.1.115-137.2000 PMID: 10704476

63. de Rezende Dias G, Fujii TTS, Fogel BF, Lourenço-de-Oliveira R, Silva-do-Nascimento TF, Pitaluga AN, et al. Cryptic diversity in an Atlantic Forest malaria vector from the mountains of South-East Brazil. Parasit Vectors. 2018; 11: 36. https://doi.org/10.1186/s13071-018-2615-0 PMID: 29335015

64. Bonaldo MC, Gómez MM, dos Santos AA, Abreu FVS de, Ferreira-de-Brito A, Miranda RM de, et al. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. Mem Inst Oswaldo Cruz. 2017; 112: 447–451. https://doi.org/10.1590/0074-02760170134 PMID: 28591405

65. Brasil. Monitoramento do Período Sazonal da Febre Amarela [Internet]. 2018 pp. 1–15. Available: http://portalarquivos2.saude.gov.br/images/pdf/2018/fevereiro/21/Informe-n14-FA-20fev18-c.pdf

66. Moreno ES, Agostini I, Holzmann I, Di Bitetti MS, Oklander LI, Kowalewski MM, et al. Yellow fever impact on brown howler monkeys (Alouatta guariba clamitans) in Argentina: A metamodelling approach based on population viability analysis and epidemiological dynamics. Mem Inst Oswaldo Cruz. 2015; 110: 865–876. https://doi.org/10.1590/0074-02760150075 PMID: 26517499

67. Almeida M., Dos Santos E, Da Cruz Cardoso J, Da Fonseca DF, Noll CA, Silveira VR, et al. Yellow fever outbreak affecting Alouatta populations in southern Brazil (Rio Grande do Sul State), 2008–2009. Am J Primatol. 2012; 74: 68–76. https://doi.org/10.1002/ajp.21010 PMID: 22020690

68. Araújo FAA, Ramos DG, Santos AL, Passos PH de O, Elkhoury ANSM, Costa ZGA, et al. Epizootias em primatas não humanos durante reemergência do vírus da febre amarela no Brasil, 2007 a 2009. Epidemiol e Serviços Saúde. 2011; 20: 527–536. https://doi.org/10.5123/S1679-49742011000400012

69. Bicca-marques JC, Calegaro-marques C, Rylands AB, Karen B, Mittermeier RA, Almeida MAB De, et al. Yellow fever threatens Atlantic Forest primates Júlio. Sci Adv. 2017; 1600946: 18–20.

70. Duchiade A. Brazilian Forests Fall Silent as Yellow Fever Decimates Threatened Monkeys. In: Scientific American [Internet]. 12 May 2018 [cited 12 May 2019]. Available: https://www.scientificamerican.com/article/brazilian-forests-fall-silent-as-yellow-fever-decimates-threatened-monkeys/

71. Datasus Ministério da Saúde. TabNet Win32 3.0: MALÁRIA—Casos confirmados Notificados no Sistema de Informação de Agravos de Notificação—Rio de Janeiro [Internet]. [cited 12 May 2019]. Available: http://tabnet.datasus.gov.br/cgi/tabcgi.exe?sinannet/cnv/malarj.def

4.4 Artigo 4: *Haemagogus leucocelaenus* and *Haemagogus janthinomys* are the primary vectors in the major yellow fever outbreak in Brazil, 2016–2018.

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Haemagogus leucocelaenus and *Haemagogus janthinomys* are the primary vectors in the major yellow fever outbreak in Brazil, 2016–2018

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ABSTRACT

The yellow fever virus (YFV) caused a severe outbreak in Brazil in 2016–2018 that rapidly spread across the Atlantic Forest in its most populated region without viral circulation for almost 80 years. A comprehensive entomological survey combining analysis of distribution, abundance and YFV natural infection in mosquitoes captured before and during the outbreak was conducted in 44 municipalities of five Brazilian states. In total, 17,662 mosquitoes of 89 species were collected. Before evidence of virus circulation, mosquitoes were tested negative but traditional vectors were alarmingly detected in 82% of municipalities, revealing high receptivity to sylvatic transmission. During the outbreak, five species were found positive in 42% of municipalities. *Haemagogus janthinomys* and *Hg. leucocelaenus* are considered the primary vectors due to their large distribution combined with high abundance and natural infection rates, concurring together for the rapid spread and severity of this outbreak. *Aedes taeniorhynchus* was found infected for the first time, but like *Sabethes chloropterus* and *Aedes scapularis*, it appears to have a potential local or secondary role because of their low abundance, distribution and infection rates. There was no evidence of YFV transmission by *Aedes albopictus* and *Aedes aegypti,* although the former was the most widespread species across affected municipalities, presenting an important overlap between the niches of the sylvatic vectors and the anthropic ones. The definition of receptive areas, expansion of vaccination in the most affected age group and exposed populations and the adoption of universal vaccination to the entire Brazilian population need to be urgently implemented.

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KEYWORDS Yellow fever; Atlantic forest; *Haemagogus*; *Aedes*; *Sabethes*

Introduction

Yellow fever (YF) is a viral disease that decimated populations and harmed commercial routes in the Americas in the nineteenth century and continues to induce a heavy public health burden by annually causing thousands of cases and deaths in Africa and South America despite the existence of effective vaccines [1,2]. The etiological agent of this disease is the yellow fever virus (YFV), which has originated in Africa and spread to the Americas and the Caribbean probably during the seventeenth—nineteenth centuries. The discovery that YFV is transmitted by the bite of the domestic mosquito *Aedes* (*Stegomyia*) *aegypti* revolutionized the understanding of its epidemiology and guided control and protection measures for urban human populations early in the twentieth century [3–5].

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Since the 1930s, two main YFV transmission cycles have been described: the sylvatic, in which the virus is transmitted by arboreal mosquitoes between non-human primates (NHP) in the forest and where humans can be incidentally infected [6–8] and the urban, maintained between *Ae. aegypti* and humans [9]. An intermediate/rural cycle has been so far described only in Africa [10]. While the transmitters in the sylvatic and intermediate cycles in Africa are *Aedes* mosquitoes of the subgenera *Stegomyia* and *Diceromyia*, this role is played by species of genus *Haemagogus* and *Sabethes* in the New World, considered the primary and secondary sylvatic vectors respectively [10]. If the elimination of the urban cycle is feasible as it has occurred very rarely in the Americas since 1942, the enzootic sylvatic one is considered ineradicable [8,11]. The sylvatic cycle consists of a permanent threat both for its spillover to an urban cycle in the nearby of highly *Ae. aegypti* infested locals as well as for the emergency of epidemics where vaccination coverage in risk areas is inadequate [11,12]. This was the case of the severe outbreak recorded in southeastern Brazil in 2016–2018 [11,13].

Intriguingly, no record of YFV circulation had been detected since the 1930s in the Atlantic Forest zone in Brazil, the biome where sylvatic transmission was first discovered [14,15]. Hence, in contrast with the perennial transmission focus represented by the endemic/enzootic Amazon region and epizootic/YFV emerging areas in the *Cerrado* biome, the Brazilian health authorities have excluded this east-coastal zone from the YFV national vaccination program for decades [9,11,12]. Between the mid-twentieth century and 1999, YFV expansion and retraction waves originated in the Amazon have spread southward across the *Cerrado*, but extra-Amazon epizootics and epidemics were essentially limited to the Central-West region. However, from 2000 on, YFV expansion waves have reached the pampa biome in the southernmost Brazilian state and progressively spread eastward across the *Cerrado*. In late 2016, it spilled over from the *Cerrado* into transition zone between this biome and the Atlantic Forest in Minas Gerais state (MG) and rapidly spread across this last biome in the southeast. This region records the highest population densities in the country, but vaccination coverage against YFV was almost null at that moment. Then, the country's largest outbreak of sylvatic YF erupted, and rapidly spread in the southeast states of MG, Espírito Santo (ES), Rio de Janeiro (RJ) and São Paulo (SP) [11,16]. In less than two years, it has caused 2,058 confirmed human cases and 689 deaths, rates not observed since the first half of the twentieth century. It also caused a huge impact on NHP biodiversity as consequence of thousands of epizootic events [17,18]. The outbreak spread more than 900 km at an estimated speed of around 3 km a day [13,19]. The movement of paucisymptomatic and asymptomatic viremic humans and displacement of infected mosquitoes has been suggested as the main factors inducing this rapid spatial spread [11]. Therefore, defining the main vectors involved in the sylvatic transmission is critical in understanding the main ecological risk factors driving this unprecedented outbreak and guiding public health measures.

We conducted a comprehensive entomological survey based on a combined analysis of distribution, abundance and YFV natural infections in mosquitoes before and during the outbreak in the Brazilian states affected by this sanitary disaster in order to determine the primary vectors in the sylvatic cycle, and clarify the role of anthropic and domestic mosquitoes such as *Ae. (Stegomyia) albopictus* and *Ae. aegypti*.

Results

In total, 17,662 mosquitoes (15,398 adults and 2,264 immatures) belonging to 89 species were collected (Table 1).

Collections before the yellow fever outbreak: During this period, 5,341 mosquitoes were collected in 28 municipalities in RJ and in two bordering states (SP and MG). *Haemagogus leucocelaenus* was the most widespread species, being detected in 78.5% of sampled municipalities. In addition, it was the second most abundant species, accounting for 9.8% of total adult caught mosquitoes. Regarding other traditional YFV vectors, *Hg. janthinomys* and *Sabethes chloropterus* were detected in 39.2% and 10.7% of the municipalities surveyed before virus circulation, with a relative abundance of 1.6% and 0.21%, respectively (Figures 1 and 2, Table 1 and S1).

In addition, *Aedes scapularis* and *Ae. albopictus* were respectively the second and fourth most widespread species, present in 67.9% and 57.1% of surveyed municipalities prior the outbreak. While *Ae. scapularis* was the most abundant species (20.6%), *Ae. albopictus* accounted for 6.1% of abundance (Table 1 and S1). Remarkably, *Psorophora ferox* and *Sabethes albiprivus* were among the most captured mosquitoes prior the outbreak (Table 1), with relative abundance >2%, being detected in 42.9 and 28.6% of sampled municipalities, respectively.

Collections during the yellow fever outbreak: In this epidemiological context, 10,057 mosquitoes were collected in 21 municipalities, five of which (20%) had also been sampled before YFV transmission. Remarkably, the density and abundance of *Hg. janthinomys* tripled in relation to the pre-epidemic period, while that of *Hg. leucocelaenus* continued to be high (Table 1, S1 ad S2). These *Haemagogus* species were detected in 57% and 71% of municipalities with local active YFV transmission, respectively. Intriguingly, *Ae. albopictus* was the most widespread (present in 95% of municipalities) and the fifth most abundant species (relative

Table 1. Mosquitoes species in decreasing order of adults collected before and during the YFV outbreak, from May 2015 to May 2018, in 44 municipalities of four Southeast Brazil states and Bahiaian in the Northeast: We also present life stage, number of pool tested, number of positive pools, infection rates and percentage of presence in the sampled municipalities.

	No Adult. before	No Adult. during	No Total adult.	Relative abundance (%)[1]	No of immature	No Mosquito total	Pools tested[2] (Positives)	% of tested pools	MIR[3]	MLE[4]	Pres. bef.[5](%) $n = 28$	Pres. dur.[6](%) $n = 21$
Ae. scapularis	1096	1870	2966	19.262	0	2966	403(1)	72.9	0.54	0.54	67.9	85.7
Ae. taeniorhynchus	219	2428	2647	17.191	0	2647	199(1)	67.5	0.59	0.59	7.1	23.8
Hg. leucocelaenus	525	895	1420	9.222	1419	2839	327(41)	83.2	34.92	37.65	78.5	71.4
Ae. albopictus	329	478	807	5.241	439	1246	262(0)	87.6	0	0	57.1	95.2
Hg. janthinomys	89	527	616	4.001	27	643	162(20)	94.2	34.48	36.35	39.2	57.1
Li. durhamii	131	384	515	3.345	3	518	118(0)	63.4	0	0	60.7	76.2
Sa. albiprivus	132	302	434	2.819	42	476	173(0)	92.0	0	0	28.6	42.9
Ps. ferax	180	122	302	1.961	0	302	60(0)	69.8	0	0	42.9	57.1
Sh. fluviatilis	171	33	204	1.325	0	204	27(0)	73.0	0	0	35.7	14.3
Wy. confusa	71	121	192	1.247	0	192	41(0)	56.9	0	0	25.0	38.1
Sa. petrocchiae'	0	178	178	1.156	0	178	17(0)	39.5	0	0	0.0	4.8
Ae. serratus	67	73	140	0.909	0	140	37(0)	84.1	0	0	21.4	42.9
Wy. pilicauda	90	41	131	0.851	0	131	19(0)	59.4	0	0	39.3	23.8
Ae. aegypti	52	61	113	0.734	0	113	30(0)	75.0	0	0	10.7	28.6
Ru. humboldti	98	12	110	0.714	0	110	23(0)	82.1	0	0	25.0	19.0
Wy. aporonoma/ staminifera	26	83	109	0.708	0	109	29(0)	53.7	0	0	35.7	52.4
Ae. terrens	77	26	103	0.669	128	231	30(0)	83.3	0	0	35.7	38.1
On. personatum	79	18	97	0.630	8	105	20(0)	71.4	0	0	21.4	28.6
Ps. albipes	92	1	93	0.604	0	93	10(0)	76.9	0	0	3.6	4.8
Tr. pallidiventer	57	36	93	0.604	0	93	32(0)	72.7	0	0	42.9	38.1
Ru. frontosa	70	17	87	0.565	0	87	20(0)	69.0	0	0	39.3	23.8
Ma. indubitans	1	81	82	0.533	0	82	13(0)	81.3	0	0	3.6	4.8
Li. pseudomethisticus	59	16	75	0.487	0	75	16(0)	69.6	0	0	21.4	23.8
Wy. palmata/galvaoi	35	24	59	0.383	0	59	10(0)	66.7	0	0	17.9	14.3
Sa. fabricii/undosus	9	46	55	0.357	0	55	19(0)	79.2	0	0	10.7	28.6
Wy. medioalbipes	12	41	53	0.344	0	53	15(0)	55.6	0	0	3.6	23.8
Tr. digitatum	36	14	50	0.325	0	50	17(0)	65.4	0	0	25.0	33.3
Wy. mystes	11	32	43	0.279	0	43	18(0)	62.1	0	0	17.9	38.1
Sa. chloropterus	11	31	42	0.273	0	42	21(1)	100.0	23.8	23.2	10.7	28.6
Cx. quinquefasciatus	9	31	40	0.260	0	40	3(0)	23.1	0	0	7.1	19.0
Sa. aurescens	31	7	38	0.247	12	50	12(0)	85.7	0	0	28.6	9.5
Wy. davisi	17	13	30	0.195	0	30	6(0)	75.0	0	0	17.9	4.8
Ma. titillans	9	20	29	0.188	0	29	3(0)	33.3	0	0	7.1	14.3
Wy. bonnei/deanei	18	7	25	0.162	0	25	7(0)	70.0	0	0	10.7	14.3
Sa. melanonymphe	17	5	22	0.143	7	29	13(0)	100.0	0	0	17.9	23.8
Ru. cerqueirai	16	5	21	0.136	0	21	5(0)	55.6	0	0	10.7	14.3
Tr. castroi/similis	20	1	21	0.136	0	21	6(0)	85.7	0	0	14.3	4.8
Wy. incaudata	11	10	21	0.136	0	21	7(0)	70.0	0	0	10.7	14.3
Wy. edwardsi	15	5	20	0.130	0	20	5(0)	55.6	0	0	14.3	14.3
Cq. juxtamansonia	0	17	17	0.110	0	17	6(0)	85.7	0	0	0.0	14.3
Wy. bourrouli/forcipenis	3	14	17	0.110	0	17	8(0)	61.5	0	0	7.1	23.8
Wy. lutzi	5	12	17	0.110	6	23	4(0)	30.8	0	0	14.3	19.0
Sa. identicus	6	10	16	0.104	1	17	10(0)	76.9	0	0	17.9	28.6
Tr. compressum	2	14	16	0.104	0	16	1(0)	8.3	0	0	7.1	14.3
Ae. fluviatilis	4	10	14	0.091	9	23	6(0)	100.0	0	0	10.7	9.5
Cq. venezuelensis	3	10	13	0.084	0	13	5(0)	50.0	0	0	7.1	9.5
Sa. intermedius	8	3	11	0.071	0	11	6(0)	85.7	0	0	14.3	9.5
Ru. reversa/theobaldi	10	0	10	0.065	0	10	2(0)	50.0	0	0	14.3	0.0
Cq. nigricans	0	8	8	0.052	0	8	1(0)	50.0	0	0	0.0	4.8
Ps. lutzii/amazonica	2	6	8	0.052	0	8	5(0)	100.0	0	0	3.6	9.5
Sa. purpureus'	1	7	8	0.052	0	8	3(0)	100.0	0	0	3.6	9.5
Li. flavisetosus	0	7	7	0.045	0	7	0(0)	0.0	0	0	0.0	14.3
Ae. fulvithorax	4	2	6	0.039	0	6	3(0)	60.0	0	0	10.7	4.8
Wy. pallidoventer	6	0	6	0.039	0	6	2(0)	100.0	0	0	3.6	0.0
Cq. albicosta	0	5	5	0.032	0	5	3(0)	100.0	0	0	0.0	9.5
Sa. soperi	2	3	5	0.032	0	5	4(0)	80.0	0	0	7.1	9.5
Sa. whitmani	0	5	5	0.032	0	5	2(0)	66.7	0	0	0.0	9.5
Sa. xyphydes	2	3	5	0.032	0	5	4(0)	100.0	0	0	3.6	14.3
Wy. dyari	0	5	5	0.032	0	5	1(0)	50.0	0	0	0.0	9.5
Ae. condolences'	0	4	4	0.026	0	4	1(0)	100.0	0	0	0.0	4.8
Ae. rhyacophilus	0	4	4	0.026	0	4	2(0)	50.0	0	0	0.0	14.3
An. fluminensis	4	0	4	0.026	0	4	0	0.0	0	0	3.6	0.0
Wy. theobaldi	4	0	4	0.026	0	4	2(0)	100.0	0	0	7.1	0.0
Wy. antunesi	3	0	3	0.019	0	3	2(0)	100.0	0	0	3.6	0.0
Wy. oblita	2	1	3	0.019	26	29	1(0)	50.0	0	0	7.1	4.8
Ae. argyrothorax	0	2	2	0.013	0	2	2(0)	100.0	0	0	0.0	9.5
Tr. soaresi	0	2	2	0.013	0	2	0	0.0	0	0	0.0	9.5
Wy. codiocampa	0	2	2	0.013	0	2	2(0)	100.0	0	0	3.6	0.0
Wy. longirostris	2	0	2	0.013	0	2	2(0)	100.0	0	0	7.1	0.0
Wy. melanocephala	1	1	2	0.013	0	2	2(0)	100.0	0	0	3.6	4.8
Cq. hermanoi'	0	1	1	0.006	0	1	1(0)	100.0	0	0	0.0	4.8
Cq. shannoni	0	1	1	0.006	0	1	1(0)	100.0	0	0	0.0	4.8
Ps. pseudomelanota'	1	0	1	0.006	0	1	0	0.0	0	0	3.6	0.0
Sa. quasicyaneus	0	1	1	0.006	0	1	1(0)	100.0	0	0	0.0	4.8
Wy. arthrostigma'	0	1	1	0.006	0	1	0	0.0	0	0	0.0	4.8
Wy. cerqueirai	1	0	1	0.006	0	1	1(0)	100.0	0	0	0.0	4.8
Wy. exallos'	1	0	1	0.006	0	1	1(0)	100.0	0	0	3.6	0.0
Wy. knabi'	0	1	1	0.006	0	1	1(0)	100.0	0	0	0.0	4.8
Wy. shannoni	0	1	1	0.006	0	1	0	0.0	0	0	0.0	4.8
Other taxa[7]	1276	1799	3075	19.970	137	3212	405 (0)	55.5	0	0	–	–
Total	5341	10057	15398	100	2264	17662	2738(64)	71.3	–	–	–	–

[1]Relative abundance is calculated by dividing the number of adults of one species by the number of adults of all species × 100. [2]Number of adult pools tested; [3]Minimum Infection Rate = No of positive pools/No of same species adults analyzed × 1000; [4]Maximum Likelihood Estimate per 1000 mosquitoes = $1-(1-Y/X)^{1/m}$ where Y is the number of positive pools, X is the total number of pools, and m is the size of each tested pool; [5]Percentual of municipalities where each species was found before the outbreak; [6]Percentage of municipalities where each species was found during the outbreak. [7]Other taxa were represented by: *Culex sp.; Wyeomyia sp.; An. cruzii; Cx. nigripalpus; Sabethes sp.; Runchomyia sp.; Aedes sp.; Coquillettidia sp.; Limatus sp.; Psorophora sp.; Anopheles sp.; Shannoniana sp.; Mansonia sp.; Trichoprosopon sp.; Cx. declarator'; Aedeomyia sp.; Lutzia sp.'; Culex coronator'; An. bellator; An. lutzi; An. mediopunctatus; An. neivai; Haemagogus sp.*
We marked with ' those taxa with ambiguous classification, due to the existence of complex of cryptic species.

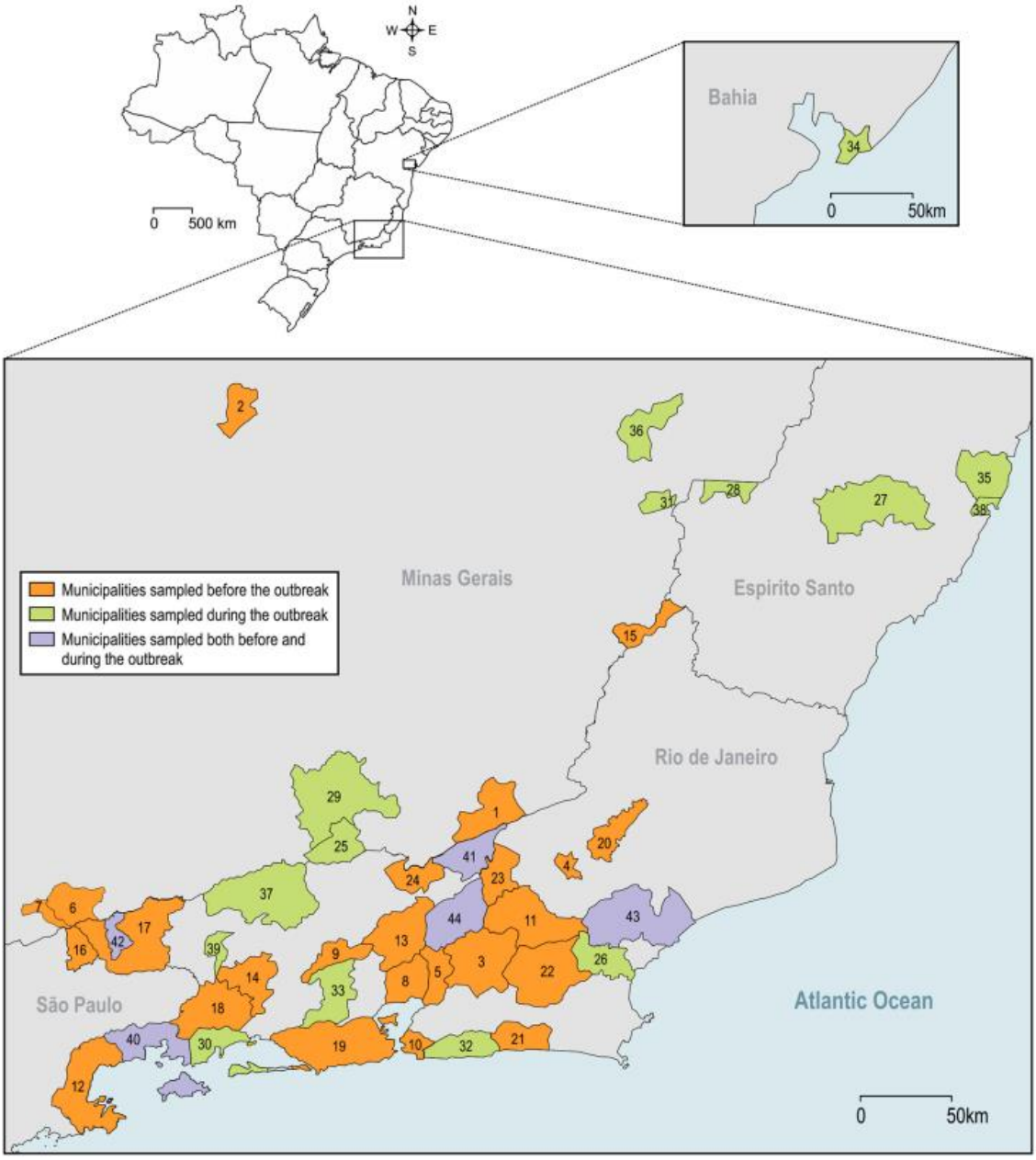

Figure 1. Brazilian municipalities sampled before, during and both before and during local YFV transmission. 1 – Além Paraíba; 2 – Belo Horizonte; 3 – Cachoeiras de Macacu; 4 – Cordeiros; 5 – Guapimirim; 6 – Itamonte; 7 – Itanhandu; 8 – Magé; 9 – Miguel Pereira; 10 – Niterói; 11 – Nova Friburgo; 12 – Paraty; 13 – Petrópolis; 14 – Piraí; 15 – Porciúncula; 16 – Queluz; 17 – Resende; 18 – Rio Claro; 19 – Rio de Janeiro; 20 – Silva Jardim; 21 – São Sebastião do Alto; 22 – Saquarema; 23 – Sumidouro; 24 – Três Rios; 25 – Belmiro Braga; 26 – Casimiro de Abreu; 27 – Domingos Martins; 28 – Ibatiba; 29 – Juiz de Fora; 30 – Mangaratiba; 31 – Manhumirim; 32 – Maricá; 33 – Nova Iguaçu; 34 – Salvador; 35 – Serra; 36 – Simonésia; 37 – Valença; 38 – Vitória; 39 – Volta Redonda; 40 – Angra dos reis; 41 – Carmo; 42 – Itatiaia; 43 – Macaé; 44 – Teresópolis.

abundance = 4.75%) during the outbreak (Tables 1 and S1). Although *Ae. taeniorhynchus* was the most abundant species in the total collections made during the outbreak (24.1%), its distribution was restricted to five coastal lowland municipalities under influence of the Atlantic Forest biome. *Sabethes albiprivus* was the most widespread and abundant species of the genus, while *Sa. petrocchiae* although abundant, was collected in only one affected municipality (Simonésia – MG) located in the transition between the Atlantic Forest and *Cerrado* biomes (Tables 1 and S2, Figure 3).

Virus detection: RNA of 2,738 pools, containing 10,537 adult mosquitoes from 85 species, was extracted and tested for YFV. Virus genome was detected in 64 pools (2.3%) containing 323 mosquitoes belonging to five species: *Hg. janthinomys*, *Hg. leucocelaenus*,

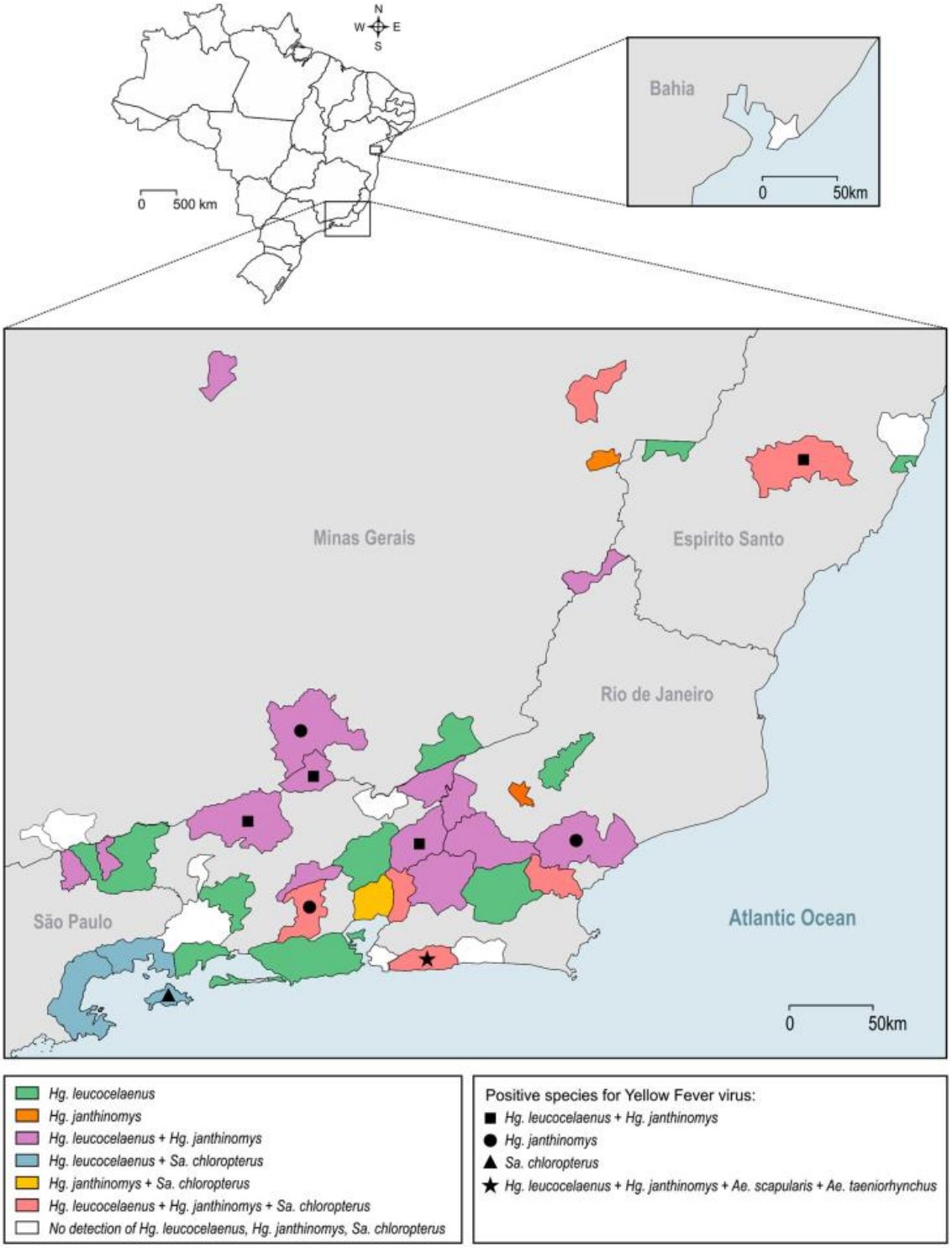

Figure 2. *Haemagogus leucocelaenus*, *Hg. janthinomys* and *Sabethes chloropterus* distribution along sampled municipalities. Geometric forms represented species found positive for yellow fever virus per municipality.

Sa. chloropterus, *Ae. scapularis* and *Ae. taeniorhynchus* (Tables 1 and 2). Positive mosquitoes were detected in 42.8% of surveyed municipalities (9/21) in three Brazilian states: 6 out of 17 from RJ (59 positive pools), two out of five from MG (3 positive pools) and one out of four from ES (2 positive pools) (Tables 2 and S3). Positive pools were found in mosquitoes captured in 2017 and 2018. No positive mosquito was found prior the detection of signals of YFV circulation in the respective municipalities. Also, YFV was not found in any pool of

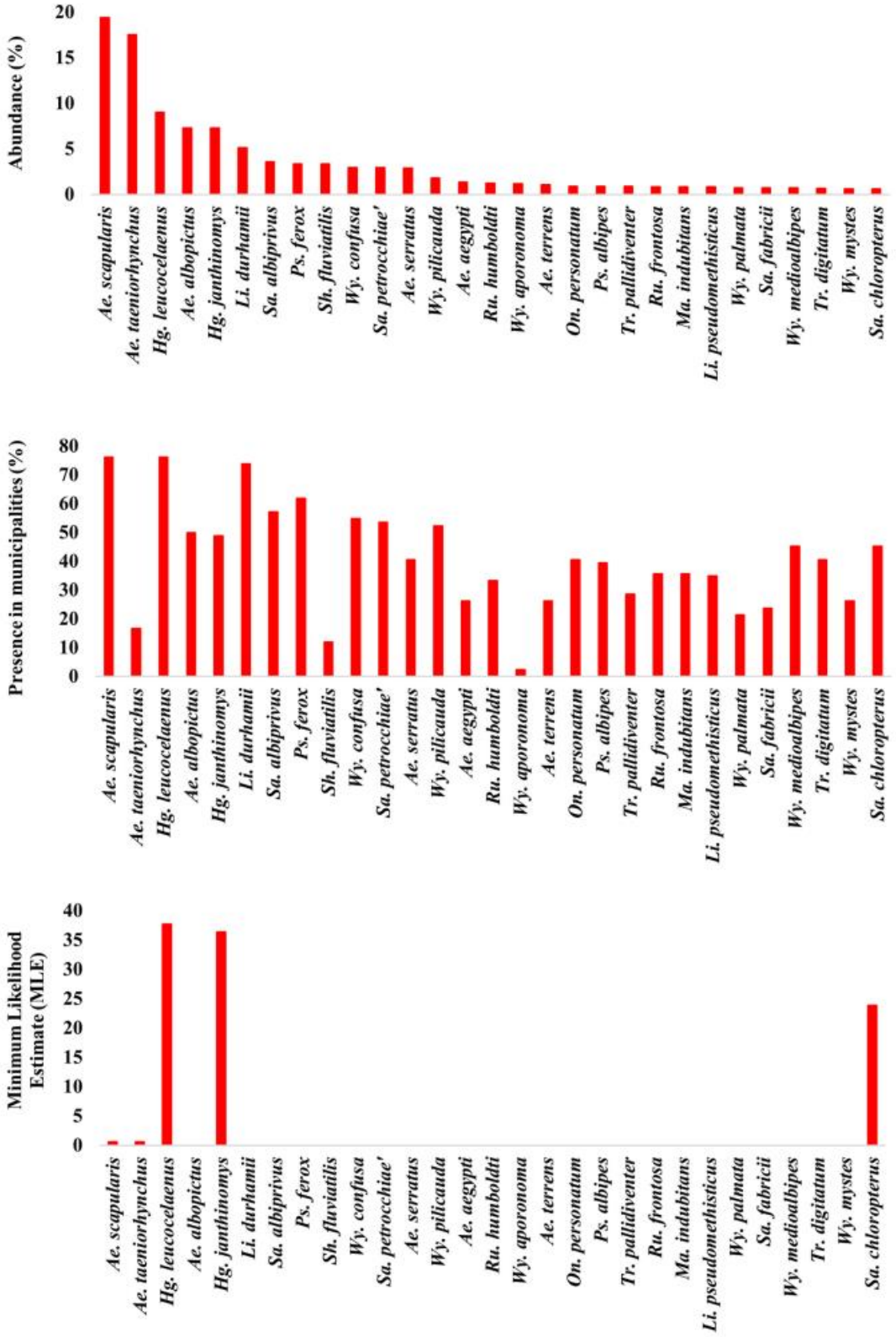

Figure 3. Percentage of abundance (1st graph) and presence (2nd graph) of the 29 most abundant species considering both before (between may/2015 and Jan/2017) and during YFV outbreak (between fev/2017 and may/2018) moments. 3rd graph shows the Maximum Likelihood Estimate (MLE) from the same species. Name of cryptic species e.g. *Hg. janthinomys*, *Wy. aporonoma/staminifera*, *Wy. palmata/galvaoi*, *Sa. fabricii/undosus* are abbreviated.

Table 2. Description of YFV-positive mosquito pools. ES: Espírito Santo; RJ: Rio de Janeiro; MG: Minas Gerais.

State	Municipality (Positive pools)	Species	Tested pools (Positive)	MIR[1]	MLE[2]	Relative abund.[3] (%)	Collection date	Human cases[4]	Days between first YFV signals[5] and mosq. collections
ES	Domingos_Martins (2)	*Hg. janthinomys*	1 (1)	1000	_	3.9	2/23/17	25	3 (M)
		Hg. leucocelaenus	4 (1)	66.6	67.6	7.1	2/23/17		
RJ	Macaé (1)	*Hg. janthinomys*	6 (1)	58.8	55.5	17	4/26/17	5	22 (M)
	Maricá (39)	*Hg. janthinomys*	5 (2)	142.8	153.5	0.4	5/5 and 8/5/17	3	18 and 21 (H/M)
		Hg. leucocelaenus	100 (35)	81.9	101.6	12.2	5/4-8/17		17-21 (H/M)
		Ae. scapularis	72 (1)	2.1	2.1	21.6	5/6/17		19 (H/M)
		Ae. taeniorhynchus	164 (1)	0.6	0.6	52.6	5/6/17		20 (H/M)
	Teresópolis (2)	*Hg. janthinomys*	19 (1)	13.1	13.1	18.7	12/19/17	23	5 (M)
		Hg. leucocelaenus	18 (1)	19.6	19.1	15.7	12/19/17		5 (M)
	Nova Iguaçú (1)	*Hg. janthinomys*	8 (1)	29.4	29.4	21.6	1/9/18	0	4 (M)
	Valença (15)	*Hg. janthinomys*	40 (12)	70.5	80.1	36.6	1/18,19,24,26/18	40	11, 12, 17, 19 (H)
		Hg. leucocelaenus	33 (3)	23.2	23.7	23.6	1/24/18		17 (H)
	Angra dos Reis (Ilha Grande) (1)	*Sa. chloropterus*	1 (1)	1000	_	0.7	2/7/18	57	3 (M)
MG	Belmiro Braga (2)	*Hg. janthinomys*	24 (1)	10.8	10.9	24.5	1/29/18	1	19 (M)
		Hg. leucocelaenus	18 (1)	13.5	13.3	23.8	1/18/18		8 (M)
	Juiz de Fora (1)	*Hg. janthinomys*	1 (1)	333.3	_	17.9	1/27/18	43	24 (M)

[1]Minimum Infection Rate = No of positive pools/No of same species adults analyzed × 1000; [2]Maximum Likelihood Estimate per 1000 mosquitoes = $1-(1-Y/X)^{1/m}$ where Y is the number of positive pools, X is the total number of pools, and m is the size of each tested pool; [3]Relative abundance = (Number of adults of the same species/No total of adults) × 100; [4]YF human cases detected in the same epidemic period in which mosquito collections were carried out. [5]Epizootic events in NHPs (M) and/or human (H) hospitalizations with clinical suspicion of YF.

mosquitoes caught in Salvador (BA) where YFV epizootics were confirmed just prior collection (Figures 2 and 3).

Haemagogus janthinomys and/or *Hg. leucocelaenus* were the species found infected in all localities where positive mosquitoes were detected, except in Angra dos Reis – Ilha Grande, RJ, where *Sa. chloropterus* was the only positive species although *Hg. leucocelaenus* was present (Figure 2). *Haemagogus janthinomys* displayed higher infection rates (MIR and MLE) than *Hg. leucocelaenus* in most localities (Table 2). *Aedes scapularis* (MIR = 2.1, MLE = 2.1) and *Ae. taeniorhynchus* (MIR = 0.6, MLE = 0.6) were found infected only once, coincidently in the same municipality (Maricá, RJ), where *Hg. leucocelaenus* (MIR = 81.9, MLE = 101.6) and *Hg. janthinomys* (MIR = 142.8, MLE = 153.5) were also detected naturally infected (Table 2, Figure 3).

Additionally, we tested 976 adult *Hg. leucocelaenus* and 19 *Hg. janthinomys* obtained from eggs collected in the same areas (Domingos Martins, Macaé, Maricá, Valença, Teresópolis and Belmiro Braga) and time in which infected females were detected, but all were negative, providing no evidence of vertical transmission.

Although widespread in municipalities suffering YFV outbreaks, all *Ae. aegypti* specimens were found negative, even when collected around houses inhabited by viremic dwellers infected in the sylvatic cycle.

Discussion

At the beginning of 2017, YFV reached Brazilian coastal states both in the southeast and northeast (BA), Atlantic Forest regions considered YFV-free and without vaccine recommendation for decades [9,17,20], causing a major sylvatic outbreak and devastating epizootics among NHPs in 2017–2018. During almost 80 years without YFV circulation, the southeast region under influence of the Atlantic Forest underwent significant environmental changes and a remarkable 368% increase in human population density that potentially influenced mosquito fauna distribution, diversity and abundance [11]. Changes in mosquito communities would potentially govern vector species status and might shape arbovirus transmission patterns [21].

Our results were based on extensive mosquito sampling, which covered approximately 1300 km between the northernmost and southernmost surveyed municipalities (Salvador – BA and Paraty – RJ), in the four most affected states, through a combined analysis of mosquito distribution, abundance and YFV natural infections before and during the outbreak. Although there had been no evidence of YFV circulation for nearly 80 years at 28 municipalities where we sampled mosquitoes before the outbreak, traditional YFV vectors were alarmingly detected in most of them (82%), revealing the high local receptivity to sylvatic YFV transmission. Therefore, its establishment in the coastal Atlantic Forest was only a matter of time.

Altogether, results obtained during the outbreak indicate *Hg. janthinomys* and *Hg. leucocelaenus* as the main sylvatic YFV vectors in the region. The genome sequencing of viral RNA detected in these mosquitoes (e.g. GenBank accession numbers MF423373 and MF423374) confirmed the occurrence of a unique molecular signature of fixed amino acid mutations in highly conserved positions at NS3 and NS5 proteins in YFV causing the current Brazilian outbreak [13,16]. Other taxa found naturally infected, such as *Sa. chloropterus*, *Ae. scapularis* and *Ae. taeniorhynchus* appear to have a local or secondary role and, therefore, low epidemiological importance either because of

reduced abundance and distribution (*Sa. chloropterus*), or as for the low infection rates (*Ae. scapularis*) combined with distribution limited to coastal lowlands (*Ae. taeniorhynchus*). Noteworthy, natural infections were detected only in mosquitoes captured between 3 and 24 days after glimpsing the first signal of YFV circulation (mostly epizootics) in the respective area, and no vertical transmission in mosquitoes was detected. A total of 5,703 mosquitoes belonging to 84 other species tested negative and showed no obvious role in YFV transmission in this outbreak.

Haemagogus janthinomys has been found several times infected with YFV in Brazil and other American countries and is considered the primary vector across Brazilian biomes for the last decades, namely the Amazon endemic region, the emergence zones in the transition between Amazonia and *Cerrado*, as well as in the *Cerrado stricto sensu* [21,22]. It had been also recognized as primary vector in the 1930–1940s epidemics in the Atlantic Forest [23]. Our current data reinforce the key role of *Hg. janthinomys* in the 2016–2018 outbreak, that is: density, abundance and distribution increasing during the outbreak (3.0 fold), highest displayed infection rates and favorable behavior, as discussed hereafter. *Haemagogus leucocelaenus*, whose role in sylvatic YFV transmission in the Americas was almost neglected until last decade, is herein considered as primary vector due to its very high distribution and abundance in surveyed municipalities during the outbreak as well as the noteworthy natural infection rates. During the investigation of YFV epidemics in inland southern Brazil, where *Hg. janthinomys* was not found [24,25], *Hg. leucocelaenus* was considered to play an important role in the transmission, although still regarded as secondary vector. The species was also found naturally infected in São Paulo during the sylvatic YF outbreak in 2009, when *Hg. janthinomys* and *Sa. chloropterus* were tested negative [26]. Although, *Hg. janthinomys* had higher infection rate values than *Hg. leucocelaenus* in most surveyed YFV foci as it is usually described in the literature [22], the latter occurred in greater abundance and was more distributed across the southeastern YFV transmission territory, which reinforces its importance in the maintenance and dissemination of YFV in this region. In all municipalities where YFV was detected in mosquitoes, *Hg. janthinomys* or both *Hg. janthinomys* and *Hg. leucocelaenus* (56%) were found infected, except for Ilha Grande where only *Sa. chloropterus* was found carrying the virus. Curiously, natural infections of both species in the same transmission area was described in one RJ site during the 1930s [27]. This suggests that the concurrence of the two species in YFV transmission may be typical and recurrent in the coastal Atlantic Forest, distinctly from other YFV endemic or epidemic South American biomes like the Amazon and *Cerrado*.

Species of *Haemagogus* exhibit primatophilic habits [28], which facilitates their contact with YFV infected NHPs and virus transmission to both human and NHPs. Collectively, their competence to amplify, disseminate and transmit the virus [28–30] as well as the high abundance, distribution, primatophilic behavior, arboreal feeding habits and large flight range may have contributed to the magnitude, severity and rapidity of spread of the 2016–2018 outbreak in this region [31,32]. *Haemagogus leucocelaenus* seems to exhibit greater plasticity of habitats and blood feeding patterns than *Hg. janthinomys* [24,33]. *Haemagogus leucocelaenus* may colonize secondary and modified forest patches, while *Hg. janthinomys* would be more stringent in terms of climatic and environmental conditions [24,34]. While *Hg. janthinomys* bites much more frequently at the canopy level, *Hg. leucocelaenus* usually attacks on lower forest strata, although both mosquitoes may disperse vertically in the forest [30,31]. Indeed, we captured an impressive number of *Hg. janthinomys* (4th most abundant species during the outbreak) at the ground level in the forest, as well as in the forest fringe and in open fields. The lower mean tree canopy height and vegetation density in most surveyed municipalities may sustain less amount and diversity of vertebrate hosts, which may have forced *Hg. janthinomys* to explore the ground level and the open fields. In addition, the topography of forest fragments in most YFV affected municipalities in the southeast may further influence this behavior, as previously suggested to occur with the canopy feeder mosquito *Anopheles cruzii* in the region [35,36]. The combination of behavioral and biological characteristics of these two *Haemagogus* species may also help to understand the estimates of virus spread of 3.5–5 km/day in this Brazilian outbreak [11,13,37]. In fact, *Hg. janthinomys* and *Hg. leucocelaenus* have high dispersion capacity between forest fragments, and may bite distant from the woods and even indoors [11,37,38]. It is important to mention that we found infected *Hg. janthinomys* in forest fragments as small as 7 hectares, some of which are contiguous to urban neighborhoods and remote urbanized high income borough settled in recently cut forest valleys. The growth of cities and the search for a more bucolic life have put people closer to fragments of forest increasing the exposure of people to the sylvatic mosquito bites in this region [11]. Therefore, taking into account the estimated flight range of these main vectors [37], vaccination campaigns in affected municipalities must consider not only people visiting or living in the close vicinity of forest, but also those who live or circulate at distances as large as 12 km from early detected epizooty.

This is the first time that *Sa. chloropterus* is found infected with YFV in the Atlantic Forest biome. The infected *Sa. chloropterus* was recovered when the outbreak reached a large island around 2 km apart from

the continent (Ilha Grande, in Angra dos Reis) where we did not find any *Hg. janthinomys,* both before and during the outbreak. At the time of mosquito collection, we found 10 carcasses and 5 dying howler-monkeys on the inspected trails. Interestingly we had found *Hg. leucocelaenus* in collections made before the outbreak. *Sabethes chloropterus* showed low abundance and was limited in distribution throughout the sampled area and time, a characteristic previously described [39,40]. Its canopy-feeding behavior and essentially sylvatic distribution may facilitate the contact with infected NHPs. But, our findings suggest a secondary role of *Sa. chloropterus* as YFV vector in this region of the country.

In contrast to the above-mentioned *Haemagogus* and *Sabethes* mosquitoes, *Aedes scapularis* and *Ae. taeniorhynchus* are opportunistic mosquitoes, whose biting peak occurs primarily in twilight at ground level of fragmented forest, the forest edge and open fields [41]. Thus, the opportunities of these *Aedes* mosquitoes to bite NHPs in the forest canopy are limited. *Aedes scapularis* is experimentally competent to transmit YFV [42,43], and occasionally becomes infected in nature [21]. Nevertheless, its role in the current outbreak, even as a secondary vector, seems to be little relevant as we found only one positive pool despite this species' great abundance and distribution across YFV foci, both in the lowlands and mountain slopes. We believe our report of YFV natural infection in *Ae. taeniorhynchus* is novel. However, as we examined whole bodies of non-blood-fed mosquitoes, we cannot ensure whether YFV replication would be only limited to the midgut or already present in the salivary glands of the infected specimens. While YFV outbreak affected sites of various reliefs including mostly mountain valleys and slopes, the distribution of *Ae. taeniorhynchus* is limited to the coastal lowlands. Its competence to experimentally transmit YFV is controversial to null [43,44]. Our detections of natural infections in these *Aedes* species occurred exclusively in a forest fragment undergoing a sylvatic transmission of great force illustrated by records of very recently confirmed epizootics in howler-monkeys and human fatal cases nearby and where we collected 40 positive pools of *Hg. janhtinomys* and *Hg. leucocelaenus* in one week. So, it is likely that *Ae. scapularis* and *Ae. taeniorhynchus* only become infected in environments and moments when the availability of viremic hosts infected by the bite of *Haemagogus* mosquitoes is very high. Thus, natural infections in these mosquitoes, especially in *Ae. taeniorhynchus,* should be considered with caution as they do not assure this species playing any important role in the spread of YFV.

Despite our large sample effort, species considered as potential vectors and found naturally infected with YFV in other areas (e.g. *Ae. serratus* [24], *Ae. albopictus* [43], *Sa. albiprivus* and *Psorophora* species [29,45–48]) were negative in the present study, even when captured in large quantities and in sympatry with the infected *Haemagogus*. *Sa. albiprivus* was the most abundant and well distributed species of *Sabethes*, both in arid areas of *Cerrado*, in dense Atlantic Forest as well as in transition between these biomes, and has proved to be competent to experimentally transmit YFV [29]. Therefore, *Sa. albiprivus* may play a very secondary role on YFV maintenance, even if not detected in the present study.

We did not find any natural infection when analyzing numerous *Ae. albopictus* from areas with large numbers of human cases and/or confirmed epizootics (e.g. Ilha Grande, Valença and Juiz de Fora). Brazilian *Ae. albopictus* may experimentally transmit YFV of the South American genotype, and it has been shown that YFV has the potential for adaptation to this mosquito with augmentation of virus titers in the saliva following successive contacts [29,49–51]. Additionally, this species was the most disseminated in the municipalities sampled during the outbreak in the southeast, the Brazilian region most infested by this mosquito [52]. Several authors warn that *Ae. albopictus* may act as bridge vector and would represent a threat of YFV reurbanization or facilitating enzootic spillovers with establishment of YFV into an intermediate/rural cycle due to its ecological plasticity and ubiquitous environmental distribution [11,52,53]. An important overlap of expanded niches of the sylvatic primary vectors, *Hg. janthinomys* and *Hg. leucocelaenus,* and the anthropic ones, like *Ae. albopictus,* in the Atlantic Forest biome has been observed in the last decades [11]. Therefore, it is advised to urgently design and apply surveillance and control measures concerning this mosquito in areas with transmissions and at risk.

Urban YF has not been recorded in Brazil since 1942. However, there is a great concern about urban YFV reemergence due to high infestation indices of *Ae. aegypti* in periurban and urban areas very close to the sylvatic cycles in the low vaccination coverage municipalities, such as those affected by the outbreak in southeastern Brazil [12,15,20]. All *Ae. aegypti* specimens we captured during the outbreak, including those sampled around houses inhabited by viremic humans infected in the sylvatic cycle, tested negative for YFV. However, the low vaccination coverage, the presence of *Hg. leucocelaenus* in several urban parks, the proximity of NHPs in several cities with the arrival of YFV-viremic humans seeking medical care in urban centers infested with *Ae. aegypti* are among the factors that may increase the risks of YFV reurbanization in the south and southeast, the most populated region of the country [11]. Most surveyed municipalities in southeastern Brazil have frequently endured urban epidemics of other *Ae. aegypti*-transmitted viruses, including dengue, chikungunya and Zika. It fact, YFV circulated intensely in 2017–2018 where *Ae. aegypti* is

very active. Together, these facts indicate that reducing *Ae. aegypti* populations and vaccinating urban populations near sylvatic outbreaks is more critical than ever.

The 2016–2018 sylvatic outbreak was the most severe in the last eight decades. Understanding the causes of this severity needs virological, primatological, ecological, epidemiological and immunological studies [11,13,16,54]. YFV transmission is a complex and multifactorial phenomenon involving social, ecological and biological issues, among which the entomological component is crucial. Here, by describing the distribution and abundance of potential transmitters and defining the primary vectors throughout the region touched by the outbreak, we could advise proper control measures as well as assemble essential knowledge on this intricate epidemiological event.

Entomological and virological surveillance must be urgently and permanently considered from northeast to south Brazil to rapidly define receptive and vulnerable areas as well as early detection of virus circulation, for better assessment of the risk areas and prediction of future spread, and thus target a quick extension of vaccination in expanded risk areas and prioritize the most affected age group using mobile immunization units, simultaneously moving toward the universal routine YFV vaccination for the entire Brazilian population.

Material and methods

Study chronology: Mosquitoes collections were performed in 44 municipalities in two distinct epidemiological situations: before and during the YFV outbreak. From May/2015 to June/2017, 12–15 days mosquito samplings were carried out in 28 municipalities before any local identification of YFV transmission, in order to evaluate receptivity for YF reemergence and early detect this arbovirus circulation. From Jan/2017 to May/2018, 1–8 days mosquito collections were conducted in 21 municipalities with suspected or confirmed YFV foci, i.e. where human cases or epizootic events had just been locally recorded. Among these 21 municipalities, 16 were surveyed for the first time and 5 had already been sampled before the outbreak (Figure 1).

Study areas: The criteria for selection of sampling municipalities were distinct according to the above-mentioned aims and epidemiological situations, i.e. before or during local YFV circulation (Figure 1). In the first situation, surveyed municipalities ($N = 28$) belonged to RJ and to bordering states (MG and SP). These municipalities were selected to include a variety of ecological and environmental conditions.

Twenty-one municipalities with YFV transmission locally confirmed were surveyed, being 20 in the three most affected states (MG, ES and RJ), that recorded a total of 73.5% of confirmed human cases, and one in Bahia (BA) where only NPH infections were confirmed. We selected five out these municipalities because they had been surveyed prior to local YFV transmission, and the remaining 16 municipalities were chosen taking into account the local incidence of human cases and epizootic records and the proximity to the great metropolitan areas in RJ, ES, MG and BA (Figure 1).

Entomological surveys: In all 44 surveyed municipalities (Figure 1), adult mosquitoes were caught with aspirators and nets during incursions into forest patches and their edges as well as near houses. Specimens were frozen in liquid N_2 or dry ice in the field, and kept under the same conditions of freezing during transport to our laboratory at Instituto Oswaldo Cruz (IOC) in Rio de Janeiro.

In seven municipalities (Belo Horizonte and Simonésia – MG, Domingos Martins and Serra – ES, Maricá and Casimiro de Abreu – RJ, and Salvador – BA), adult mosquitoes were also collected using BG-Sentinel traps (Biogentes) baited with CO_2. Twelve BG traps were continuously operated per area for 3–5 days. They were installed at each 100 m (0, 100, 200, and 300 m) along three transects from the edge to deep into the forest fragments, as described in more details elsewhere [53].

Ovitraps baited with an infusion of dry leaves found on forest ground and three wooden paddles each were settled for 7–12 days at the canopy of 15–30 trees per area. Paddles found with eggs were immerged in dechlorinated water for egg hatching, and larvae were reared until adult stage in the laboratory. Larvae were also sampled from all detected natural larval habitats (e.g. bromeliads, trees-holes, bamboo internodes) and reared until adult.

In the laboratory, field and laboratory emerged adult mosquitoes were identified under a stereomicroscopy on a cold table [55]. Voucher specimens were deposited at the CCuli Collection, at IOC (url: http://cculi.fiocruz.br/index). *Haemagogus janthinomys*, a common species widely distributed across South America, and *Haemagogus capricornii* are sympatric in southeast Brazil. Their adult females and immature forms are morphologically indistinguishable, the few distinctive characters are in male genitalia [56]. When available, male genitalia of *Haemagogus* collected in all municipalities were examined. However, males with the phenotype corresponding to *Hg. capricornii* were found only in Valença (RJ). Therefore, we used the name *janthinomys* to refer to specimens potentially belonging to either taxa.

The major part of captured adults was pooled (≤10 individuals each) according to species, sampling locality and date, and subsequently homogenized in 250–1000 µL of L-15 culture medium by using the Precellys 24® tissue homogenizer in bead tubes. Homogenates were kept at −80°C for posterior viral genome

detection. Only non-blood-fed mosquitoes were analyzed.

Virus detection: After centrifugation (9600 *g*, 10 min, 4°C), RNA was extracted from 140 µL of supernatant using the Qiagen RNA Viral Kit following the manufacturer's recommendations. RNA from mosquitoes obtained in the first half 2017 were screened by conventional RT-PCR while the remaining samples were examined by RT-qPCR. Details for RT-PCR protocol were previously described [16]. Briefly, the set of primers utilized in the conventional PCR were 5′-CTGTGTGCTAATTGAGGTGCATTG-3′ and 5′-ATGTCATCAGGCTCTTCTCT-3′, targeting nucleotides 9 to 663, between 5′ and PrM regions of the YFV genome. Infections were diagnosed by the specific detection of this single amplicon with the likely YFV amplicon size of 650 bp. Obtained amplicons were purified using Qiagen QIAquik PCR purification kit following the manufacturer's recommendation. For confirmation, the amplicons were directly sequenced without molecular cloning. Nucleotide sequencing reactions were performed using the ABI BigDye terminator V3.1 Ready Reaction Cycle Sequencing Mixture (Applied Biosystems) according to manufacturer's recommendations. Nucleotide sequence was determined by capillary electrophoresis at the sequencing facility of Fiocruz-RJ (RPT01A – Sequenciamento de DNA – RJ). Raw sequence data were aligned and edited using the SeqMan module of LaserGene (DNASTAR Inc.). Edited nucleic acid sequences were compared to other YFV strains available at the Gen-Bank database using The Basic Local Alignment Search Tool (BLAST) (url: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For RT-qPCR, viral RNA was reverse transcribed and amplified using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems) in an Applied Biosystems StepOnePlus Instrument. For each reaction we used 300 nM forward primer (5′-GCTAATTGAGGTGCATTGGTCTGC-3′, genome position 15–38), 600 nM reverse primer (5′-CTGCTAATCGCTCAACGAACG-3′, genome position 83–103) and 250 nM probe (5′FAM-ATCGAGTTGCTAGGCAATAAACAC-3′TAMRA, genome position 41–64). Samples were run in duplicate, with deionized water serving as negative control for extraction and PCR reactions. The reverse transcription was performed at 50°C for 5 min. The qPCR conditions were 95°C for 20 s, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. Copy numbers of YF genomic RNA were calculated by absolute quantitation using a standard curve for each run. To construct the standard curve, an amplicon was cloned comprising the genomic region 1 to 865 of the isolate ES-504 (GeneBank accession number: KY885000) using pGEM-T Easy Vector (Promega) to serve as a template for *in vitro* transcription. The RNA was transcript with mMessage mMachine High Yield Capped RNA Transcription Kit (Invitrogen) using SP6 enzyme and purified using MEGAclear Kit (Ambion) according to manufacturer's instructions. The purity of the transcript was verified using NanoDrop 8000 Spectrophotometer (Thermo Scientific), and the concentration of the RNA was determined using Qubit 2.0 Fluorometer (Invitrogen). The standard curve was generated by serial ten-fold dilution (ranging from 10 to 10^9 copies/reaction) of the transcript. The limit of detection under standard assay conditions was approximately 40 viral RNA copies/mL. Confirmation of YFV diagnose was done by amplifying and sequencing the 650 bp amplicon as described above.

Standardization of RT-qPCR assays was done from serial dilutions (10^{-1} to 10^{-10}) of one confirmed wild mosquito positive sample, mixed or not with homogenates of negative *Ae. aegypti* from our laboratory colony. The sensitivity of the test was compared in triplicate with that of conventional RT-PCR. Conventional RT-PCR and RT-qPCR detected the virus genome until 10^{-7} and 10^{-9} dilution, respectively.

Data analyses: Quantitative and qualitative fauna data were analyzed in Microsoft Excel software. Two infections rates were used: Minimum Infection Rate (MIR) was calculated by dividing the number of infected pools by the total number of adult mosquitoes of the same species collected in the same area, multiplied per 1000. Maximium Likelihood Estimate (MLE) per 1000 adult mosquitoes was obtained using the formula $1-(1-Y/X)^{1/m}$ where Y is the number of positive pools, X is the total number of pools, and m is the size of each tested pool [57]. Maps were constructed in Arcgis webmap version.

Ethical statements: Mosquito collections in the Atlantic Forest were approved by local environmental authorities (SISBIO-MMA licenses 54707–2 and 52472–2, INEA license 012/2016012/2016 and PNMNI license 001/14–15). This study did not involve endangered or protected species.

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Author contributions

R.L.O. and F.V.S.A. conceived the study. M.Q.G., J.L.S., R.G.C., R.F.C.S, R.C.L.J., F.V.S.A. were involved in mosquito samplings. C.P. and T.P.S. designed mosquito collection in transects with traps. M.I.L.B. M.A.M., M.S.A.S.N. and I.S.B.A. identified mosquitoes. M.G.C., R.M.M., I.S.B prepared mosquito pools and extracted RNA. R.L.O., F.V.S.A, R.G.C., R.F.C.S, R.C.L.J., A.F, A.P.M.R., E.O.L.F. and M.S.R. organized field collections and defined sampling areas. A.F.B., A.A.C.S., A-B.F., S.M., F.V.S.A., I.P.R. and M.G.C. were involved in virus screening in mosquitoes. M.C.B, M.M.G., A.F.B., I.P.R., A.A.C.S. sequenced and analyzed viral genomes. F.V.S.A, I.P.R. and R.L.O. wrote the manuscript. All authors reviewed the paper.

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References

[1] Vasconcelos PFC, Monath TP. Yellow fever remains a potential threat to public health. Vector-Borne Zoonotic Dis. 2016;16:566–567.
[2] Garske T, et al. Yellow fever in Africa: estimating the burden of disease and impact of mass vaccination from outbreak and Serological data. PLoS Med. 2014;11:e1001638.
[3] Finlay C. Yellow fever: its transmission by means of the *Culex* mosquito. Am J Med Sci. 1886;92:395–409.
[4] Reed W, Carrol J, Agramonte A. The etiology of yellow fever. J Am Med Assoc. 1901;36:415–440.
[5] Clements AN, Harbach RE. History of the discovery of the mode of transmission of yellow fever virus. J Vector Ecol. 2017;42:208–222.
[6] Lutz A. Reminiscencias da febre amarella no Estado de São Paulo. Erinnerungen aus der Gelbfieberzeit im Staate São Paulo. Mem Inst Oswaldo Cruz. 1930;24:127–142.
[7] Soper FL, Penna H, Cardoso E, et al. Yellow fever without *Aedes aegypti*. study of a rural epidemic in the Valle do Chanaan, Espirito Santo, Brazil, 1932. Am J Hyg. 1933;18:555–587.
[8] Soper FL. Jungle yellow fever. A new epidemiological Entity in South America. Rev Hig e Saude Publica. 1937;10.
[9] Monath TP, Vasconcelos PFC. Yellow fever. J Clin Virol. 2015;64:160–173.
[10] Hanley KA, et al. Fever versus fever: the role of host and vector susceptibility and interspecific competition in shaping the current and future distributions of the sylvatic cycles of dengue virus and yellow fever virus. Infect Genet Evol. 2013;19:292–311.
[11] Possas C, et al. Yellow fever outbreak in Brazil: the puzzle of rapid viral spread and challenges for immunisation. Mem Inst Oswaldo Cruz. 2018;113:1–12.
[12] Possas C, et al. Urgent call for action: avoiding spread and re-urbanisation of yellow fever in Brazil. Mem Inst Oswaldo Cruz. 2017;113:1–2.
[13] Gómez MM, et al. Genomic and structural features of the yellow fever virus from the 2016-2017 Brazilian outbreak. J Gen Virol. 2018;99:536–548.
[14] Kumm HW, Laemmert HW. The geographical distribution of immunity to yellow fever among the primates of Brazil. Am. J. Trop. Med. Hyg. 1950;30:733–748.
[15] Franco O. História da Febre Amarela no Brasil. Ministério da Saúde. 1969;1:210.
[16] Bonaldo MC, et al. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. Mem Inst Oswaldo Cruz. 2017;112:447–451.
[17] Brasil. Monitoramento Dos Casos E Óbitos De Febre Amarela No Brasil - Informe – Nº 43/2017. 1–7 (2017). Available at: http://portalarquivos.saude.gov.br/images/pdf/2017/junho/02/COES-FEBRE-AMARELA—INFORME-43—Atualiza—-o-em-31maio2017.pdf.
[18] Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. *Monitoramento do Período Sazonal da Febre Amarela*. (2018).
[19] Fioravanti C. O alerta dos macacos. Pesquisa FAPESP. 2018;263:19–23.
[20] Romano APM, et al. Yellow fever outbreaks in unvaccinated populations, Brazil, 2008-2009. PLoS Negl Trop Dis. 2014;8:18–21.
[21] Vasconcelos PFC, et al. Epidemic of jungle yellow fever in Brazil, 2000: Implications of climatic alterations in disease spread. J Med Virol. 2001;65:598–604.
[22] Vasconcelos PFC, et al. An epidemic of sylvatic yellow fever in the southeast region of Maranhao state, Brazil, 1993-1994: Epidemiologic and entomologic findings. Am J Trop Med Hyg. 1997;57:132–137.
[23] Shannon RC, Whitman L, Franca M. Yellow fever virus in jungle mosquitoes. Science. 1938;88:110–111.
[24] Cardoso JDC, et al. Yellow fever virus in *Haemagogus leucocelaenus* and *Aedes serratus* mosquitoes, Southern Brazil, 2008. Emerg Infect Dis. 2010;16:1918–1924.

[25] Vasconcelos PFC, et al. Isolations of yellow fever virus from *Haemagogus leucocelaenus* in Rio Grande do Sul State, Brazil. Trans R Soc Trop Med Hyg. 2003;97:60–62.
[26] Mascheretti M, et al. Yellow fever: Reemerging in the state of Sao Paulo, Brazil, 2009. Rev Saude Publica. 2013;47:881–889.
[27] Soper FL. Yellow fever: the present situation (October 1938) with special reference to South America. Trans R Soc Trop Med Hyg. 1938;32:297–322.
[28] Laemmert HW, Ferreira LDC, Taylor RM. Part II—investigations of vertebrate hosts and arthropod vectors. Am J Trop Med Hyg. 1946;s1-26:23–69.
[29] Couto-Lima D, et al. Potential risk of re-emergence of urban transmission of yellow fever virus in Brazil facilitated by competent *Aedes* populations. Sci Rep. 2017;7:4848.
[30] Bates M. The natural history of mosquitoes. New York (NY): Macmillan; 1949.
[31] Lourenço-de-Oliveira R, Failloux A-B. High risk for chikungunya virus to initiate an enzootic sylvatic cycle in the tropical Americas. PLoS Negl Trop Dis. 2017;11:e0005698.
[32] Arnell HJ. Mosquito studies (Diptera, Culicidae) XXXIIL A revision of the *Scapularis* group of *Aedes* (*Ochlerotatus*). Contrib Am Entomol Inst. 1976;13:1–144.
[33] Alencar J, et al. Diversity of yellow fever mosquito vectors in the Atlantic forest of Rio de Janeiro, Brazil. Rev Soc Bras Med Trop. 2016;49:351–356.
[34] Alencar J, et al. Influence of climatic factors on the population dynamics of *Haemagogus janthinomys* (Diptera: Culicidae), a vector of sylvatic yellow fever. Entomol News. 2010;121:45–52.
[35] Pina-Costa Ad, et al. Malaria in Brazil: what happens outside the Amazonian endemic region. Mem Inst Oswaldo Cruz. 2014;109:618–633.
[36] Deane LM, Ferreira Neto JA, Lima MM. The vertical dispersion of *Anopheles* (*Kerteszia*) *cruzi* in a forest in southern Brazil suggests that human cases of malaria of simian origin might be expected. Mem Inst Oswaldo Cruz. 1984;79:461–463.
[37] Causey OR, Laemmert HW, Kumm HW. Dispersion of forest mosquitoes in Brazil: further studies 1. Am J Trop Med Hyg. 1950;s1-30:301–312.
[38] Pinheiro FP, Travassos da Rosa A, Moras MAP. An epidemic of yellow fever in central Brazil, 1972-1973: II Aspectos Ecológicos. Inst Evandro Chagas 50 anos Contrib. às ciências biológicas e à. Med Trop. 1986;1:409–437.
[39] Dos Santos Silva J, Lopes CM, Guimarães AÉ, et al. Diversity of mosquitoes at the Itatiaia National Park, state of Rio de Janeiro, Brazil. J Am Mosq Control Assoc. 2017;33:270–275.
[40] Guimarães AÉ, Arlé M, Guimarães AÉ, et al. Mosquitos no Parque Nacional da Serra dos Órgãos, estado do Rio de Janeiro, Brasil: I – distribuição estacional. Mem Inst Oswaldo Cruz. 1984;79:309–323.
[41] Forattini OP, Gomes AdC. Biting activity of *Aedes scapularis* (Rondani) and *Haemagogus* mosquitoes in Southern Brazil (Diptera: Culicidae). Rev Saude Publica. 1988;22:84–93.
[42] Davis NC, Shannon RC. Studies on yellow fever in South America : iv. Transmission experiments with *Aedes aegypti*. J Exp Med. 1929;50:793–801.
[43] Whitman L, Antunes PCA. Studies on the capacity of various Brazilian mosquitoes, representing the Genera Psorophora, *Aedes*, Mansonia, and *Culex*, to transmit yellow fever 1. Am J Trop Med Hyg. 1937;s1-17:803–823.
[44] Dégallier N, et al. A comparative study of yellow fever in Africa and South America. Cienc Cult. 1992;44:143–151.
[45] Goenaga S, Fabbri C, Dueñas JC, et al. Isolation of yellow fever virus from mosquitoes in Misiones Province, Argentina. Vector-Borne Zoonotic Dis. 2012;12:986–993.
[46] Davis NC, Shannon RC. The location of yellow fever virus in infected mosquitoes and the possibility of hereditary transmission. Am J Epidemiol. 1930;11:335–344.
[47] Davis NC, Shannon RC. Further attempts to transmit yellow fever with mosquitoes of South America*. Am J Epidemiol. 1931;14:715–722.
[48] Vasconcelos PFdC. Febre amarela yellow fever. Rev Soc Bras Med Trop. 2003;36:275–293.
[49] Lourenco de Oliveira R, Vazeille M, de Filippis AMB, et al. Large genetic differentiation and low variation in vector competence for dengue and yellow fever viruses of Aedes albopictus from Brazil, the United States, and the Cayman Islands. Am J Trop Med Hyg. 2003;69:105–114.
[50] Amraoui F, et al. Experimental adaptation of the yellow fever virus to the mosquito *Aedes albopictus* and potential risk of urban epidemics in Brazil, South America. Sci Rep. 2018;8:14337.
[51] Miller BR, Ballinger ME. *Aedes albopictus* mosquitoes introduced into Brazil: vector competence for yellow fever and dengue viruses. Trans R Soc Trop Med Hyg. 1988;82:476–477.
[52] Carvalho RG, Lourenço-de-Oliveira R, Braga IA. Updating the geographical distribution and frequency of *Aedes albopictus* in Brazil with remarks regarding its range in the Americas. Mem Inst. Oswaldo Cruz. 2014;109:787–796.
[53] Pereira dos Santos T, et al. Potential of *Aedes albopictus* as a bridge vector for enzootic pathogens at the urban-forest interface in Brazil. Emerg Microbes Infect. 2018;7:191.
[54] Moreira-Soto A, Torres MC, Lima de Mendonça MC, et al. Evidence for multiple sylvatic transmission cycles during the 2016–2017 yellow fever virus outbreak, Brazil. Clin Microbiol Infect. 2018;24(9):1019.e1–1019.e4.
[55] Consoli RAGB, Oliveira RLd. Principais mosquitos de importância sanitária no Brasil. Rio de Janeiro (RJ): Editora Fiocruz; 1994.
[56] Arnell JH. Mosquito studies (Diptera, Culicidae) XXXII. A revision of the genus *Haemagogus*. Contrib Am Entomol Inst. 1973;10:1–174.
[57] Biggerstaff BJ. Confidence intervals for the difference of two proportions estimated from pooled samples. J Agric Biol Environ Stat. 2008;13:478–496.

4.5 Artigo 5: Genomic and structural features of the yellow fever virus from the 2016–2017 Brazilian outbreak

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Genomic and structural features of the yellow fever virus from the 2016–2017 Brazilian outbreak

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Abstract

Southeastern Brazil has been suffering a rapid expansion of a severe sylvatic yellow fever virus (YFV) outbreak since late 2016, which has reached one of the most populated zones in Brazil and South America, heretofore a yellow fever-free zone for more than 70 years. In the current study, we describe the complete genome of 12 YFV samples from mosquitoes, humans and non-human primates from the Brazilian 2017 epidemic. All of the YFV sequences belong to the modern lineage (sub-lineage 1E) of South American genotype I, having been circulating for several months prior to the December 2016 detection. Our data confirm that viral strains associated with the most severe YF epidemic in South America in the last 70 years display unique amino acid substitutions that are mainly located in highly conserved positions in non-structural proteins. Our data also corroborate that YFV has spread southward into Rio de Janeiro state following two main sylvatic dispersion routes that converged at the border of the great metropolitan area comprising nearly 12 million unvaccinated inhabitants. Our original results can help public health authorities to guide the surveillance, prophylaxis and control measures required to face such a severe epidemiological problem. Finally, it will also inspire other workers to further investigate the epidemiological and biological significance of the amino acid polymorphisms detected in the Brazilian 2017 YFV strains.

INTRODUCTION

Yellow fever (YF) is a viral disease that is transmitted by the bite of infected mosquitoes in Africa and South America. It affects around 200 000 people annually, mostly in Africa [1–3]. There are two main epidemiological cycles: (1) the enzootic sylvatic cycle, in which the virus is transmitted between non-human primates (NHPs) and wild arboreal mosquitoes of genus *Aedes, Haemagogus* and *Sabethes* can accidentally infect man, and (2) the urban cycle, in which inter-human transmission is ensured by the domestic and anthropophilic mosquito *Aedes aegypti* [3]. While only the sylvatic cycle has been reported in Brazil and most South American countries

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Keywords: yellow fever virus; 2016-2017 Brazilian outbreak; amino acid changes; genetic diversity; evolution.
Abbreviations: BD, Brownian diffusion; DENV, dengue virus; DOPE, discrete optimized protein energy; ES, Espírito Santo; ESS, effective sample size; GTR, general time-reversible; HKY, Hasegawa, Kishino and Yano; HPD, highest probability density; I, isoleucine; IBGE, Brazilian Institute of Geography and Statistics; K, lysine; MCC, maximum clade credibility; MCMC, Markov chain Monte Carlo; MD, molecular dynamics; MEGA, molecular evolutionary genetics analysis; MG, Minas Gerais; MRCA, most recent common ancestor; MTase, methyltransferase; NHP, non-human primates; NS3pro, NS3 protease domain; PS, path sampling; PDB, Protein Data Bank; PP, posterior probability; R, arginine; RJ, Rio de Janeiro; RDP, Recombination Detection Program; RdRp, RNA-dependent RNA polymerase; RRW, relaxed random walk; S, serine; SS, stepping stone; T, threonine; TMRCA, time to the most recent common ancestor; V, valine; VTFM, variable target function method; YF, yellow fever; YFV, yellow fever virus; ZIKV, zika virus.
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Four supplementary tables and ten supplementary figures are available with the online version of this article.

in the last 75 years, in Africa people may acquire the infection in both cycles, as well as in an intermediate cycle occurring in rural areas close to forests [2, 4].

The aetiological agent is the yellow fever virus (YFV) (genus *Flavivirus*, family *Flaviviridae*), presenting a single-positive-sense RNA genome containing a 5′ end cap structure that is translated in a single immature polyprotein precursor. The precursor polyprotein is cleaved by viral and cellular proteases into structural proteins (capsid, C; envelope, E; and membrane protein, M) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [2]. The currently circulating YFV strains probably arose within the last 1500 years in Africa, where five genotypes have been documented, with two coming from West Africa (West Africa I and II) and three coming from East and Central Africa (East Africa, East/Central Africa and Angola) [5, 6]. Together with the invasive mosquito *Ae. aegypti*, the YFV spread from Africa to the Americas, probably during the 17th and 18th centuries, generating two genotypes from the Western African ancestor (South America I and II) [5–7]. South American genotype I is the most disseminated and frequently detected genotype during epizootic and epidemic waves in Brazil and the other countries of South America [8, 9]. Until the 1990s, the transmission area in Brazil was primarily limited to the Amazon forest in the North region and the savanna-like cerrado in the Central-West region. However, in two decades, the YFV territory has progressively expanded southward and eastward, reaching the Atlantic forest and other biomes of the country's most populated regions [10]. During this boundary expansion, five viral sub-lineages (1A to 1E) emerged successively within genotype I, as distinguished by analysis of partial nucleotide sequencing of the YFV genome [8, 9, 11]. Most recently, South America genotype I was divided into two major lineages, denominated as the old (sub-lineages: Old Para, 1A, 1B and 1C) and the modern lineages (sub-lineages: Trinidad and Tobago, 1D and 1E) [11, 12].

A rapid expansion of the YFV area has been reported since late 2016 in southeastern Brazil [13, 14]. From December 2016 to May 2017, the YFV spread quickly from the transition zone between the cerrado and the inland Atlantic forest of Minas Gerais state (MG) to the coastal areas in the Espírito Santo (ES) and Rio de Janeiro (RJ) states, and then approached densely populated areas close to the rain forest that had insignificant vaccination coverage. In a few months, around 3850 NHPs died, and nearly 800 human cases with 435 deaths were registered, of which 274 had been confirmed as YF by July 2017 [13, 14]. Interestingly, the analysis of two genome sequences obtained from howler monkeys from ES state during the ongoing outbreak confirmed that they cluster within the sub-lineage 1E. Furthermore, these strains revealed new polymorphisms comprising several amino acid substitutions, mainly located in the components of the viral replicase complex, in the protease domain of the NS3 protein (NS3pro) and in the methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) domain of the NS5 protein [15]. It was unclear, however, whether the observed amino acid substitutions are genetic signatures of the most recent YF outbreaks, as few complete genomes of current circulating viral strains were available [8, 15].

In this study, we elucidated the complete genome sequence of 12 YFV strains from 3 hosts (NHPs, mosquitoes and humans) involved in the transmission cycle of the current Brazilian outbreak in two southeastern states (RJ and ES). Since only a few complete YFV genomes are available, these new YFV sequences will contribute to a better understanding of the natural history of YF [16, 17]. Further, the YFV sequences were analysed to establish whether previously observed specific amino acid changes are fixed in other recent YFV samples and therefore constitute a molecular signature of the 2017 YFV. To determine the position of these amino acid substitutions in NS3pro and NS5 proteins, we created homology models for NS3 and NS5. Moreover, we performed phylogenetic and evolutionary studies to determine the genotypes of the YFV strains, estimate the time to the most recent common ancestor (TMRCA) of Brazilian YFV 2017 samples obtained from RJ and ES and research the main routes of viral dissemination in RJ state.

RESULTS

YFV samples' geographical distribution and genome characterization

To provide a substantial insight into the epidemiology of the outbreak, over a short period (from February to June 2017) we collected YFV samples from 5 distinct infected host species, including mosquitoes, NHPs and humans, from 11 localities belonging to 3 main river basins throughout the current epidemic/epizootic territory of the ES and RJ Atlantic coast (Table 1, Fig. 1). We analysed the whole YFV genome of 12 samples: 2 from mosquitoes belonging to 2 species [*Haemagogus leucocelaenus* (pool of 5 mosquitoes) and *Hg. Janthinomys* (1 mosquito)], 6 from NHPs (4 howler monkeys and 2 marmosets) and 4 from human cases (Table 1).

A comparison of all the southeast Brazilian outbreak YFV genomes reveals low genetic variation, with an average nucleotide identity of 99.8 % and amino acid identity values ranging from 99.9 to 100 %. However, some of the nucleotide variations are non-synonymous, leading to new amino acid substitutions in the polyprotein sequence (Fig. S1, Table S1, available in the online version of this article). Regardless of the host, all of the analysed 2017 YFV Brazilian genomes display a set of nine unique amino acid substitutions. Remarkably, a comparison with all of the complete YFV genome sequences available to date confirmed that these polymorphisms are only present in the Brazilian strains from the ongoing outbreak (Fig. S2). They localize at the C protein (V108I; C residue 108), at NS3pro (E1572D; R1605K; NS3 residues 88 and 121, respectively), at NS5 in MTase domain (K2607R; V2644I; G2679S; NS5 residues 101, 138 and 173, respectively) and at NS5 in the RdRp domain (V3149A; N3215S; NS5 residues 643 and 709, respectively) (Fig. S1). Nevertheless, the partial nucleotide sequence from H189 also displayed all of the amino acid

Table 1. Yellow fever virus samples collected in the 2017 Brazilian outbreak

Host	Sample ID (Genbank accession number)	Date of collection	Location	Geographical coordinates
Hg. leucocelaenus	PA193 - (MF423373)	21/02/2017	Areinha, Domingos Martins – ES	20° 17′ 08″ S 40° 50′ 15″ W
Hg. janthinomys	PA196 - (MF423374)	23/02/2017	Areinha, Domingos Martins – ES	20° 17′ 08″ S 40° 50′ 15″ W
Alouatta clamitans (howler-monkey)	ES-504* - (KY885000)	20/02/2017	Areinha, Domingos Martins – ES	20° 17′ 08″ S 40° 50′ 15″ W
	ES-505* - (KY885001)	22/02/2017	Areinha, Domingos Martins – ES	20° 17′ 08″ S 40° 50′ 15″ W
	RJ87 - (MF423375)	04/04/2017	Atalaia, Macaé – RJ	22° 18′ 31.6″ S 42° 00′ 01.7″ W
	RJ94 - (MF423376)	13/04/2017	Cabeceira do Sana, Macaé – RJ	22° 14′ 23.1″ S 42° 09′ 05.0″ W
	RJ95 - (MF423377)	19/04/2017	Santa Fé, Carmo – RJ	21° 53′ 05.0″ S 42° 32′ 29.7″ W
	RJ96 - (MF423378)	19/04/2017	Santa Fé, Carmo – RJ	21° 53′ 05.0″ S 42° 32′ 29.7″ W
Callithrix jacchus/penicillata (marmoset)	RJ97- (MF538785)	21/04/2017	Araras, Petrópolis – RJ	22° 23′ 51.1″ S 43° 10′ 56.5″ W
	RJ104 - (MF538786)	05/06/2017	Caneca Fina, Guapimirim – RJ	22° 29′ 35.9″ S 42° 56′ 58.9″ W
Human cases	H189† (MG550109)	18/04/2017	Bananal, Maricá – RJ	22° 55′ 25.3"S 42° 43′ 17.1"W
	H190 - (MF538782)	16/03/2017	São Fidélis – RJ	21° 38′ 17.2″ S 41° 45′ 49.2″ W
	H191 - (MF538783)	18/03/2017	Casimiro de Abreu – RJ	22° 29′ 10.5″ S 42° 12′ 06.2″ W
	H196 - (MF538784)	26/02/2017	Porciúncula – RJ	20° 49′ 17.5″ S 41° 54′ 38.6″ W
	H199 - (MF434851)	25/04/2017	Silva Jardim – RJ	22° 27′ 42.9″ S 42° 18′ 28.6″ W

*YFV ES504 and ES505 have been previously described [13].

†Partial genome sequence.

changes detected in the other sequences, except for the mutation in the C protein. Moreover, all 2017 Brazilian YFV sequences also share an amino acid change at position N/D2803S (NS5 residue 297). This amino acid substitution was also present in a unique Venezuelan strain isolated in 2006 (GenBank accession no. KM388818). We also identified additional amino acid substitutions that are not present in all of the 2017 Brazilian genomes analysed in this work (Fig. S1). Accordingly, the YFV H199 sequence shows a change from an alanine (A) to a serine (S) at the 826 amino acid position, corresponding to the NS1 protein (position 48). The YFV genomes RJ95, RJ96, RJ97, H191 and PA193 present a substitution from an isoleucine (I) to a valine (V) at position 2176 (NS4A, position 77).

Modelling and structural analysis of NS2B/NS3 and NS5 proteins

To gain some insight into the structural location and functional effects of the amino acid substitutions in the YFV NS3 and NS5 proteins, we built tridimensional models based on the crystallographic structures of flavivirus homologue proteins. The complete YFV NS3 model was constructed by utilizing the crystal structure of the DENV NS3 protease/helicase (PDB: 2VBC), displaying 51 % identity and 96 % coverage; the Zika virus NS3 protease domain (PDB: 5GJ4), with 56 % identity and 27 % coverage; and the YFV NS3 helicase domain (PDB: 1YKS), as aligned in Fig. S3. The three different templates were adopted as a consequence of the need to build a model of the complete protein, joining together the protease and helicase domains, focusing on their correct relative orientation, which was mainly achieved through the hinge domain featured in the DENV template structure. The NS2B/NS3 interface was based upon ZIKV crystallographic structure 5GJ4. The three active site residues of the NS3 protease domain were conserved in the YFV target sequence and the DENV and ZIKV template sequences. The YFV NS5 models were built based on the Japanese encephalitis virus NS5 protein (PDB ID: 4K6M), presenting 60 % identity and 98 % coverage (Fig. S4).

The effect of the amino acid substitutions on both NS3 and NS5 proteins was evaluated through hydrogen bond formation and electrostatic analysis. The two substitutions found in NS3, E88D and R121K (polyprotein positions: E1572D and R1605K, respectively) are conservative and, as such, they showed little impact on the surface electrostatic potential (Fig. 2c, d) when we compared the 2010 and 2017 YFV NS3 proteins. For the E88D substitution, a small structural

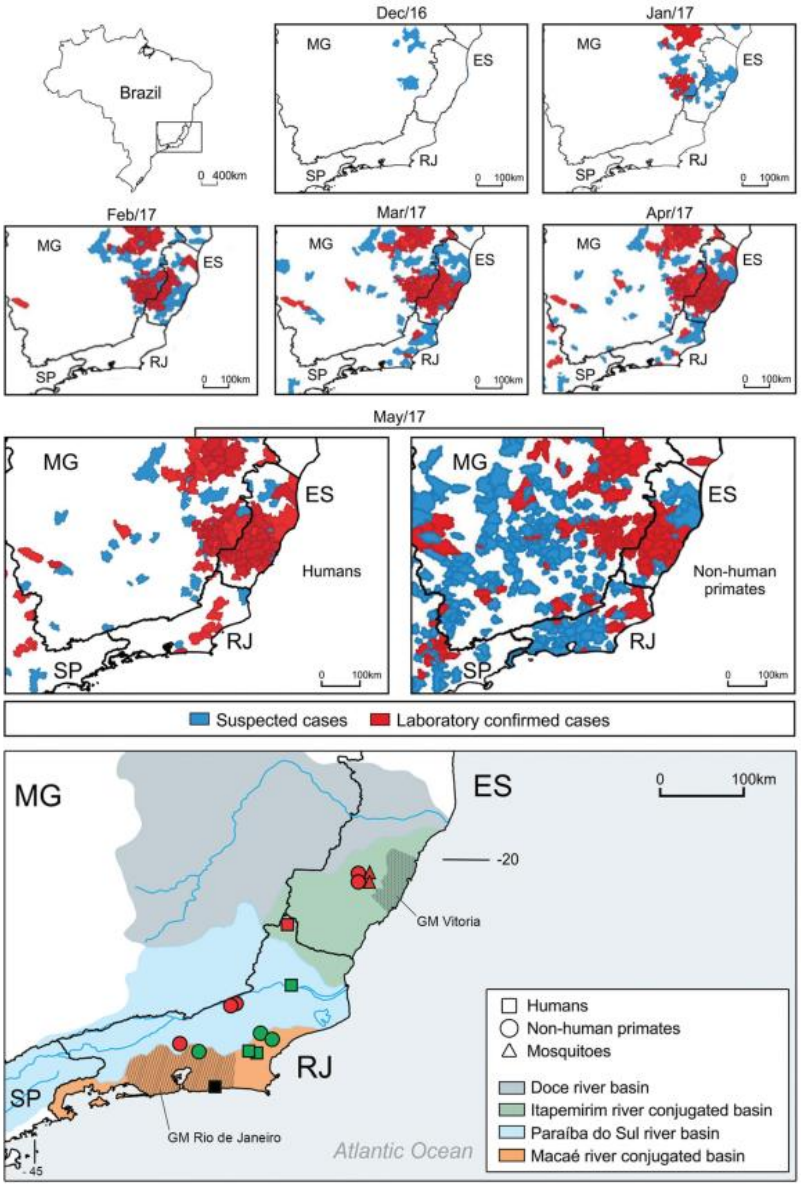

Fig. 1. The spatiotemporal spread of the yellow fever outbreak in southeast Brazil from December 2016 to May 2017 according to the Brazilian Ministry of Health [13], and geographical origins of the yellow fever virus (YFV) samples according to states, river basins, hosts and YFV sub-clades A (red) and B (green). The black square corresponds to the H189 sample, for which only partial sequences of the YFV genome were obtained. Brazilian states: ES, Espírito Santo; MG, Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo. The hatched areas correspond to the great metropolitan (GM) areas of Rio de Janeiro and Vitória.

change was observed, which mainly consisted of lysine 174 (polyprotein position: K1658) side-chain displacement due to the loss of a hydrogen bond with the protein backbone (Fig. 2e, f). On the other hand, the R121K substitution is located near the NS2B binding groove and might influence the interaction between these two molecules. Hydrogen bond analysis indicated that K121 could favour the formation of a hydrogen bond with threonine 77 of NS2B (polyprotein position: T1431), whereas such interaction was not identified in the 2010 model (Fig. 2a, b). This interaction

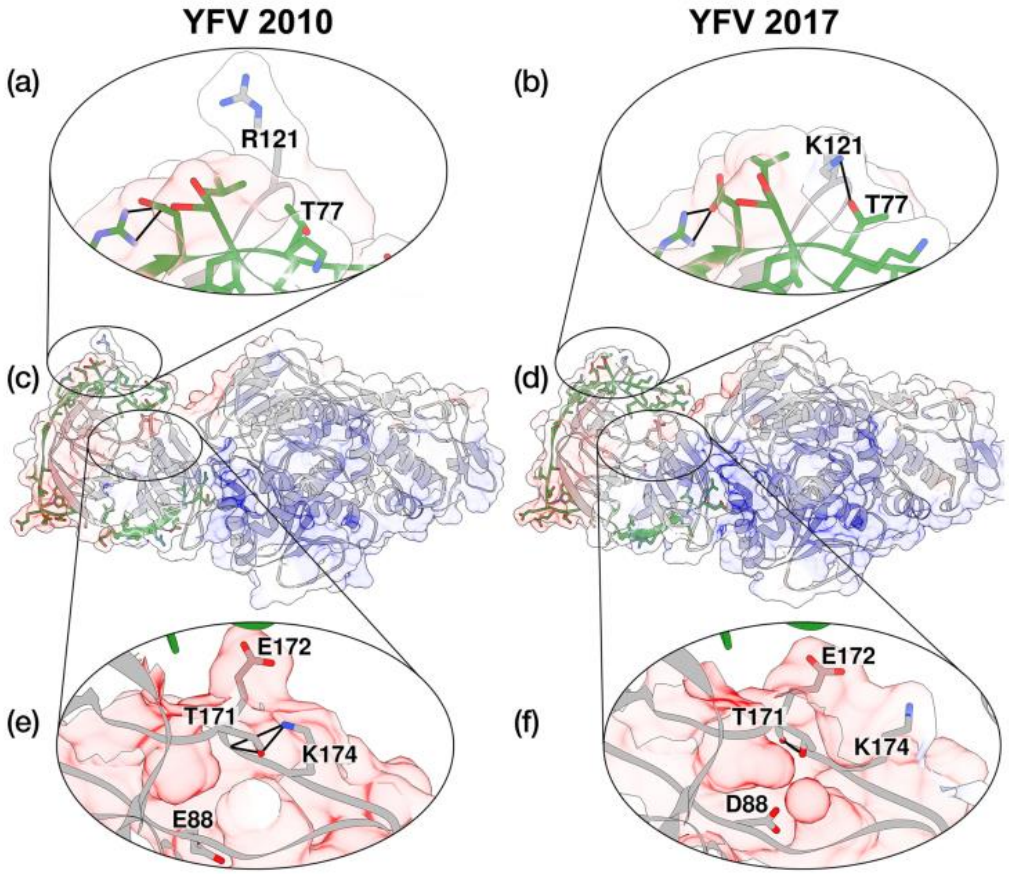

Fig. 2. Tridimensional models of the 2017 YFV and 2010 Venezuelan NS2B–NS3 protein complex developed by comparative modelling. Cartoon and surface electrostatic potential representation of the R121K (polyprotein residue 1605) substitution (a, b), whole complex (c, d), and E88D (polyprotein residue E1572D) substitution (e, f). The molecular surface is coloured according to the electrostatic potential, where red, white and blue correspond to acidic, neutral and basic potentials, respectively. NS2B is shown in green. Thick black lines represent hydrogen bonds (a, b, e, f).

could, in turn, modulate the NS3–NS2B binding affinity, and thus the protease efficiency.

The three first amino acid substitutions in NS5 are clustered in the MTase domain, whereas the remaining ones are in the RdRp domain (Fig. 3a). All amino acid substitutions at the MTase domain are conservative, but they are spatially adjacent. The arginine 101 (polyprotein position: R2607) alpha carbon is 9.7 Å away from that of the isoleucine 138 (polyprotein position: I2644), which in turn is 10.6 Å away from the serine 173 alpha carbon (polyprotein position: S2679). These three residues face the RdRp domain tunnel opening (Fig. 3b), which presents a basic electrostatic profile to accommodate the YFV RNA molecule (Fig. S5), which suggests that they might influence the enzyme activity. Additionally, the N297S substitution (polyprotein position: N/D/S 2803) from the RdRp domain, although conservative, is located near the hinge domain. The remaining two amino acid alterations – V643A (polyprotein position: V/A3149) and N709S (polyprotein position: N/S3215) – are located at the protein surface and are also conservative. Molecular dynamics simulation analysis revealed that mutations in the NS5 protein from the 2017 sample show a decrease in fluctuation around the hinge region (Fig. S6).

Evolutionary and phylogeographical analyses

Phylogenetic analysis of prM/E sequences indicated that all of the 2017 YFV Brazilian strains cluster inside sub-lineage 1E of the modern lineage of South America genotype I in a monophyletic clade with high support (bootstrap=87 %) (Fig. S7). The same clustering pattern was obtained when we performed phylogenetic analyses on either NS3 or NS5 nucleotide sequences (Fig. S8).

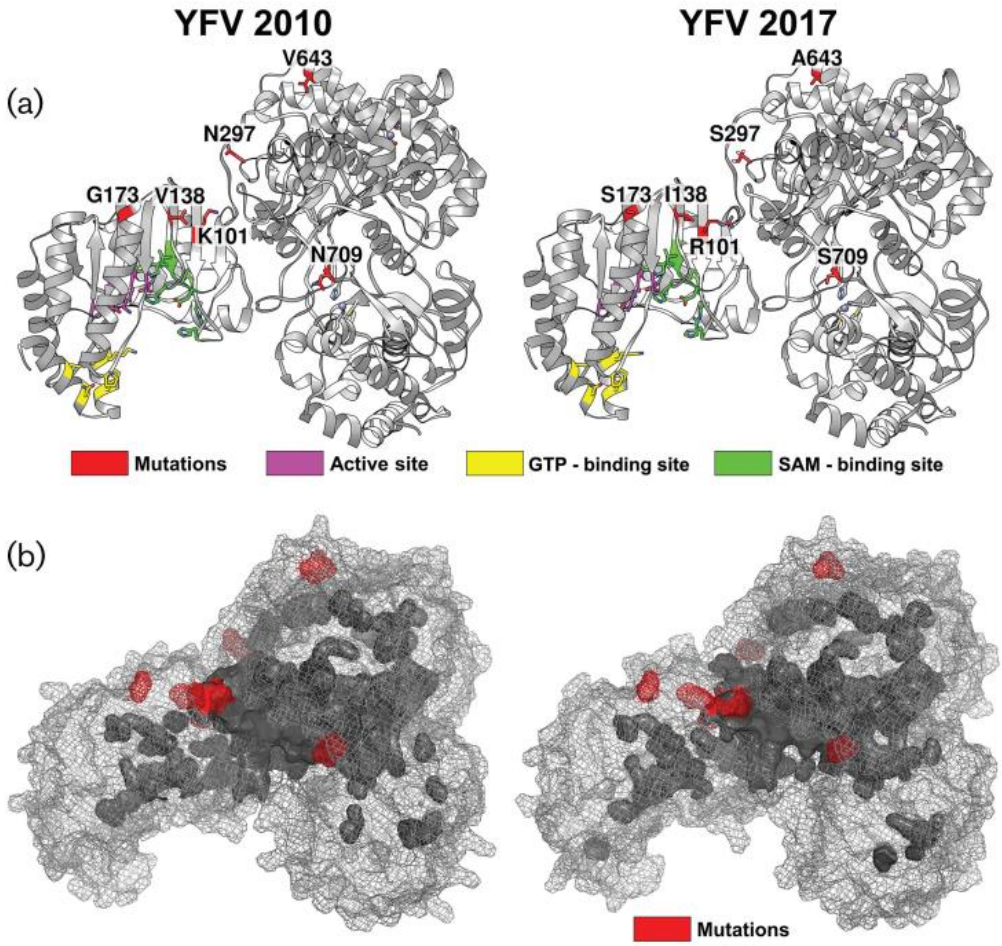

Fig. 3. Tridimensional models of the 2017 YFV and 2010 Venezuelan NS5 proteins established by comparative modelling. (a) Cartoon representation of NS5 protein. Amino acid substitutions and binding site residues are shown in the sticks and are coloured according to the legend. (b) Cavities of NS5 protein. Amino acid substitution sites are shown in red. The amino acid alterations showed in the models at the MTase domain are K101R, V138I and G173S (polyprotein residues 2607, 2644, and 2679, respectively), while those at the RdRp domain are N297S, V643A and N709S (polyprotein residues 2803, 3149, and 3215, respectively).

The Bayesian maximum clade credibility (MCC) tree inferred from the 34 complete open reading frame (ORF) sequences of YFV South American genotypes I and II disclosed that YFV strains from the ongoing outbreak in southeast Brazil grouped in a highly supported [posterior probability (PP)=1] monophyletic cluster nested within sub-lineage E strains of the modern lineage (Fig. 4). The median substitution rate of the complete ORF of the YFV South American genotypes was estimated at 3.5×10^{-4} substitution/site/year (95 % HPD: 2.4–4.8×10^{-4} substitution/site/year), in agreement with previous estimations, while the median TMRCA for all Brazilian 2017 YFV strains was estimated in April 2016 (95 % HPD: July 2015–October 2016). Ancestral ORFs were reconstructed at the key nodes of YFV genotype I phylogeny corresponding to the MRCA of all South American I genotype sequences (N1), all modern lineage sequences (N2), modern sub-clade 1E (N3), Venezuelan strains plus 2017 YFV strains (N4) and 2017 YFV strains (N5). Amino acid substitutions between ancestral key nodes were inferred and are shown in relation to the polyprotein positions. As previously described [12], six non-synonymous mutations were detected between nodes N1 and N3 (Figs 4 and S9). No amino acid substitutions were detected between nodes N3 and N4, whereas nine non-synonymous mutations (corresponding to the amino acid signatures of the 2017 YFV Brazilian strains) were detected between nodes N4 and N5 (Figs 4 and S9).

The inferred Bayesian tree further revealed that 2017 YFV Brazilian strains from RJ were intermixed with sequences

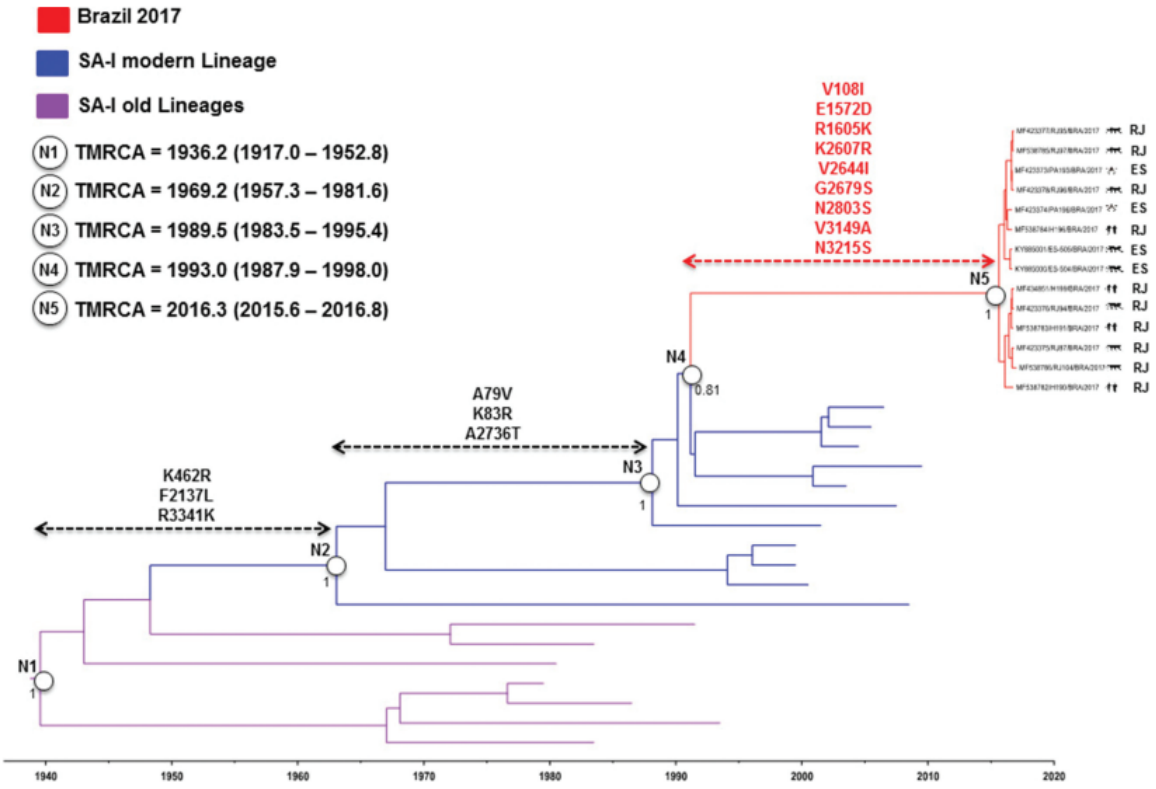

Fig. 4. Time-scaled Bayesian maximum clade credibility (MCC) based on the complete ORF of 34 YFV American strains. The tree branches are coloured according to the legend at the upper left. The names and accession numbers of the strains are provided in Table S2. Reconstructed ancestral key nodes of the South American genotype I (N1), modern-lineage sequences (N2), modern sub-clade 1E (N3), Venezuelan strains plus 2017 YFV Brazilian strains (N4) and 2017 YFV Brazilian strains (N5) are shown. Inferred amino acid substitutions between ancestral key nodes are shown in relation to the polyprotein positions. The amino acid substitutions shown in black have been described previously [12]. The state of origin (ES or RJ) of the 2017 Brazilian strains is indicated, together with the host source of the viruses. Posterior supports are shown at key nodes. All horizontal branch lengths are drawn to a scale of years. The tree is automatically rooted under the assumption of a relaxed molecular clock.

from ES, consistent with multiple viral exchanges between both Brazilian states (Fig. 4). To better understand the spatiotemporal dynamics of YFV dissemination in southeastern Brazil, we inferred the unobserved locations of sequence ancestors of the 14 Brazilian 2017 YFV sequences using a variety of continuous phylogeographical models, assuming homogeneous [Brownian diffusion (BD) model] and heterogeneous [relaxed random walk (RRW)model] dispersion rates among lineages. The BD model was only moderately rejected (log Bayes factor=3.6) in favour of the (RRW model with Cauchy distribution (Table 2), thus supporting no great variation in the dispersion rate among the branches. Consistent with this notion, the average diffusion rate value for the Brazilian YFV epidemic was roughly similar among all models, ranging from 3.0 to 3.4 km day^{-1} (Table 2). The continuous diffusion inference places the root location of Brazilian 2017 YFV sequences in ES and supports at least three independent introductions into RJ between late February and early April, followed by a rapid southward dissemination in the direction of the great metropolitan area of RJ (Fig. 5). According to this inference, the virus was spread within RJ following two main routes that correspond to highly supported ($PP \geq 0.98$) viral sub-clades exclusively composed of sequences from RJ (Fig. S10). In the first route, the virus first reached the municipality of São Fidelis (in the northeast of RJ) around late February and later advanced southward along the coastal side of the Serra do Mar, a 1500 km long system of mountain ranges and escarpments parallel to the Atlantic coast in southeastern Brazil, finally reaching the great metropolitan region (municipality of Guapimirim) in early June. In the second route, the virus first reached the municipality of Carmo on the northern state border with MG around early April and later continued southward along the northern side of the Serra do Mar, reaching the great metropolitan region (municipality of Petrópolis) in late April.

Table 2. Comparison of how continuous spatial models fit the 2017 Brazilian yellow fever virus dataset and estimated dispersal rate under the different models

	Homogeneous BD	Heterogeneous cauchy RRW	Heterogeneous gamma RRW	Heterogeneous lognormal RRW
PS – log ML*	−14395.7	−14392.1	−14396.3	−14394.8
PS – log BF†	3.6	Best-fitting model	4.2	2.7
SS – log ML*	−14395.8	−14392.0	−14396.5	−14394.6
SS – log BF†	3.8	Best-fitting model	4.5	2.6
Dispersal rate (km day^{-1})‡	3.0 (1.2–5.1)	3.4 (1.7–4.9)	3.3 (1.2–5.0)	3.4 (1.3–5.2)

*Log marginal likelihood (ML) estimates for the different continuous phylogeographical models obtained using the path sampling (PS) and stepping-stone sampling (SS) methods.

†The log Bayes factor (BF) is the difference of the log ML between alternative (H1) and null (H0) models (H1/H0). Log BFs >3 indicates that model H1 is more strongly supported by the data than model H0.

‡Posterior mean and 95 % HPD (in parentheses) estimates of the dispersal rate.

DISCUSSION

In the 2016–2017 YFV outbreak in Southeast Brazil, most of the epizootics and human cases originally occurred in inland rural areas of MG and subsequently in eastern Atlantic coastal sites under the influence of the ES rain forest segments. After that, the spreading YFV approached the great metropolitan areas of Vitoria (ES) and Rio de Janeiro (RJ) contiguously, where nearly 1.8 and 12.3 million unvaccinated inhabitants, respectively, resided. Alarmingly, these densely populated areas possess some of the busiest South American airports, ports and road networks, are highly infested by YFV-competent urban vectors (*Ae.aegypti* and *Ae.albopictus*) and have repeatedly experienced severe dengue epidemics. Altogether, these ecological and sanitary marks raised concern about the potential risk of YFV reemerging in an urban cycle in Brazil, in addition to the risk of rapid transgression to other countries and continents [18, 19].

The YFV samples analysed in the current study are almost identical at the nucleotide and amino acid levels, regardless of having been derived from 5 distinct host species infected in a 5-month lag from 11 sites dispersed along 350 km of the southeast Brazilian coast across the outbreak territory, including the great metropolitan area of Rio de Janeiro. They also share a molecular signature represented by nine amino acids, with eight in highly conserved positions at NS3 and NS5 proteins and one in the structural capsid protein. A previous analysis of 2017 YFV from two howler monkeys from a single locality in ES did not consider the substitution at position 2803 inside the NS5 protein. However in the current study, the analysis of a higher number of samples confirmed this substitution in all analysed 2017 YFV strains. This molecular signature represented by nine amino acids has never been documented before in YFV strains of South American and African origin [15].

The main source of the variability of the modern YFV strains isolated in Trinidad and Tobago, Brazil and Venezuela during the 2000s in relation to ancestral lineages circulating in previous decades is the occurrence of several amino acid substitutions, particularly within non-structural viral proteins [12]. Interestingly, eight out of the nine amino acid substitutions representing the molecular signature of the 2017 YFV Brazilian strains analysed here localized inside the NS3 and NS5 proteins. Hypothetically, amino acid changes at conserved protein positions may play a role in the capacity for viral infection to vertebrate and invertebrate hosts, thus accelerating the spread of the ongoing outbreak. The NS3 and NS5 proteins have multiple enzymatic activities that are essential for viral RNA replication and 5′-capping [20–22]. Several studies have demonstrated the significance of non-structural proteins interacting with other viral proteins and host molecules [22–25], including those involved in the host innate immune response against flavivirus infection [24, 26–28]. Thus, amino acid changes at those conserved protein positions may have an impact on the capacity for viral infection to vertebrate and invertebrate hosts, which may in turn accelerate the ongoing outbreak. Furthermore, previous arbovirus studies have demonstrated that, despite evolutionary constraints, changes in the host range and/or the efficiency of infection and replication in key amplification hosts or vectors may occur via simple point mutations [29]. Hence, the conservative substitution A226V in the E1 protein of the Chikungunya virus appears to be an early event in the multistep process of CHIKV adaptation to *Ae. albopictus* [30].

For these reasons, we investigated the potential impact of the identified amino acid substitutions through structural analysis of protein models. All of the amino acid substitutions occur close to domains that might be affected by these subtle modifications. In the NS3 protein, the R121K substitution is located in the region responsible for the interaction with NS2B, the cofactor for the proteolytic activity of this enzyme. Although both residues bear a positive charge, lysine was shown to potentially establish a hydrogen bond with NS2B, due to the less bulky side-chain. Looking at the NS5 protein, we found that the three amino acid substitutions located at the MTase domain were spatially contiguous and could influence the relative orientation between the two domains. The N297S substitution might also have a significant role in enzymatic efficiency, since it is located near the hinge domain. Hence, molecular dynamics simulations have shown that all these substitutions combined may have a stabilizing effect on the linker domain, which has been demonstrated to influence

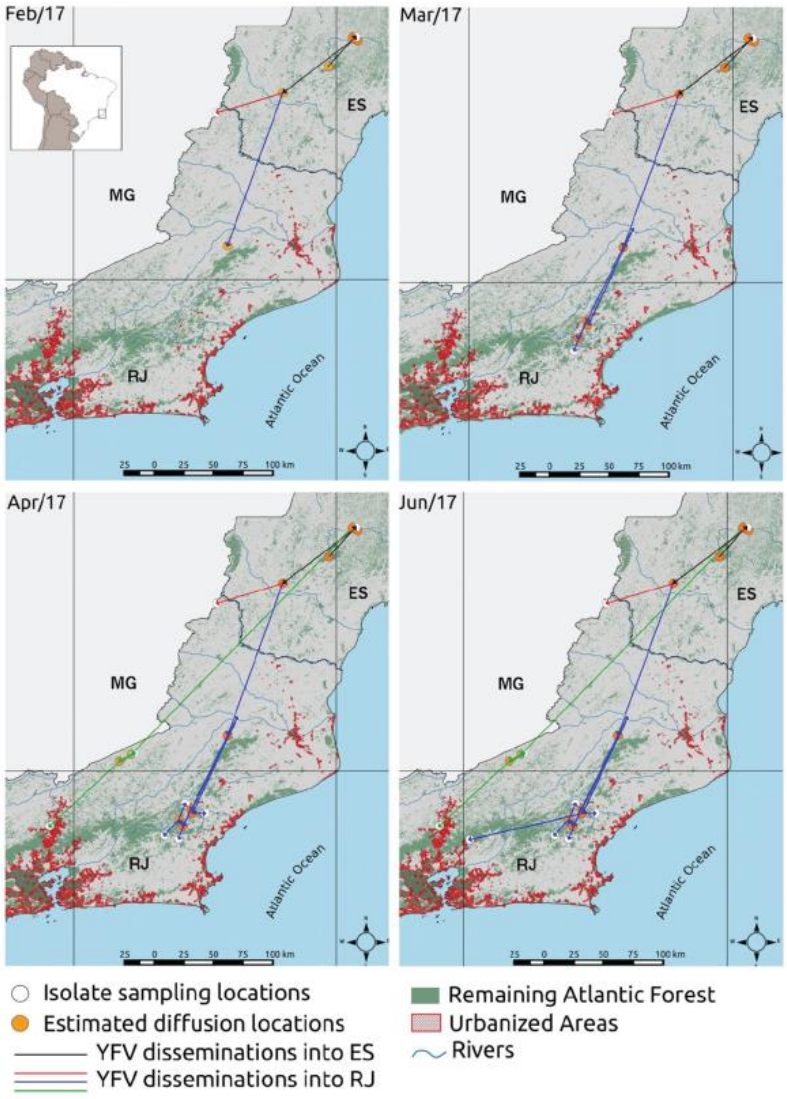

Fig. 5. Reconstructed spatiotemporal diffusion of the yellow fever outbreak in southeast Brazil from December 2016 to May 2017 under the heterogeneous Cauchy relaxed random walk (RRW) model. The arches represent a spatial projection of a representative phylogeny, with each node being mapped to its known (external node with a white circle) or estimated (internal node) location (orange circles). The black arches indicate dissemination events within ES, while the green, blue and red arches denote the three inferred YFV disseminations into RJ. Brazilian states: ES, Espírito Santo; MG, Minas Gerais; RJ, Rio de Janeiro. The hatched areas correspond to the great metropolitan (GM) areas of Rio de Janeiro and Vitória.

the enzyme processivity directly and viral replication in dengue 4 virus *in vitro* models [31, 32]. These findings shall be further addressed in future studies considering the unrevealed diversity of the YFV in cell culture and animal models.

All of the 2017 Brazilian YFV belonged to sub-lineage 1E and clustered with the Venezuelan 2004–2010 YFV strains. Although this is consistent with the notion that the ancestral YFV strain responsible for the ongoing Brazilian outbreak would have originated in Venezuela [12], the 2017 Brazilian and Venezuelan YFV strains share a relatively distant common ancestor with 2004–2010 Venezuelan YFV strains that can be traced to 1993 (95 % HPD: 1987–1997). This indicates that the YFV modern lineage may have been circulating in endemic Brazilian regions for a long period before

being introduced into southeast Brazil, but the precise pathway of viral dissemination is difficult to elucidate because of the scarcity of Brazilian YFV sequences sampled from endemic regions over the last 10–15 years. We estimate that the median TMRCA for the Brazilian 2017 YFV strains is early 2016, suggesting that the virus circulated for several months in the southeastern region before the ongoing outbreak was first recognized in December 2016.

The reconstruction of ancestral ORFs at key internal nodes of the YFV genotype I phylogeny showed a fast rate of amino acid fixation over the last few decades of YFV evolution in the Americas. Over an estimated interval of 55 years (1930s–1990s) between the most recent common ancestors (MRCAs) of genotype I and modern sub-clade 1E six amino acid substitutions were fixed in the YFV genome, as previously observed by Mir and colleagues [12]. During the last 25 years (1990s–2010s), spanning the period between the MRCAs of modern sub-clade 1E and the 2017 Brazilian lineage, nine amino acid substitutions were fixed, representing the 2017 YFV molecular signature. Another important difference between both periods is that the amino acid substitutions fixed between the 1930s and the 1990s were equally distributed among structural and non-structural viral proteins, while most (89 %) of amino acid substitutions established in the last 25 years were mapped in non-structural proteins. The potential association between the characterized amino acid substitutions, changes in YFV evolutionary dynamics and the recent emergence of severe outbreaks deserves further attention.

Our Bayesian phylogeographical analysis supports the contention that YFV first emerged in ES, which is consistent with epidemiological data, and later spread southward towards the great metropolitan area of RJ. Our continuous phylogeographical reconstruction supports multiple independent introductions into RJ between late February and early April and subsequent dissemination within the state following two main routes along the coastal and northern sides of the Serra do Mar mountain system. Both dissemination routes seem to converge in the great metropolitan region, reaching municipalities (Guapimirim and Petropolis) located only 70–85 km away from the city of Rio de Janeiro. According to our estimates, YFV spread southward from ES to the great metropolitan area of RJ with an average dispersal rate of 3.4 km day^{-1} (95 % HPD: 1.7–4.9 km day^{-1}), which is fully consistent with the expected rate of viral dissemination due to the dispersion of sylvatic mosquitoes and NHPs [33, 34], and with the epidemiological models based on the dates and location of monkey deaths that estimated the speed of displacement of the YFV in southeastern Brazil at 2.7 km day^{-1} during the warmer months [35].

The lack of YFV sequences from several heavily affected regions from ES and MG imposes an important limitation to our evolutionary and phylogeographical inference and the inclusion of more complete 2017 YFV genome sequences from ES and MG would help to better define the onset date and dispersion pathways of the 2017 YFV Brazilian lineage. Future analysis involving more YFV genomes from Brazilian endemic and non-endemic regions, as well as from neighbouring countries, from the last few years will be also crucial to better understand the evolutionary dynamics and epidemiology of YFV in recent years in South America. Studies based on reverse genetic approaches will also be important to establish whether the amino acid substitutions present in the Brazilian 2017 YFV lineage affect viral fitness and/or transmissibility.

METHODS

Mosquito samples

Adult mosquitoes were collected with BG-Sentinel adult traps baited with dry ice as a source of CO_2, as well as with an insect net when in proximity to humans in the forest, at the forest fringe and in the modified environment. The insects were immediately frozen in dry ice or N_2 and transported to the laboratory, where the species were identified according to Consoli and Lourenço-de-Oliveira [36], and processed as described elsewhere [37].

Non-human primate samples

Blood samples were taken from the femoral vein of dying NHPs or the cardiac cavity of recently dead NHPs. Samples from howler monkeys were obtained as previously described [15].

Human samples

Blood samples were obtained from patients for diagnostic procedures carried out at their respective municipal public health ambulatories. Serum samples of YFV laboratory-confirmed cases, negative for Zika, Chikungunya and dengue virus infections, were provided by the LACEN-RJ staff and stored at −80 °C until RNA extraction.

YFV RNA extraction, screening for YFV infection by RT-PCR and nucleotide sequencing

The detection of the YFV genome was performed as described elsewhere [15]. The sets of primers utilized in the PCR and sequencing procedures are listed in Tables S2 and S3, respectively. Complete genome sequences were deposited in the GenBank database (Table 1). Amplified products were sequenced as previously described [15]. The sequences were assembled with SeqMan Pro version 8.1.5 (DNASTAR, Madison, WI, USA). The Molecular Evolutionary Genetics Analysis (MEGA) 7.0 program was adopted to calculate nucleotide and amino acid distances, as well as to explore the amino acid differences.

Comparative modelling, optimization and MD simulation of NS3 pro and NS5 proteins

We modelled the NS2B–NS3 protein complex and the NS5 protein of the 2017 outbreak YFV prototype (Genbank, KY885001) together with the 2010 Venezuelan 10A strain (GenBank accession no. KM388816). Initially, we used the BLASTP program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), defining the Protein Data Bank (PDB) as a search set to select the template structures for comparative

modelling. Three PDB structures were incorporated as templates for the NS2B–NS3 protein complex. Template 2VBC (51 % identity and 96 % of coverage) corresponds to the crystal structure of the NS3 protease/helicase from dengue virus [38]. Template 1YKS (96 % identity and 70 % of coverage) comprises the NS3 helicase domain of the YFV [39]. Template 5GJ4 comprises the NS3 protease domain (56 % identity and 27 % of coverage) and the NS2B peptide cofactor (42 % identity and 31 % of coverage) of Zika virus [40]. For the NS5 model generation, a single PDB structure from the Japanese encephalitis virus (4K6M) [41] was employed as a template, sharing 60 % identity with the YFV sequence. The template and target sequences were then aligned with the PSI-Coffee mode of the T-Coffee program [42]. One hundred homology models were generated with the standard 'auto model' routine of Modeller version 9.18 [43] for each target sequence. Each model was optimized via the variable target function method (VTFM) until 300 iterations were achieved. Molecular dynamics (MD) optimization was conducted in the slow level mode. The full cycle was repeated twice to produce an optimized conformation of the model. The resulting modelled structures were selected according to their discrete optimized protein energy (DOPE) score. The GROMACS version 5.1.2 package [44] was used to carry out minimization using the AMBER99SB ILDN force field [45]. A short minimization procedure of 150 steps (100 steps of steepest descent plus 50 steps of conjugate gradient) was performed. Initial and optimized models were evaluated by DOPE, Ramachandran plot and QMean server (Table S4) [46]. Electrostatic potential analysis was performed with the APBS program [47]. Atomic partial charges and atomic radii parameters from the Amber force field were assigned with the PDB2PQR server [48]. The figures generated from sequence alignments were rendered using ALINE [49], and three-dimensional structures were generated with UCSF Chimera and PyMol.

Molecular dynamics simulations were carried out with the GROMACS package, with the AMBER99SB-ILDN force field. Protonation states were assigned using PDB2PQR software, and the zinc-coordinating cysteine residues were manually deprotonated. The models were further optimized prior to the MD runs through 5.0×106 steps of the steepest-descent (with and without heavy atom restraints) and conjugate-gradient algorithms. The systems were then run under an NPT ensemble, for 500 ps with restraints and 2.0×105 ps without restraints. The V-rescale thermostat and Berendsen barostat were adopted for temperature and pressure control, respectively. The 2010 and 2017 strains and replicas were simulated at 297 and 310 K for a total of eight runs and 1.6×106 ps. Analysis was achieved over the final 150 ns of the production runs.

Evolutionary and phylogeographical analyses

A 666-nucleotide sequence consisting of the last 108 nucleotide terminal flank of the prM gene, the entire M gene (225 nucleotides) and the first 333 nucleotides of the E gene was enlisted to genotype the YFV strains [5]. The phylogenetic tree was generated by the neighbour-joining method [50] with a matrix of genetic distances established under the Kimura two-parameter model [51]. The robustness of each node was assessed by bootstrap resampling (2000 replicates) [52]. The homologous region (prM/E) of a dengue virus strain available at the GenBank database (AF349753) was used as an outgroup.

The open reading frame (ORF; 10 239 nt-long coding region) of the YFV sequences of American origin (South America genotypes I and II) with a known date of isolation were obtained from the GenBank database (www.ncbi.nlm.nih.gov). The retrieved sequences (n=22) were aligned together with the sequences from the current study (n=12) and submitted to recombination analysis by applying the Recombination Detection Program (RDP) version 4.95 [53]. All of the analysed YFV sequences were confirmed as non-recombinant according to the RDP [54], GENECONV [55], MaxChi [56], Chimaera [57], BootScan [58], SiScan [59] and 3Seq [60] methods with their default settings. Only statistically significant (P<0.05) events supported by at least two methods were considered. The nucleotide substitution rate and time to the most recent common ancestor (TMRCA) of all 34 American YFV strains were estimated with respect to the Markov chain Monte Carlo (MCMC) algorithm implemented in the BEAST version 1.8.3 package [61, 62] with BEAGLE [63] to improve run time. In order to analyse fixed amino acid changes during the evolution of the YFV sequences, complete ORFs were reconstructed at key internal nodes of the American YFV complete genome phylogeny with the BEAST version 1.8.3 package. The consensus complete ORF sequence for each key ancestral node was computed with the R package SeqinR [51].

The BEAST version 1.8.3 package was also adopted to reconstruct the spatiotemporal dynamics of the 14 YFV complete ORFs from the Brazilian 2016–2017 YF epidemic in continuous space, utilizing the latitude and longitude of each Brazilian 2017 YFV isolate. The unobserved locations of the sequence ancestors in continuous space were estimated with a homogenous Brownian diffusion (BD) model and the heterogeneous Cauchy, Gamma and lognormal relaxed random walk (RRW) models [64]. The diffusion models were compared in accordance with log marginal likelihood estimation based on the path sampling (PS) and stepping-stone (SS) sampling methods [65]. The best-fit nucleotide substitution model (GTR+I+Γ4 for the American YFV dataset and HKY for the Brazilian YFV dataset), a relaxed uncorrelated lognormal molecular clock model [66] and a Bayesian skyline coalescent tree prior [67] were employed in all analyses. The uncertainty of the parameter estimates was assessed after the initial 10 % of the run was excluded by calculating the effective sample size (ESS) and the 95 % highest probability density (HPD) values, respectively, with the TRACER version 1.6 program [68]. TreeAnnotator version 1.7.5 [62] and FigTree version 1.4.0 [69] programs were adopted to summarize the posterior tree distribution and visualize the annotated maximum clade credibility (MCC) tree, respectively.

Maps

The maps were generated with QGIS GIS software using public access data collected from the Brazilian Institute of Geography and Statistics (IBGE) [70], the Ministry of the Environment [71] and the Brazilian Ministry of Health [13] websites.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was reviewed and approved by the Ethics Committee for human research at the Instituto Oswaldo Cruz (IOC) (CAAE 69206217.8.0000.5248), which exempted the need for specific written informed consent from patients or their legal representatives. The protocols for mosquito rearing as well as handling and blood sampling of NHPs were approved by the Institutional Ethics Committee of Animal use at IOC (CEUA licenses LW-34/2014 and L037/2016, respectively). Capture of wild NHPs and mosquitoes was approved by the Brazilian environmental authorities: SISBIO-MMA licenses 54707-137362-2 and 52472-1, and INEA license 012/2016012/2016.

References

1. **Barnett ED.** Yellow fever: epidemiology and prevention. *Clin Infect Dis* 2007;44:850–856.
2. **Monath TP, Vasconcelos PF.** Yellow fever. *J Clin Virol* 2015;64: 160–173.
3. **Weaver SC, Reisen WK.** Present and future arboviral threats. *Antiviral Res* 2010;85:328–345.
4. **Vasconcelos PF, Monath TP.** Yellow Fever remains a potential threat to public health. *Vector Borne Zoonotic Dis* 2016;16:566–567.
5. **Bryant JE, Holmes EC, Barrett AD.** Out of Africa: a molecular perspective on the introduction of yellow fever virus into the Americas. *PLoS Pathog* 2007;3:e75.
6. **Mutebi JP, Wang H, Li L, Bryant JE, Barrett AD.** Phylogenetic and evolutionary relationships among yellow fever virus isolates in Africa. *J Virol* 2001;75:6999–7008.
7. **Staples JE, Monath TP.** Yellow fever: 100 years of discovery. *JAMA* 2008;300:960–962.
8. **Nunes MR, Palacios G, Cardoso JF, Martins LC, Sousa EC *et al.*** Genomic and phylogenetic characterization of Brazilian yellow fever virus strains. *J Virol* 2012;86:13263–13271.
9. **Vasconcelos PF, Bryant JE, da Rosa TP, Tesh RB, Rodrigues SG *et al.*** Genetic divergence and dispersal of yellow fever virus, Brazil. *Emerg Infect Dis* 2004;10:1578–1584.
10. **Romano AP, Costa ZG, Ramos DG, Andrade MA, Jayme VS *et al.*** Yellow fever outbreaks in unvaccinated populations, Brazil, 2008–2009. *PLoS Negl Trop Dis* 2014;8:e2740.
11. **de Souza RP, Foster PG, Sallum MA, Coimbra TL, Maeda AY *et al.*** Detection of a new yellow fever virus lineage within the South American genotype I in Brazil. *J Med Virol* 2010;82:175–185.
12. **Mir D, Delatorre E, Bonaldo M, Lourenço-de-Oliveira R, Vicente AC *et al.*** Phylodynamics of yellow fever virus in the Americas: new insights into the origin of the 2017 Brazilian outbreak. *Sci Rep* 2017;7: 7385.
13. **Health BMo.** 2017. Emergência epidemiológica de febre amarela no Brasil, no período de dezembro de 2016 a julho de 2017. http://portalarquivos.saude.gov.br/images/pdf/2017/setembro/06/2017_027.pdf [accessed 2017].
14. **Pan American Health Organization.** 2017. Epidemiological update -yellow fever. www.paho.org/hq/index.php?option=com_docman&-task=doc_view&Itemid=270&gid=40841g=en [accessed].
15. **Bonaldo MC, Gómez MM, dos Santos AA, Abreu FVS, Ferreira-de-Brito A *et al.*** Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. *Mem Inst Oswaldo Cruz* 2017;112:447–451.
16. **Beasley DW, Mcauley AJ, Bente DA.** Yellow fever virus: genetic and phenotypic diversity and implications for detection, prevention and therapy. *Antiviral Res* 2015;115:48–70.
17. **Carrington CV, Auguste AJ.** Evolutionary and ecological factors underlying the tempo and distribution of yellow fever virus activity. *Infect Genet Evol* 2013;13:198–210.
18. **Couto-Lima D, Madec Y, Bersot MI, Campos SS, Motta MA *et al.*** Potential risk of re-emergence of urban transmission of yellow fever virus in Brazil facilitated by competent Aedes populations. *Sci Rep* 2017;7:4848.
19. **Dorigatti I, Hamlet A, Aguas R, Cattarino L, Cori A *et al.*** International risk of yellow fever spread from the ongoing outbreak in Brazil, December 2016 to May 2017. *Euro Surveill* 2017;22:30572.
20. **Carpp LN, Galler R, Bonaldo MC.** Interaction between the yellow fever virus nonstructural protein NS3 and the host protein Alix contributes to the release of infectious particles. *Microbes Infect* 2011; 13:85–95.
21. **Patkar CG, Kuhn RJ.** Yellow Fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions. *J Virol* 2008;82:3342–3352.
22. **Teramoto T, Balasubramanian A, Choi KH, Padmanabhan R.** Serotype-specific interactions among functional domains of dengue virus 2 nonstructural proteins (NS) 5 and NS3 are crucial for viral RNA replication. *J Biol Chem* 2017;292:9465–9479.
23. **Le Breton M, Meyniel-Schicklin L, Deloire A, Coutard B, Canard B *et al.*** Flavivirus NS3 and NS5 proteins interaction network: a high-throughput yeast two-hybrid screen. *BMC Microbiol* 2011;11:234.
24. **Wu RH, Tsai MH, Tsai KN, Tian JN, Wu JS *et al.*** Mutagenesis of dengue virus protein NS2A revealed a novel domain responsible for virus-induced cytopathic effect and interactions between NS2A and NS2B transmembrane segments. *J Virol* 2017;91:e01836-16.

25. **Zou J, Lee L, Wang QY, Xie X, Lu S *et al.*** Mapping the Interactions between the NS4B and NS3 proteins of dengue virus. *J Virol* 2015; 89:3471–3483.
26. **Cumberworth SL, Clark JJ, Kohl A, Donald CL.** Inhibition of type I interferon induction and signalling by mosquito-borne flaviviruses. *Cell Microbiol* 2017;19:e12737.
27. **Mathew A, Townsley E, Ennis FA.** Elucidating the role of T cells in protection against and pathogenesis of dengue virus infections. *Future Microbiol* 2014;9:411–425.
28. **Schlesinger JJ, Foltzer M, Chapman S.** The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. *Virology* 1993;192:132–141.
29. **Coffey LL, Forrester N, Tsetsarkin K, Vasilakis N, Weaver SC.** Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease. *Future Microbiol* 2013;8:155–176.
30. **Tsetsarkin KA, Chen R, Leal G, Forrester N, Higgs S *et al.*** Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes. *Proc Natl Acad Sci USA* 2011;108:7872–7877.
31. **Lim SP, Koh JH, Seh CC, Liew CW, Davidson AD *et al.*** A crystal structure of the dengue virus non-structural protein 5 (NS5) polymerase delineates interdomain amino acid residues that enhance its thermostability and de novo initiation activities. *J Biol Chem* 2013;288:31105–31114.
32. **Zhao Y, Soh TS, Zheng J, Chan KW, Phoo WW *et al.*** A crystal structure of the dengue virus NS5 protein reveals a novel interdomain interface essential for protein flexibility and virus replication. *PLoS Pathog* 2015;11:e1004682.
33. **Causey OR, Kumm HW, Laemmert HW.** Dispersion of forest mosquitoes in Brazil; further studies. *Am J Trop Med Hyg* 1950;30: 301–312.
34. **Jung L, Mourthe I, Grelle CE, Strier KB, Boubli JP.** Effects of local habitat variation on the behavioral ecology of two sympatric groups of brown howler monkey (*Alouatta clamitans*). *PLoS One* 2015;10: e0129789.
35. **Fioravanti C.** O alarme dos macacos. *Revista Pesquisa Fapesp* 2018.
36. **Consoli R, Oliveira RL.** *Principais Mosquitos de Importância Sanitária no Brasil.* Rio de Janeiro: Editora FIOCRUZ; 1994.
37. **Ferreira-de-Brito A, Ribeiro IP, Miranda RM, Fernandes RS, Campos SS *et al.*** First detection of natural infection of Aedes aegypti with Zika virus in Brazil and throughout South America. *Mem Inst Oswaldo Cruz* 2016;111:655–658.
38. **Luo D, Vasudevan SG, Lescar J.** The flavivirus NS2B-NS3 protease-helicase as a target for antiviral drug development. *Antiviral Res* 2015;118:148–158.
39. **Wu J, Bera AK, Kuhn RJ, Smith JL.** Structure of the Flavivirus helicase: implications for catalytic activity, protein interactions, and proteolytic processing. *J Virol* 2005;79:10268–10277.
40. **Phoo WW, Li Y, Zhang Z, Lee MY, Loh YR *et al.*** Structure of the NS2B-NS3 protease from Zika virus after self-cleavage. *Nat Commun* 2016;7:13410.
41. **Lu G, Gong P.** Crystal structure of the full-length Japanese encephalitis virus NS5 reveals a conserved methyltransferase-polymerase interface. *PLoS Pathog* 2013;9:e1003549.
42. **di Tommaso P, Moretti S, Xenarios I, Orobitg M, Montanyola A *et al.*** T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res* 2011;39:W13–W17.
43. **Webb B, Sali A.** Protein structure modeling with MODELLER. In: Kihara D (editor). *Protein Structure Prediction (Methods in Molecular Biology)*. New York: Springer; 2014. pp. 1–15.
44. **Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC *et al.*** GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* 2015;1-2:19–25.
45. **Lindorff-Larsen K, Piana S, Palmo K, Maragakis P, Klepeis JL *et al.*** Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins* 2010;78:1950–1958.
46. **Benkert P, Künzli M, Schwede T.** QMEAN server for protein model quality estimation. *Nucleic Acids Res* 2009;37:W510–W514.
47. **Baker NA, Sept D, Joseph S, Holst MJ, Mccammon JA.** Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci USA* 2001;98:10037–10041.
48. **Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA.** PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res* 2004;32:W665–W667.
49. **Bond CS, Schüttelkopf AW.** ALINE: a WYSIWYG protein-sequence alignment editor for publication-quality alignments. *Acta Crystallogr D Biol Crystallogr* 2009;65:510–512.
50. **Saitou N, Nei M.** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.
51. **Kimura M.** A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980;16:111–120.
52. **Felsenstein J.** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783–791.
53. **Martin DP, Murrell B, Golden M, Khoosal A, Muhire B.** RDP4: detection and analysis of recombination patterns in virus genomes. *Virus Evol* 2015;1:vev003.
54. **Martin D, Rybicki E.** RDP: detection of recombination amongst aligned sequences. *Bioinformatics* 2000;16:562–563.
55. **Padidam M, Sawyer S, Fauquet CM.** Possible emergence of new geminiviruses by frequent recombination. *Virology* 1999;265:218–225.
56. **Smith JM.** Analyzing the mosaic structure of genes. *J Mol Evol* 1992;34:126–129.
57. **Posada D, Crandall KA.** Evaluation of methods for detecting recombination from DNA sequences: computer simulations. *Proc Natl Acad Sci USA* 2001;98:13757–13762.
58. **Martin DP, Posada D, Crandall KA, Williamson C.** A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. *AIDS Res Hum Retroviruses* 2005;21:98–102.
59. **Gibbs MJ, Armstrong JS, Gibbs AJ.** Sister-scanning: a Monte Carlo procedure for assessing signals in recombinant sequences. *Bioinformatics* 2000;16:573–582.
60. **Boni MF, Posada D, Feldman MW.** An exact nonparametric method for inferring mosaic structure in sequence triplets. *Genetics* 2007;176:1035–1047.
61. **Drummond AJ, Rambaut A.** BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007;7:214.
62. **Drummond AJ, Suchard MA, Xie D, Rambaut A.** Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* 2012;29: 1969–1973.
63. **Suchard MA, Rambaut A.** Many-core algorithms for statistical phylogenetics. *Bioinformatics* 2009;25:1370–1376.
64. **Lemey P, Rambaut A, Welch JJ, Suchard MA.** Phylogeography takes a relaxed random walk in continuous space and time. *Mol Biol Evol* 2010;27:1877–1885.
65. **Baele G, Lemey P, Bedford T, Rambaut A, Suchard MA *et al.*** Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Mol Biol Evol* 2012;29:2157–2167.
66. **Drummond AJ, Ho SY, Phillips MJ, Rambaut A.** Relaxed phylogenetics and dating with confidence. *PLoS Biol* 2006;4:e88.
67. **Drummond AJ, Rambaut A, Shapiro B, Pybus OG.** Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol Biol Evol* 2005;22:1185–1192.
68. **Rambaut A, Suchard M, Drummond A.** 2013. Tracer v1.6. http://tree.bio.ed.ac.uk/software/tracer/.
69. **Rambaut A.** 2014. FigTree v1.4. http://tree.bio.ed.ac.uk/software/figtree/.
70. **(IBGE) BloGaS.** MAPAS. 2017.
71. **Environment BMot.** Monitoramento de biomas. 2017.

4.6 Artigo 6: Distinct YFV lineages co-circulated in the Central-Western and Southeastern Brazilian regions from 2015 to 2018

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Distinct YFV Lineages Co-circulated in the Central-Western and Southeastern Brazilian Regions From 2015 to 2018

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The current outbreak of yellow fever virus (YFV) that is afflicting Brazil since the end of 2016 probably originated from a re-introduction of YFV from endemic areas into the non-endemic Southeastern Brazil. However, the lack of genomic sequences from endemic regions hinders the tracking of YFV's dissemination routes. We assessed the origin and spread of the ongoing YFV Brazilian outbreak analyzing a new set of YFV strains infecting humans, non-human primates (NHPs) and mosquitoes sampled across five Brazilian states from endemic and non-endemic regions between 2015 and 2018. We found two YFV sub-clade 1E lineages circulating in NHP from Goiás state (GO), resulting from independent viral introductions into the Araguaia tributary river basin: while one strain from 2017 clustered intermingled with Venezuelan YFV strains from 2000, the other YFV strains sampled in 2015 and 2017 clustered with sequences of the current YFV outbreak in the Brazilian Southeastern region (named $YFV_{2015-2018}$ lineage), displaying the same molecular signature associated to the current YFV outbreak. After its introduction in GO at around mid-2014, the $YFV_{2015-2018}$ lineage followed two paths of dissemination outside GO, originating two major YFV sub-lineages: (1) the $YFV_{MG/ES/RJ}$ sub-lineage spread sequentially from the eastern area of Minas Gerais state to Espírito Santo and then to Rio de Janeiro states, following the Southeast Atlantic basin; (2) the $YFV_{MG/SP}$ sub-lineage spread from the southwestern area of Minas Gerais to the metropolitan

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region of São Paulo state, following the Paraná basin. These results indicate the ongoing YFV outbreak in Southeastern Brazil originated from a dissemination event from GO almost 2 years before its recognition at the end of 2016. From GO this lineage was introduced in Minas Gerais state at least two times, originating two sub-lineages that followed different routes toward densely populated areas. The spread of YFV outside endemic regions for at least 4 years stresses the imperative importance of the continuous monitoring of YFV to aid decision-making for effective control policies aiming the increase of vaccination coverage to avoid the YFV transmission in densely populated urban centers.

Keywords: yellow fever virus, Brazilian outbreak, lineages, amino acid changes, phylogeography

INTRODUCTION

In Brazil, the yellow fever virus (YFV) have been sporadically detected in human and non-human primates (NHPs) populations from the enzootic/endemic Northern (Amazon) and epidemic Central-Western regions during the second half of the 20th century (Carrington and Auguste, 2013; Monath and Vasconcelos, 2015). Since the early 2000s, the virus has progressively expanded to the Southeastern and Southern Brazilian regions and in December 2016 began the largest epizootic/epidemic of sylvatic YF registered in the country over the last 50 years (Vasconcelos, 2010; Possas et al., 2018a,b). Between December 2016 and June 2018, a total of 2,139 YF human cases were confirmed in all Southeastern Brazilian states of Minas Gerais (n = 997), São Paulo (n = 577), Rio de Janeiro (n = 307) and Espírito Santo (n = 258) with 735 deaths (case-fatality, 34%). Moreover, in 2019 YFV transmission continues in São Paulo and is emerging in the north of Paraná state from South region of Brazil (**Supplementary Figure 1**; Secretaria de Vigilância em Saúde, 2019).

Sequential YF outbreaks reported in the Southeastern and Southern Brazilian regions in 2000–2001, 2008–2009 and 2016–2018 were more likely caused by single independent events of re-introduction of YFV strains from endemic areas (Mir et al., 2017). A recent study speculated that the YFV strain causing the current outbreak would have been originated in the Brazilian Central-West region. This conjecture was based on the date of the most recent common ancestor of the 2016–2018 Brazilian YFV, which was estimated in a period (July 2014 to January 2016) when YFV circulation was reported in the state of Goiás (Central-Western region; Rezende et al., 2018). However, the precise routes of dissemination of YFV strains from endemic to non-endemic areas observed in Brazil in the last 15–20 years are difficult to elucidate because of the scarcity of sequences sampled from endemic regions in that period.

The spatiotemporal dynamics of dissemination of the 2016–2018 Brazilian YFV lineage within the Southeastern region also remained unclear. The first study considering full-genome YFV sequences based on samples from Espírito Santo and Rio de Janeiro states from 2017 placed the origin of the Southeastern outbreak in Espírito Santo in April 2016 (July 2015 to October 2016) and supported a rapid southward viral dissemination in direction to the great metropolitan area of Rio de Janeiro (Gomez et al., 2018). A second study also comprising full-genome YFV sequences sampled from Minas Gerais, Espírito Santo and Rio de Janeiro in 2017 supports that the outbreak arose in Minas Gerais in July 2016 (March to November 2016) and was then southerly disseminated toward Espírito Santo and Rio de Janeiro (Faria et al., 2018). A third study, based on partial genome sequences, showed that YFV strains isolated in the state of São Paulo in 2016 branched in basal position relatively to those isolated in Minas Gerais and Espírito Santo at 2017–2018 and traced the origin of current outbreak to July 2015 (July 2014 to January 2016), thus suggesting that the 2016–2018 YFV Brazilian lineage may have circulated in São Paulo state before spread to Minas Gerais and Espírito Santo (Rezende et al., 2018).

Therefore, to define in greater detail the geographic origin and subsequent dissemination routes of the 2016–2018 Brazilian YFV clade, we generated and analyzed 13 YFV new genomes obtained from humans, NHPs and mosquitoes, between 2015 and 2018, from all Southeast Brazilian states (Minas Gerais, Espírito Santo, Rio de Janeiro e São Paulo) and from Central-West region (Goiás). Interestingly, we identified two YFV lineages circulating in Central-West (Goiás) during 2015–2017, one (GO27/2015 and GO21/2017) displaying the nine amino acid signature characteristic of the 2016–2018 YFV Southeastern outbreak (Bonaldo et al., 2017) and the other (GO05/2017) isolated 2 year later that displayed an amino acid pattern typical of older YFV sub-clade 1E strains sampled in Brazil and Venezuela between 2000 and 2010 (De Souza et al., 2010). Besides, these 13 new YFV complete coding region sequences (CDS) were combined with previously described YFV CDS from Brazil (n = 67), Venezuela (n = 5) and Trinidad and Tobago (n = 1) and then subjected to phylogeographic analyses to get a better picture of the ongoing YFV epidemic in Brazil.

MATERIALS AND METHODS

YFV Samples

Viral samples from 13 infected hosts from distinct biomes and river basins in the states of Goiás (n = 3), Central-Western region, and Rio de Janeiro (n = 2), Minas Gerais (n = 3), Espírito Santo

(n = 3) and São Paulo (n = 2), Southeastern region of Brazil, were analyzed (**Table 1**). Serum samples of human cases, liver samples of human and NHP, and homogenates of entire bodies of pooled adult female mosquitoes were collected and processed as previously described (Ferreira-De-Brito et al., 2016; Bonaldo et al., 2017; Gomez et al., 2018; Abreu et al., 2019). The YFV isolates ABR1005 and ABR1009 were obtained from the sera of a 64 and 30-year-old male patients, respectively. The ABR1005 patient was hospitalized 4 days after onset of symptoms and died due to multiple organ failure. The ABR1009 patient was hospitalized 4 days after onset of symptoms but fully recovered from the infection.

YFV Genome Detection and Nucleotide Sequencing

The sera from the individuals from São Paulo state (ABR1005 and ABR1009) and an NHP liver homogenate sample from Goiás (GO27) were employed to obtain first-passage YFV isolates by infection of monolayer cell cultures of the C6/36 clone of *Aedes albopictus*. Viral RNA was obtained from cell cultures or directly from samples as described elsewhere (Bonaldo et al., 2017). The set of primers utilized in PCR and sequencing procedures followed a previous report (Gomez et al., 2018). Nucleotide sequences were determined by capillary electrophoresis at the sequencing facility of Fiocruz-RJ (RPT01A – Sequenciamento de DNA – RJ). The sequences were assembled with SeqMan Pro version 8.1.5 (DNASTAR, Madison, WI, United States). The Molecular Evolutionary Genetics Analysis 7.0 program (Kumar et al., 2016) was adopted to explore the amino acid differences as well as to calculate nucleotide and amino acid distances.

Evolutionary and Phylogeographic Analyses

Complete CDS (10,239 nt in length) of the 13 newly generated YFV genomes were combined with CDS of YFV American sequences available in GenBank[1] according to the following inclusion criteria: (1) link to a publication, (2) coverage of at least 99% of the viral CDS, and (3) known date and country of collection. The sequences were aligned with MAFFT (Katoh and Standley, 2013) and viral phylogenies were reconstructed by maximum likelihood (ML) analysis implemented in PhyML (Guindon et al., 2010) applying the best substitution model selected by jModelTest v1.6 (Darriba et al., 2012). The temporal signal of different combinations of sequences representing the South American genotypes I and II (SA-I+II), the South American genotype I (SA-I), and the Modern lineage of SA-I (Mir et al., 2017) were examined with TempEst v1.5.1 (Rambaut et al., 2016). The dataset with the best temporal structure (SA-I) was chosen for the subsequent time tree reconstructions by Bayesian method (**Supplementary Table 1**).

The time scale of the SA-I and 2016–2018 Brazilian YFV datasets were estimated using the Markov chain Monte Carlo algorithms implemented in the BEAST v1.8.4 package (Drummond and Rambaut, 2007; Drummond et al., 2012) with BEAGLE (Ayres et al., 2012) to improve running time. The evolutionary process was estimated using the best-fit nucleotide substitution model (GTR+I+Γ4), a relaxed uncorrelated lognormal molecular clock model (Drummond et al., 2006) and the non-parametric Bayesian Skyline coalescent tree prior (Drummond et al., 2005). A CTMC rate reference prior (Ferreira and Suchard, 2008) and a normal prior (mean = 4.5×10^{-4} substitution/site/year, standard deviation = 1.0×10^{-4}) in the evolutionary rate were used for the analysis of the SA-I and 2016–2018 Brazilian YFV datasets, respectively.

The reconstruction of migration events throughout the phylogeny for the 2016–2018 Brazilian YFV lineage also employed the BEAST package using discrete and continuous models. The discrete phylogeographic analysis was performed using reversible (symmetric) and nonreversible (asymmetric) discrete phylogeographic models (Lemey et al., 2009), assigning discrete traits for each sequence representing the Brazilian state of isolation, except for MG, in which further geographic subdivision were employed. The spatiotemporal reconstruction in continuous space utilizing the geographic coordinates (latitude and longitude) of each YFV isolate was estimated with a homogenous Brownian diffusion (BD) model and the heterogeneous Cauchy, Gamma and Lognormal relaxed random walk (RRW) models (Lemey et al., 2010). Comparisons among the different discrete and continuous phylogeographic models were performed using the log marginal likelihood estimation (MLE) based on path sampling (PS) and stepping-stone sampling (SS) methods (Baele et al., 2012). Bayesian analyses were run for 10^8 generations and convergence (effective sample size > 200) was inspected using TRACER v1.7 (Rambaut et al., 2018) after discarding 10% burn-in. The maximum clade credibility (MCC) trees were summarized using TreeAnnotator v.1.8.4 (Drummond et al., 2012) and visualized with FigTree v.1.4.4.[2] The viral spatio-temporal diffusion was analyzed and visualized in SPREAD (Bielejec et al., 2011) and further projected in maps generated with QGIS software[3] using public access data collected from the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística [IBGE], 2019) and National Water Agency (Agência Nacional de Águas [ANA], 2019).

RESULTS

Identification of Two YFV Lineages Circulating in the State of Goiás During 2015–2017

Initially, we sequenced the complete genomes of YFV obtained at two Brazilian biomes to infer the origin and dissemination routes of YFV during the 2016–2018 outbreak in the country. Ten YFV strains were sampled from human, NHP and mosquitoes in all

[1] www.ncbi.nlm.nih.gov

[2] http://tree.bio.ed.ac.uk

[3] http://qgis.org

TABLE 1 | YFV samples from Brazil sampled in 2015, 2017, and 2018.

State (Region)	Biome	Primary river basin (Tributary basin)	Locality	Geographic coordinate	Date of collection	Sample ID	Host	Genbank accession number
Goiás (CW)	*Cerrado*	Tocantins-Araguaia (Araguaia)	Novo Brasil	16°02′12″S 50°43′24″W	17/08/2015	GO27	*Sapajus libidinosus* (capuchin-monkey)	MK333804
		Tocantins-Araguaia (Araguaia)	Nova Crixás	13°40′39″S 50°44′55″W	20/04/2017	GO05	*Alouatta caraya* (howler-monkey)	MK333803
		Tocantins-Araguaia (Araguaia)	Amorinópolis	16°36′19″S 51°05′03″W	03/04/2017	GO21	*Homo sapiens*	MK728873
Minas Gerais (SE)	Inland Atlantic Forest	Southeast Atlantic (Paraíba do Sul)	Belmiro Braga	22°00′56″S 43°22′19″W	18/01/2018	MG3121	*Haemagogus. Leucocelaenus* (3 mosquitoes)	MK333807
			Belmiro Braga	22°04′50″S 43°33′33″W	29/01/2018	MG3155	*Haemagogus janthinomys* (5 mosquitoes)	MK333808
			Juiz de Fora	21°45′14″S 43°19′42″W	27/01/2018	JF2793	*Haemagogus janthinomys* (3 mosquitoes)	MK333806
Espírito Santo (SE)	Coastal Atlantic Forest	Southeast Atlantic (Itapemirim conjugated)	Domingos Martins	20°16′52″S 40°49′39″W	20/02/2017	ES03	*Alouatta clamitans* (howler-monkey)	MK333800
			Santa Leopoldina	20°05′12″S 40°31′07″W	05/04/2017	ES2750	*Haemagogus. leucocelaenus* (1 mosquito)	MK333802
			Santa Leopoldina	20°04′51″S 40°35′48″W	05/04/2017	ES2682	*Haemagogus janthinomys* (2 mosquitoes)	MK333801
Rio de Janeiro (SE)	Inland Atlantic Forest	Southeast Atlantic (Paraíba do Sul)	Valença	22°10′26″S 43°43′25″W	19/01/2018	VL2926	*Haemagogus janthinomys* (1 mosquito)	MK333809
	Coastal Atlantic Forest	*Sea Island*	Ilha Grande	23°11′01″S 44°11′56″W	07/02/2018	IG3036	*Sabethes chloropterus* (1 mosquito)	MK333805
São Paulo (SE)	Inland Atlantic Forest	Paraná (Tietê)	Metropolitan São Paulo	23°23′S 46°34′W	08/01/2018	ABR1005	*Homo sapiens*	MK333798
	Inland Atlantic Forest	Paraná (Tietê)	Metropolitan São Paulo	23°23′S 46°34′W	08/01/2018	ABR1009	*Homo sapiens*	MK333799

CW, Central-West region; SE, Southeast region.

Southeastern Brazilian states: Minas Gerais, Espírito Santo, Rio de Janeiro and São Paulo, located in the Atlantic Forest biome and where massive epizootics and human cases occurred. Three other YFV genomes were recovered from human and NHP sampled in Goiás, a state of the Brazilian Central-Western region whose predominant vegetation is the *Cerrado*, a savanna-like biome occupying the territory between the Amazon and Atlantic rain forest of the Southeast region (**Table 1**). Both YFV strains from Goiás were sampled from sites located in the Tocantins-Araguaia river basin. The ML and Bayesian phylogenetic analyses placed the newly generated YFV genomes inside the sub-clade 1E (De Souza et al., 2010) of the Modern lineage of SA-I (Mir et al., 2017), with high support [aLRT/posterior probability (*PP*) = 1] (**Figure 1** and **Supplementary Figure 2**). Two viral samples from Goiás (GO27/2015 and GO21/2017) infecting, respectively a capuchin-monkey from Novo Brasil on August 2015 and a person from Amarinópolis on April 2017, clustered in a highly supported (*PP* = 1) clade ($YFV_{2015-2018}$) with all Brazilian YFV sequences from the Southeastern region associated with the current outbreak. By contrast, the other strain from Goiás (GO05/2017) that infected a howler-monkey from Nova Crixás on April 2017, was intermingled among Venezuelan YFV genomic sequences from the 2000s. The mean evolutionary rate for the YFV SA-I was estimated at 4.3×10^{-4} substitution/site/year (**Supplementary Table 2**), fully consistent with that previously reported (Nunes et al., 2012; Gomez et al., 2018), while the time of the most recent common ancestor (T_{MRCA}) of the $YFV_{2015-2018}$ clade was estimated on October 2013 [95% Bayesian credible interval (BCI): May 2012 to December 2014].

Analysis of amino acid signatures showed the frequent presence of the nine unique amino acid substitutions previously described (Bonaldo et al., 2017; Gomez et al., 2018) in almost all new YFV Brazilian genomes from the Southeastern region and in one sample from Goiás (GO27/2015; **Supplementary Table 3** and **Supplementary Figure 3**). Distinctly, the other YFV strain from Goiás (GO05/2017), exhibited an amino acid pattern more similar to older YFV sub-clade 1E strains sampled in Brazil and Venezuela between 2000 and 2010 (**Supplementary Table 3** and **Supplementary Figure 3**). These results clearly support the circulation of at least two YFV sub-clade 1E lineages in the *Cerrado* between 2015 and 2017 that probably resulted from independent viral introductions in the Araguaia tributary basin of the Tocantins-Araguaia primary watershed. Interestingly, the third sample from Goiás state (GO21/2017) displayed eight out of nine aminoacidic set of alterations (**Supplementary Table 3**). Moreover, these results revealed that the molecular signature previously associated with the 2016–2017 YFV Southeastern Brazilian strains was already present in a YFV strain isolated in the *Cerrado* biome in 2015.

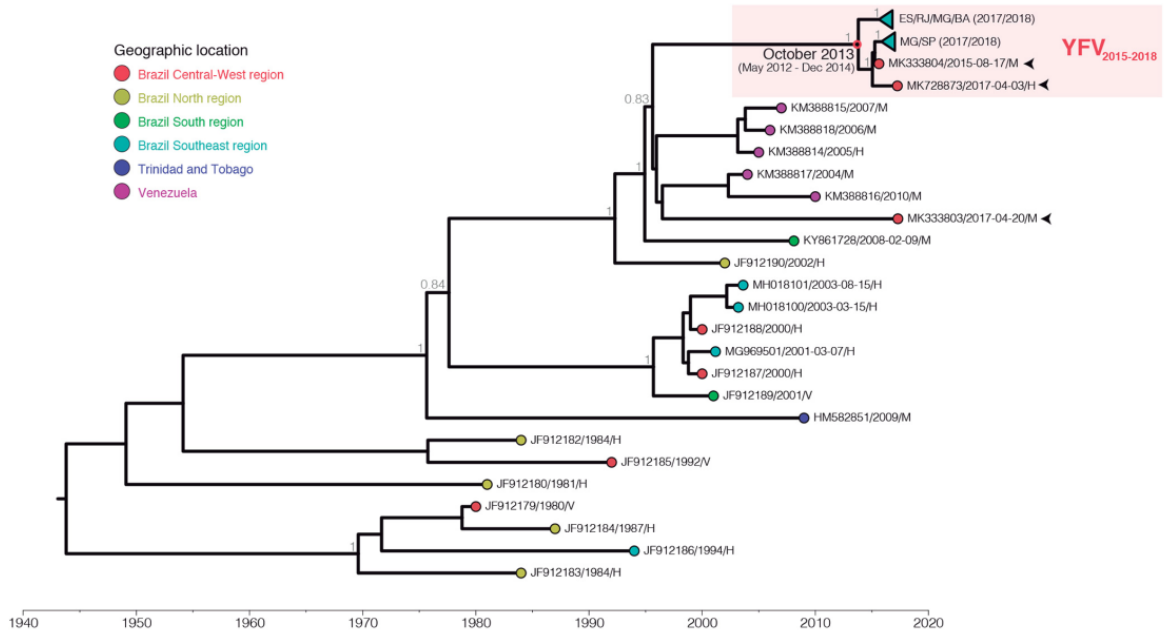

FIGURE 1 | Bayesian maximum clade credibility phylogeny of the YFV South American genotype I. Only posterior probabilities (*PP*) of key nodes are denoted. The external node color indicates sample location (geographic region or country) accordingly to the legend. The samples obtained from NHP from Goiás state are indicated by arrows. The clade comprising the GO27/2015 and GO21/2017 samples and all YFV genomes from the ongoing outbreak ($YFV_{2015-2018}$) is highlighted by a red box, and inner clades were collapsed for clarity. The node representing the most recent common ancestor of $YFV_{2015-2018}$ lineage is indicated by a red circle along with its temporal origin estimate. All horizontal branch lengths are drawn to a scale of years. Sequences names are coded as accession number/date of collection/host (H – human, V – mosquito vector, M – NHP.

The $YFV_{2015-2018}$ Lineage Likely Arose in Goiás in 2014 and Was Disseminated to the Southeastern Region Following Two Major Routes

To determine with more precision the geographic origin and dissemination routes of the $YFV_{2015-2018}$ lineage, we first applied discrete Bayesian symmetric and asymmetric phylogeographic models. The Bayes Factor test showed a strong support in favor of the discrete asymmetric phylogeographic model (**Supplementary Table 4**), presented in **Figure 2**. According to this analysis, the $YFV_{2015-2018}$ lineage likely originated in the state of Goiás [posterior state probability (*PSP*) = 0.57] in May 2014 (95% BCI: October 2012 to July 2015). From there, it followed two paths of dissemination toward the state of Minas Gerais, originating two major YFV sub-lineages in the Southeastern region here called $YFV_{MG/ES/RJ}$ and $YFV_{MG/SP}$.

The $YFV_{MG/ES/RJ}$ sub-lineage probably reached initially the eastern region of Minas Gerais (MG-E, *PSP* = 0.51) on October 2015 (95% BCI: February 2015 to April 2016), from where it most likely spread to Espírito Santo state (*PSP* = 0.99) on March 2016 (95% BCI: September 2015 to August 2016) and to the northern area of Minas Gerais (MG-N, *PSP* = 0.47) on July 2016 (95% BCI: January 2016 to October 2016). From MG-N, the YFV spread to the south of the state of Bahia, in the Northeastern Brazilian region. From ES, the YFV was introduced in Rio de Janeiro at least four times, generating two successful intrastate transmission chains that advanced southwards: (1) one chain followed the northern side of the Serra do Mar along the Paraíba do Sul tributary basin (**Supplementary Figure 4**), bypassing the Rio de Janeiro metropolitan region toward the south (Valença municipality) and affecting the southeastern region of Minas Gerais (MG-SE, *PSP* = 0.57); (2) the other transmission chain spread through the coastal area South of Serra do Mar (Macaé

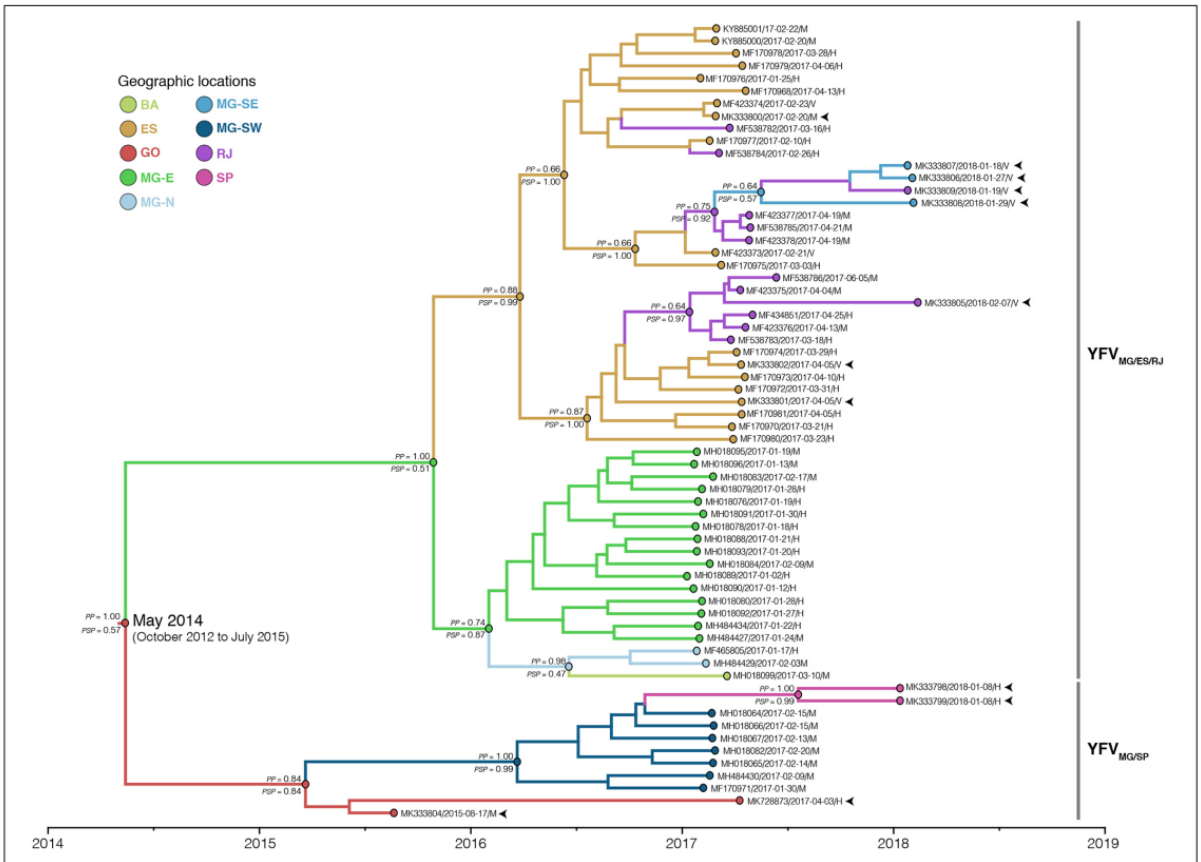

FIGURE 2 | Time-scaled maximum clade credibility phylogeny of the $YFV_{2015-2018}$ lineage. The branches' colors represent the most probable location of their descendent nodes as indicated in the legend. The posterior (*PP*) and posterior state (*PSP*) probabilities are denoted above and below key branches, respectively. The time for the most common ancestor of lineage $YFV_{2015-2018}$ is indicated at the basal node (red circle) with the 95% BCI in parenthesis. Tips names were codified as accession number/date/region/host. The tips corresponding to the samples sequenced in this study are indicated by arrows. All horizontal branch lengths are drawn to a scale of years. BA, Bahia; ES, Espírito Santo; GO, Goiás; MG-E, Eastern Minas Gerais; MG-N, Northern Minas Gerais; MG-SE, Southeastern Minas Gerais; MG-SW, Southwest Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo.

tributary basin), crossing the Rio de Janeiro state metropolitan region and reaching an island located in the southern region of Rio de Janeiro (Ilha Grande).

The $YFV_{MG/SP}$ sub-lineage most likely spread from Goiás into the southwestern area of Minas Gerais (MG-SW, *PSP* = 0.99) on March 2016 (95% BCI: September 2015 to August 2016) and then disseminated from MG-SW to the metropolitan region of the state of São Paulo. Remarkably, four YFV genomes from MG-E and one from MG-SW displayed differences in the molecular amino acid signature (D15762E; R2607Q). Nevertheless, the variations were not fixed in 2018 YFV samples as identified in the position I2176V clustering in $YFV_{MG/ES/RJ}$, which were collected in Paraíba tributary basin in 2017 and 2018, suggesting the maintenance of the YFV polymorphism in this region. Overall, these results expanded the geographical and temporal edge of the $YFV_{2015-2018}$ lineage and further revealed a very low degree of phylogenetic intermixing of $YFV_{2015-2018}$ strains from different Brazilian states during viral dissemination in the Southeastern region.

The $YFV_{2015-2018}$ Lineage Was Disseminated Following Primary River Basins in the Southeastern Region

To get some insight regarding the spatiotemporal dynamics of dissemination of the $YFV_{2015-2018}$ lineage, we applied different continuous phylogeographical models, assuming homogeneous (BD) and heterogeneous (RRW) dispersion rates among lineages. The RRW model with Lognormal distribution was strongly supported as the fittest diffusion model (**Supplementary Table 5**), indicating significant variation in the diffusion rate among the branches. The phylogeographic continuous model (**Figure 3**) changed slightly the epicenter of the $YFV_{2015-2018}$ lineage but supports the existence of two main routes of dissemination within the Brazilian Southeastern region and few viral migrations between different states, consistent with the discrete phylogeographic reconstruction. This analysis also supports that the YFV was disseminated following major river basins.

According to the continuous model, the origin of the $YFV_{2015-2018}$ lineage would be the central region of Minas Gerais, within the São Francisco watershed, from where it would have independently spread to the west, east and south, reaching the Tocantins-Araguaia basin in the Goiás in 2015, the Southeast Atlantic hydrographic region in Minas Gerais at the end of 2015, and the Paraná hydrographic region of that state in the middle of 2016, respectively. From the eastern of Minas Gerais, the virus moved southward following the Southeast Atlantic watersheds distributed among the states of Minas Gerais, Espírito Santo, and Rio de Janeiro (an area covered by the Atlantic forest biome) and northward, returning to the São Francisco river basin and south of Bahia.

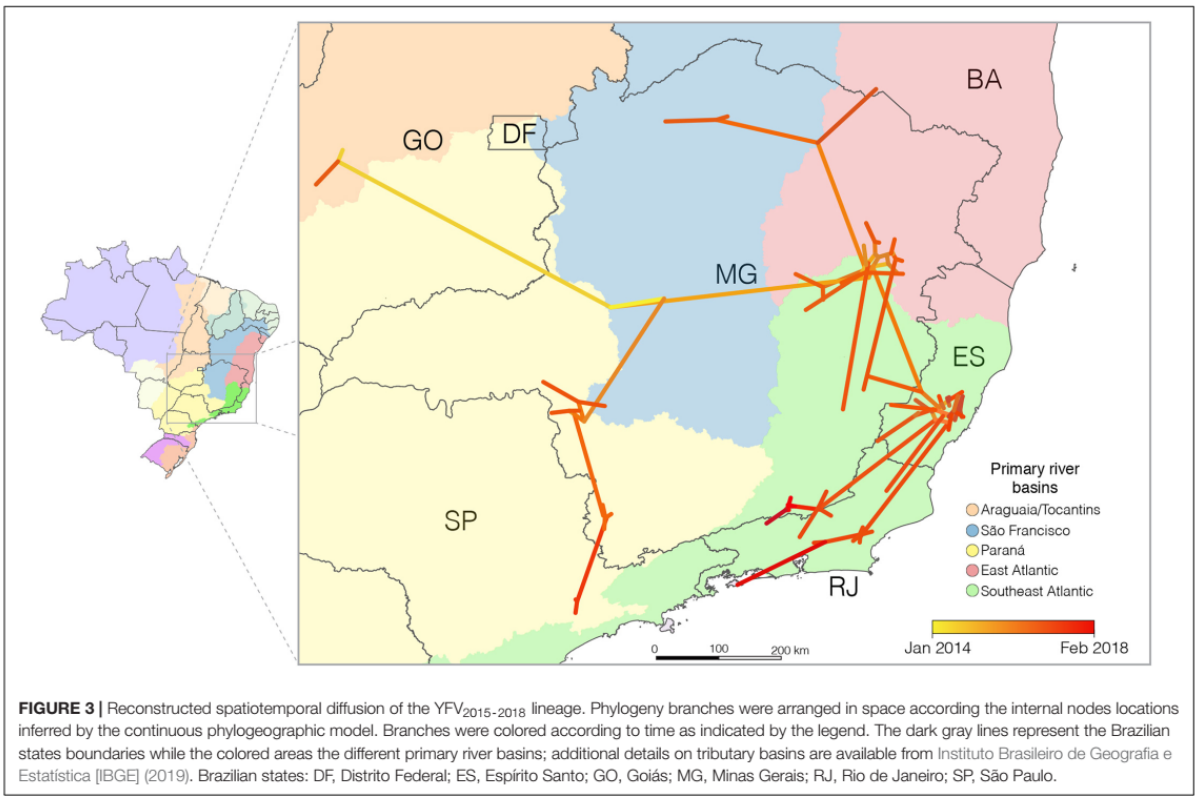

FIGURE 3 | Reconstructed spatiotemporal diffusion of the $YFV_{2015-2018}$ lineage. Phylogeny branches were arranged in space according the internal nodes locations inferred by the continuous phylogeographic model. Branches were colored according to time as indicated by the legend. The dark gray lines represent the Brazilian states boundaries while the colored areas the different primary river basins; additional details on tributary basins are available from Instituto Brasileiro de Geografia e Estatística [IBGE] (2019). Brazilian states: DF, Distrito Federal; ES, Espírito Santo; GO, Goiás; MG, Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo.

Simultaneously, the YFV lineage showed southerly dissemination from the southwestern region of Minas Gerais toward the São Paulo state, following the Paraná basin. We estimate that YFV lineages moved, on average, 0.5 km/day (95% BCI: 0.4 to 0.7 km/day).

DISCUSSION

The current re-emergence of YFV in the Southeastern Brazilian region resulted in the largest outbreak of sylvatic YF observed in South America in the last decades. The transmission has been expanding southward in Brazil reaching sites considered YFV-free areas for 80 years, and therefore with scanty YFV vaccination coverage. As a result, 38 cases and nine deaths have been reported in January 2019, around 1,160 km from the first signal of increased incidence of YF in the Southeast (north-western region of Minas Gerais) in late 2016 (Secretaria de Vigilância em Saúde, 2019).

Previous studies pointed out that the ongoing YFV outbreak in the Brazilian Southeastern region resulted from a single introduction event of a YFV Modern lineage strain from an endemic area (Mir et al., 2017; Faria et al., 2018; Gomez et al., 2018; Rezende et al., 2018). However, the precise route of viral dissemination was not achieved due to the scarcity of Brazilian YFV sequences sampled from endemic regions over the last years. Here, the analysis of two YFV samples from Goiás (Central-Western region) obtained in 2015 and 2017 revealed that they are phylogenetically related with and carry the same amino acid signature of the YFV strains causing the current outbreak in the Southeastern region. Moreover, the discrete phylogeographic analysis showed that the YFV causing the current Brazilian outbreak probably originated in Goiás at around mid-2014, a result congruent with epidemiological reports on human and NHP infections (**Supplementary Figure 1**). Altogether, our data stressed the origin of the current YFV outbreak in the Central-western region and expanded the estimated T_{MRCA} of the ongoing YFV outbreak to almost 2 years before it gained epidemiological visibility in the end of 2016 (Secretaria de Vigilância em Saúde, 2017). Interestingly, we also identified for the first time, two YFV lineages circulating in Goiás during 2015–2017, one of which followed two independent paths of dissemination toward Minas Gerais state, originating two major YFV sub-lineages in the Southeast region responsible for the severe ongoing outbreak. Of note, the molecular clock dating approach used to infer the YFV epidemic dynamics had limitations and would be undoubtedly benefited if more genome sequences (sampled from humans and reservoir populations) were available and if multiple scales of YFV evolution (between- and within-host) were considered (Frost et al., 2015).

According to the spatiotemporal epidemiologic reports of YFV infections in both humans and NHPs in the national surveillance system, after 5 years of no records of YFV cases outside the Amazon, 31 NHPs died between May and August 2014 in seven municipalities of the Tocantins state, in the Tocantins-Araguaia basin. The only corpse found still adequate to diagnosis was positive to YFV (Secretaria de Vigilância em Saúde, 2014). The Tocantins-Araguaia basin drains the territory of Tocantins and the great northern part of the neighbor state of Goiás into de Amazon river, and the gallery forests along its tributaries consist of a large network of corridors between the Amazon and *Cerrado* biomes. Thus, soon in early 2015, besides in the Tocantins, YFV infections were detected in Goiás, and the virus spilled over from the Tocantins-Araguaia into the São Francisco and Paraná basins, with reports scattered in northwestern Minas Gerais and southern Goiás and the Federal District (Brasília; **Figure 4**).

Brazilian Health authorities consider this YFV reemergence as the start of the north-southeast virus spreading wave that has not stopped yet (Secretaria de Vigilância em Saúde, 2015, 2017, 2019). Accordingly, in late 2015 and early 2016, the circulation of YFV was detected in sites of Minas Gerais and Goiás states, but still essentially limited to the *Cerrado* biome in the three above mentioned river basins. Intriguingly, in late 2016, human cases and epizooties due to YFV were recorded simultaneously and independently in the northeast Minas Gerais as well as in southeastern Minas Gerais/northern São Paulo, where the virus has then gained the Atlantic forest ecosystem. In fact, the outbreak was officially recognized only when the epidemiological records rapidly peaked and as that the virus reached the Southeast Atlantic river basin in Espírito Santo and Rio de Janeiro, as well as continued to spread in all primary watershed of São Paulo and Minas Gerais throughout 2017. One of the causes of this delay was the introduction of Chikungunya (2014) and Zika (2015) virus in Brazil and the resulting epidemics, which probably reduced the sensitivity of surveillance and interfered with the visibility of the YFV reemergence. In 2017, scattered records of YFV circulation were also made in the states of Bahia, Goiás, Mato Grosso and even in the Amazon.

In early 2018, epidemiological data suggested that transmission was mostly concentrated in the Southeast Atlantic basin in the states of Rio de Janeiro, Minas Gerais and São Paulo and eastern Paraná basin (**Figure 4**). Then, the place and time of origin for the current YFV Brazilian outbreak here estimated fully agree with the above mentioned officially confirmed infections in NHP and humans reported by the Brazilian Ministry of Health (Secretaria de Vigilância em Saúde, 2015). Indeed, the Novo Brasil municipality in Goiás, from where the NHP YFV sample analyzed here was taken, is located about 100 km from the banks of the Araguaia river. The earlier detection of YFV in NHP from the Tocantins indicates that the state of Goiás probably acted as a staging post-during the dissemination of the $YFV_{2015-2018}$ lineage from the North (Amazon basin) to the Southeast Brazilian regions. In this sense, the Tocantins-Araguaia watersheds may have played a major role in YFV dissemination as it extends from more than 2.500 km following two river axes (Araguaia and the Tocantins), offering a contiguous connection between the *Cerrado* (southward, Goiás and Tocantins states) and Amazon (northward, Pará state) biomes (Agência Nacional de Águas [ANA], 2019).

Both discrete and continuous phylogeographic models combined with the epidemiological records support of the $YFV_{2015-2018}$ lineage moved toward densely populated

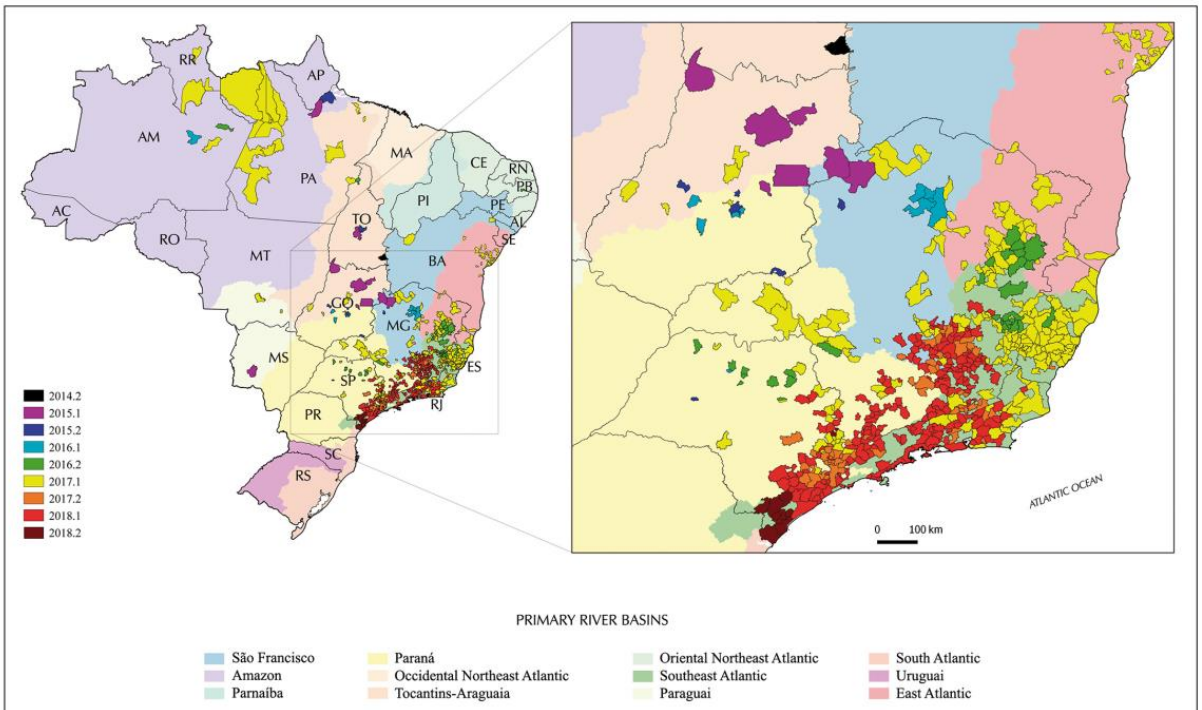

FIGURE 4 | Municipalities reporting YFV in Brazil from late 2014 to late 2018 according to epidemiological data. Colors distinguish the semester when the first confirmed human and/or non-human YFV infection was recorded in such a municipality on a half-yearly basis during the period. The dark gray lines represent the Brazilian states boundaries while the colored areas the different primary river basins; additional details on tributary basins are available from Instituto Brasileiro de Geografia e Estatística [IBGE] (2019). Brazilian states: AC, Acre; AL, Alagoas; AP, Amapá; AM, Amazonas; BA, Bahia; CE, Ceará; DF, Distrito Federal; ES, Espírito Santo; GO, Goiás; MA, Maranhão; MT, Mato Grosso; MS, Mato Grosso do Sul; MG, Minas Gerais; PA, Pará; PB, Paraíba; PR, Paraná; PE, Pernambuco; PI, Piauí; RJ, Rio de Janeiro; RN, Rio Grande do Norte; RS, Rio Grande do Sul; RO, Rondônia; RR, Roraima; SC, Santa Catarina; SP, São Paulo; SE, Sergipe; TO, Tocantins.

Southeastern urban regions with low YFV vaccine coverages following major routes along different primary river basins. The phylogeographic analyses pointed out that the $YFV_{2015-2018}$ lineage probably arrived in the Southeast region via the São Francisco watershed located in Minas Gerais and then moved to the Southeast Atlantic watersheds in the east and the Paraná hydrographic region in the southwest. The viral lineage that moved following the Southeast Atlantic watersheds reached the eastern and northern areas of Minas Gerais state, as well as the south of Bahia, Espírito Santo and Rio de Janeiro states. The viral lineage that followed the Paraná hydrographic region spread to the Southwest of Minas Gerais and São Paulo states. A previous study proposed that the $YFV_{2015-2018}$ lineage was introduced in the southeastern region through São Paulo and then moved to other Southeastern states (Rezende et al., 2018). However, this conclusion is hampered as they used only partial genomes (with a very low number of nucleotide substitutions supporting the phylogenetic relationships) and did not conduct a formal phylogeographic analysis. Although Faria et al. (2018) already described that the $YFV_{2015-2018}$ lineage displayed southward and eastward expansion from its inferred origin in Minas Gerais, our results consist of the first description of concurrent dispersion of the $YFV_{2015-2018}$ lineage following two independent routes that seems to be linked to the main hydrographical basins.

Our phylogeographic analyses also support that the rapid spread of the $YFV_{2015-2018}$ lineage in the Southeastern region seems to have resulted from a few successful viral disseminations events between states. Most YFV transmission in Espírito Santo was probably originated from a single successfully transmission from Minas Gerais, while most viral transmissions in Rio de Janeiro seems to have resulted from two independent introductions from Espírito Santo that subsequently spread along the coastal and northern sides of the Serra do Mar mountain system, as previously described (Gomez et al., 2018). We found that both transmission chains previously detected in Rio de Janeiro state continued to expand to beyond the metropolitan region, reaching municipalities close to the border with the state of São Paulo during 2018. The two YFV 2018 genomes from São Paulo analyzed here are the first described of the current YFV outbreak from that state and were the result of independent dissemination from the southwestern region of Minas Gerais, but more sequences from São Paulo are necessary to understand the epidemic dynamic in this state. It is unclear if most YFV infections in São Paulo resulted from a single or a few founder viral strains that spread from the southwest of Minas Gerais along

the Paraná hydrographic basin, or if other viral strains may have also been disseminated from Rio de Janeiro along the Southeast Atlantic watersheds. The recent detection of YFV in NHP from the coastal area of Paraná state in 2019 (Secretaria de Vigilância em Saúde, 2019) indicates continuous dissemination of YFV into the Southern region probably following the Paraná and/or the Southeast Atlantic hydrographic basins.

We estimated that the $YFV_{2015-2018}$ lineage moved on average 0.5 km/day and similar results were obtained when the $YFV_{2015-2018}$'s outgroup sequences GO27/2015 and GO21/2017 were removed from the analysis. This velocity is lower than the estimates described by Faria et al. (2018) and Gomez et al. (2018) that also analyzed the dispersion of the ongoing YFV outbreak in Brazil and found dispersion rates of 4.2 and 3.4 km/day, respectively. However, those studies analyzed sequences sampled between January-April of 2017, corresponding to the wet and warmer season, when there is an increase in the density of vectors (Alencar et al., 2018) facilitating the transmission. The primary vectors in the current outbreak in Southeast Brazil are the mosquitoes *Haemagogus leucocelaenus* and *Haemagogus janthinomys* (Abreu et al., 2019), which can disperse large distances in short time (Causey et al., 1950). Our conservative velocity of YFV dispersion fully agrees with the observed distances traveled by mosquitoes, but also with howler monkeys in Southeastern Brazil (Jung et al., 2015) and also agrees with an epidemiological model based on dates and place of reported monkey deaths, which estimated YFV displacement speeds of 2.7 km/day in the warmer months and 0.5 km/day in the coldest months (Fioravanti, 2018). Thus, the YFV dispersion velocity estimated in this study would correspond to a median value between these two speeds. Besides the role of NHPs and mosquitoes, the hypothetical potential of viremic humans in the spread of sylvatic yellow fever has yet to be confirmed (Vasconcelos, 2010; Possas et al., 2018a).

Surprisingly, we found two YFV sub-clade 1E lineages circulating in the Araguaia tributary basin, indicating at least two independent introductions of YFV in that region probably from the enzootic/endemic Amazon biome in a narrow time frame. While sample GO27/2015 isolated in 2015 displays the nine unique amino acid signatures characteristic of the 2016–2018 YFV Southeastern outbreak, sample GO05/2017 isolated 2 years later at the same watershed exhibited an amino acid pattern typical of older YFV sub-clade 1E strains sampled in Brazil and Venezuela between 2000 and 2010. According to our analysis, only YFV strains related to the GO27/2015 and GO21/2017 were able to further disseminate from Goiás toward the Southeast region. We can speculate that the different pattern of molecular signatures present in the two YFV strains from Goiás modulate the spread of each viral lineage since some of them were located in key viral enzymes (Gomez et al., 2018). Alternatively, the GO05/2017 lineage may have been introduced from the Amazon into Goiás at a later time, and its dissemination toward the Southeast was hampered due to the reduction or even exhaustion of susceptible NHP hosts caused by the previous passage of the lineage that originated the $YFV_{2015-2018}$ clade. Consistent with this last hypothesis, a recent ecological study concludes that dissemination of YFV in South America is not random, but it is influenced by key geo-environmental factors like diversity and number of susceptible NHP hosts (Hamrick et al., 2017). Curiously, epidemiological records showed YFV transmission in several sites in the Amazon in early 2017, including in the Tocantins-Araguaia basin in Pará state (**Figure 4**).

In summary, we showed that at least two different YFV lineages circulated in the *Cerrado* biome (Araguaia tributary basin) in a narrow time frame. One of these lineages further spread out the *Cerrado* biome in Goiás to the Atlantic forest biome in the Southeastern Brazilian region, originating the current Brazilian outbreak ($YFV_{2015-2018}$ lineage) at around mid-2014. The ongoing YFV outbreak in Brazil disseminated in the Southeast region following two independent routes that seem to be linked to the Paraná and Southeast Atlantic hydrographic basins, comprising densely populated regions. The spread of the YFV outside the Amazon and *Cerrado* biomes following primary hydrographic watersheds comprising large metropolis stresses the imperative importance of the continuous monitoring of YFV coupled with in-depth phylogeographic analysis to aid decision-making Health authorities for effective prophylactic and control policies aiming the increase of vaccination coverage to avoid the YFV transmission in densely populated urban centers.

DATA AVAILABILITY

All new YFV genomes generated for this study were submitted to GenBank and their accession numbers are included in the manuscript. The information of all YFV genomes used in this study is provided in the **Supplementary Table 1**.

ETHICS STATEMENT

The YFV isolates ABR1005 and ABR1009 were obtained from two patients following a study protocol approved by the institutional review boards at the Hospital das Clínicas (School of Medicine, University of São Paulo) and the Infectiology Institute Emilio Ribas (CAAE: 59542216.3.1001.0068). The analysis of human samples was carried out in accordance with the recommendations of the Ethics Committees for human research at Instituto Oswaldo Cruz (CAAE 69206217.8.0000.5248), which exempted the need of a specific written informed consent from patients or their legal representatives. Capture of NHPs and mosquitoes, as well as management of NHP samples, were carried out in accordance with the Brazilian environmental authorities (SISBIO-MMA licenses 54707-6 and 52472-2, and INEA licenses 012/2016, 019/2018) and Ethics Committee of Animal use at Instituto Oswaldo Cruz (CEUA license L037/2016).

AUTHOR CONTRIBUTIONS

ED, GB, MB, and RL-d-O conceived the study. FdA and MN carried out the collection of biological specimens. EK provided the samples. IB and MN identified the mosquito species. AF-d-B, FdA, IB, LdS, MdC, and RdM carried out viral RNA

extraction from the biological specimens and the diagnosis by RT-PCR. LdS and LR conducted the inoculation of biological specimens in cell culture. AdS, IR, and MG performed rapid viral RNA extraction and genome sequencing. AdS, MB, MG, and NF analyzed the genome sequences. ED and GB performed phylogenetic and phylogeographic analysis. ED, GB, MB, MG, NF, and RL-d-O prepared figures, tables, and supplementary material. ED, FdA, GB, IR, MB, MG, RL-d-O, EK, and AV prepared the manuscript. AR, DR, and FdA gathered, systematized, and illustrated epidemiological records. All authors critically read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01079/full#supplementary-material

REFERENCES

Abreu, F. V. S. D., Ribeiro, I. P., Ferreira-De-Brito, A., Santos, A. A. C. D., Miranda, R. M. D., Bonelly, I. D. S., et al. (2019). *Haemagogus leucocelaenus* and *Haemagogus janthinomys* are the primary vectors in the major yellow fever outbreak in Brazil, 2016–2018. *Emerg. Microb. Infect.* 8, 218–231.

Agência Nacional de Águas [ANA] (2019). *Divisões hidrográficas do Brasil.* Available at: http://www3.ana.gov.br/portal/ANA/aguas-no-brasil/panorama-das-aguas/copy_of_divisoes-hidrograficas (accessed January 31, 2019).

Alencar, J., Mello, C. F. D., Morone, F., Albuquerque, H. G., Serra-Freire, N. M., Gleiser, R. M., et al. (2018). Distribution of haemagogus and sabethes species in relation to forest cover and climatic factors in the chapada dos guimarães National Park, State of Mato Grosso, Brazil. *J. Am. Mosq. Control Assoc.* 34, 85–92.

Ayres, D. L., Darling, A., Zwickl, D. J., Beerli, P., Holder, M. T., Lewis, P. O., et al. (2012). BEAGLE: an application programming interface and high-performance computing library for statistical phylogenetics. *Syst. Biol.* 61, 170–173. doi: 10.1093/sysbio/syr100

Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M. A., and Alekseyenko, A. V. (2012). Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Mol. Biol. Evol.* 29, 2157–2167. doi: 10.1093/molbev/mss084

Bielejec, F., Rambaut, A., Suchard, M. A., and Lemey, P. (2011). SPREAD: spatial phylogenetic reconstruction of evolutionary dynamics. *Bioinformatics* 27, 2910–2912. doi: 10.1093/bioinformatics/btr481

Bonaldo, M. C., Gomez, M. M., Dos Santos, A. A., Abreu, F. V. S., Ferreira-De-Brito, A., Miranda, R. M., et al. (2017). Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. *Mem. Inst. Oswaldo Cruz* 112, 447–451. doi: 10.1590/0074-02760170134

Carrington, C. V., and Auguste, A. J. (2013). Evolutionary and ecological factors underlying the tempo and distribution of yellow fever virus activity. *Infect. Genet. Evol.* 13, 198–210. doi: 10.1016/j.meegid.2012.08.015

Causey, O. R., Kumm, H. W., and Laemmert, H. W. Jr. (1950). Dispersion of forest mosquitoes in Brazil; further studies. *Am. J. Trop. Med. Hyg.* 30, 301–312.

Darriba, D., Taboada, G. L., Doallo, R., and Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* 9:772.

De Souza, R. P., Foster, P. G., Sallum, M. A., Coimbra, T. L., Maeda, A. Y., Silveira, V. R., et al. (2010). Detection of a new yellow fever virus lineage within the South American genotype I in Brazil. *J. Med. Virol.* 82, 175–185. doi: 10.1002/jmv.21606

Drummond, A. J., Ho, S. Y., Phillips, M. J., and Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. *PLoS Biol.* 4:e88.

Drummond, A. J., and Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7:214.

Drummond, A. J., Rambaut, A., Shapiro, B., and Pybus, O. G. (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol. Biol. Evol.* 22, 1185–1192.

Drummond, A. J., Suchard, M. A., Xie, D., and Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol. Biol. Evol.* 29, 1969–1973. doi: 10.1093/molbev/mss075

Faria, N. R., Kraemer, M. U. G., Hill, S. C., Goes De Jesus, J., Aguiar, R. S., Iani, F. C. M., et al. (2018). Genomic and epidemiological monitoring of yellow fever virus transmission potential. *Science* 361, 894–899. doi: 10.1126/science.aat7115

Ferreira, M. A. R., and Suchard, M. A. (2008). Bayesian analysis of elapsed times in continuous-time Markov chains. *Can. J. Stat.* 36, 355–368.

Ferreira-De-Brito, A., Ribeiro, I. P., Miranda, R. M., Fernandes, R. S., Campos, S. S., Silva, K. A., et al. (2016). First detection of natural infection of Aedes aegypti with Zika virus in Brazil and throughout South America. *Mem. Inst. Oswaldo Cruz* 111, 655–658. doi: 10.1590/0074-02760160332

Fioravanti, C. (2018). *O Alarme Dos Macacos*. Available at: http://revistapesquisa.fapesp.br/2018/01/11/o-alarme-dos-macacos/ (accessed January 31, 2019).

Frost, S. D., Pybus, O. G., Gog, J. R., Viboud, C., Bonhoeffer, S., and Bedford, T. (2015). Eight challenges in phylodynamic inference. *Epidemics* 10, 88–92. doi: 10.1016/j.epidem.2014.09.001

Gomez, M. M., Abreu, F. V. S., Santos, A., Mello, I. S., Santos, M. P., Ribeiro, I. P., et al. (2018). Genomic and structural features of the yellow fever virus from the 2016-2017 Brazilian outbreak. *J. Gen. Virol.* 99, 536–548. doi: 10.1099/jgv.0.001033

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010

Hamrick, P. N., Aldighieri, S., Machado, G., Leonel, D. G., Vilca, L. M., Uriona, S., et al. (2017). Geographic patterns and environmental factors associated with human yellow fever presence in the Americas. *PLoS Negl. Trop. Dis.* 11:e0005897. doi: 10.1371/journal.pntd.0005897

Instituto Brasileiro de Geografia e Estatística [IBGE] (2019). *Portal De mapas*. Available at: https://mapas.ibge.gov.br/images/pdf/mapas/mappag99.pdf (Accessed January 31, 2019).

Jung, L., Mourthe, I., Grelle, C. E., Strier, K. B., and Boubli, J. P. (2015). Effects of local habitat variation on the behavioral ecology of two sympatric groups of brown howler monkey (*Alouatta clamitans*). *PLoS One* 10:e0129789. doi: 10.1371/journal.pone.0129789

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lemey, P., Rambaut, A., Drummond, A. J., and Suchard, M. A. (2009). Bayesian phylogeography finds its roots. *PLoS Comput. Biol.* 5:e1000520. doi: 10.1371/journal.pcbi.1000520

Lemey, P., Rambaut, A., Welch, J. J., and Suchard, M. A. (2010). Phylogeography takes a relaxed random walk in continuous space and time. *Mol. Biol. Evol.* 27, 1877–1885. doi: 10.1093/molbev/msq067

Mir, D., Delatorre, E., Bonaldo, M., Lourenco-De-Oliveira, R., Vicente, A. C., and Bello, G. (2017). Phylodynamics of yellow fever virus in the Americas: new insights into the origin of the 2017 Brazilian outbreak. *Sci. Rep.* 7:7385. doi: 10.1038/s41598-017-07873-7

Monath, T. P., and Vasconcelos, P. F. (2015). Yellow fever. *J. Clin. Virol.* 64, 160–173. doi: 10.1016/j.jcv.2014.08.030

Nunes, M. R., Palacios, G., Cardoso, J. F., Martins, L. C., Sousa, E. C Jr., de Lima, C. P., et al. (2012). Genomic and phylogenetic characterization of Brazilian yellow fever virus strains. *J. Virol.* 86, 13263–13271. doi: 10.1128/JVI.00565-12

Possas, C., Lourenco-De-Oliveira, R., Tauil, P. L., Pinheiro, F. P., Pissinatti, A., Cunha, R. V. D., et al. (2018a). Yellow fever outbreak in Brazil: the puzzle of rapid viral spread and challenges for immunisation. *Mem. Inst. Oswaldo Cruz* 113:e180278. doi: 10.1590/0074-02760180278

Possas, C., Martins, R. M., Oliveira, R. L., and Homma, A. (2018b). Urgent call for action: avoiding spread and re-urbanisation of yellow fever in Brazil. *Mem. Inst. Oswaldo Cruz* 113, 1–2.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., and Suchard, M. A. (2018). Posterior summarization in bayesian phylogenetics using tracer 1.7. *Syst. Biol.* 67, 901–904. doi: 10.1093/sysbio/syy032

Rambaut, A., Lam, T. T., Max Carvalho, L., and Pybus, O. G. (2016). Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol.* 2:vew007.

Rezende, I. M., Sacchetto, L., Munhoz De Mello, E., Alves, P. A., Iani, F. C. M., Adelino, T. E. R., et al. (2018). Persistence of Yellow fever virus outside the Amazon Basin, causing epidemics in Southeast Brazil, from 2016 to 2018. *PLoS Negl. Trop. Dis.* 12:e0006538. doi: 10.1371/journal.pntd.0006538

Secretaria de Vigilância em Saúde (2014). *Epizootia em Primatas Não Humanos – PNH (macacos) Confirmada Para Febre Amarela em Taguatinga, Tocantins, Agosto de 2014*. Brazil: Ministério da Saúde.

Secretaria de Vigilância em Saúde (2015). *Reemergência da Febre Amarela Silvestre no Brasil, 2014/2015: Situação Epidemiológica e a Importância da Vacinação Preventiva e da Vigilância Intensificada no Período Sazonal*. Brazil: Ministério da Saúde.

Secretaria de Vigilância em Saúde (2017). *Emergência Epidemiológica de Febre Amarela no Brasil, no Período de Dezembro de 2016 a Julho de 2017*. Brazil: Ministério da Saúde.

Secretaria de Vigilância em Saúde (2019). *Monitoramento do Período Sazonal da Febre Amarela Brasil – 2018/2019*. Brazil: Ministério da Saúde.

Vasconcelos, P. F. (2010). Yellow fever in Brazil: thoughts and hypotheses on the emergence in previously free areas. *Rev. Saude Publica* 44, 1144–1149.

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4.7 Artigo 7: Combination of surveillance tools reveals that Yellow Fever virus can remain in the same Atlantic Forest area at least for three transmission seasons

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Combination of surveillance tools reveals that Yellow Fever virus can remain in the same Atlantic Forest area at least for three transmission seasons

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BACKGROUND In Brazil, the Yellow Fever virus (YFV) is endemic in the Amazon, from where it eventually expands into epidemic waves. Coastal south-eastern (SE) Brazil, which has been a YFV-free region for eight decades, has reported a severe sylvatic outbreak since 2016. The virus spread from the north toward the south of the Rio de Janeiro (RJ) state, causing 307 human cases with 105 deaths during the 2016-2017 and 2017-2018 transmission seasons. It is unclear, however, whether the YFV would persist in the coastal Atlantic Forest of RJ during subsequent transmission seasons.

OBJECTIVES To conduct a real-time surveillance and assess the potential persistence of YFV in the coastal Atlantic Forest of RJ during the 2018-2019 transmission season.

METHODS We combined epizootic surveillance with fast diagnostic and molecular, phylogenetic, and evolutionary analyses.

FINDINGS Using this integrative strategy, we detected the first evidence of YFV re-emergence in the third transmission season (2018-2019) in a dying howler monkey from the central region of the RJ state. The YFV detected in 2019 has the molecular signature associated with the current SE YFV outbreak and exhibited a close phylogenetic relationship with the YFV lineage that circulated in the same Atlantic Forest fragment during the past seasons. This lineage circulated along the coastal side of the Serra do Mar mountain chain, and its evolution seems to be mainly driven by genetic drift. The potential bridge vector *Aedes albopictus* was found probing on the recently dead howler monkey in the forest edge, very close to urban areas.

MAIN CONCLUSIONS Collectively, our data revealed that YFV transmission persisted at the same Atlantic Forest area for at least three consecutive transmission seasons without the need of new introductions. Our real-time surveillance strategy permitted health authorities to take preventive actions within 48 h after the detection of the sick non-human primate. The local virus persistence and the proximity of the epizootic forest to urban areas reinforces the concern with regards to the risk of re-urbanisation and seasonal re-emergence of YFV, stressing the need for continuous effective surveillance and high vaccination coverage in the SE region, particularly in RJ, an important tourist location.

Key words: Yellow Fever - amino acid changes - phylogeography - *Alouatta* - mosquito vectors

Except for the rare episodes of urban Yellow Fever (YF) transmission, human infections in the Americas have been acquired from the sylvatic cycle maintained between arboreal mosquitoes and non-human primates (NHPs).(1) Forests of northern South America have been regarded as a key territory for the maintenance and emergence of YF virus (YFV) lineages.(2,3) In Brazil, the YFV is enzootic/endemic in the Amazon Region, which is in the north from where, it sometimes expands towards the central-west, south (S), and south-eastern (SE) regions, causing isolated epizootic events and human infections to severe epidemics of sylvatic origin.(1,2) Epidemiological records gathered since the discovery of the YFV sylvatic cycle in Brazil in the early 1930s, as well as recent phylogenetic analyses of South American YFV samples have reinforced this concept.(1) The epidemic character of the sylvatic YF in the extra-Amazon Brazil-

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ian territory would be a consequence of the continuous reintroductions of YFV from the endemic region in the Amazon, as the former territories could not maintain the circulation of the virus after one or two transmission seasons, due to the substantial reduction in the number of susceptible vertebrate hosts.[1,4,5]

In the last two decades, a gradual expansion of YFV has been observed towards the coast of SE and S Brazil, an area that has been considered YF-free for almost 80 years, and therefore, without vaccine recommendations.[4,6,7] An entomological and surveillance investigation conducted in 2015-2016 to determine the composition and abundance of mosquito fauna in distinct ecosystems in the coastal SE region, particularly in the state of Rio de Janeiro (RJ) and its borders, has revealed that RJ was highly receptive and vulnerable to sylvatic YFV transmission.[8] In late 2016 and early 2017, the virus re-emerged in SE Brazil, and the states of Minas Gerais (MG), Espírito Santo (ES), and RJ were affected in sequence, initiating the largest epidemic of sylvatic YFV ever recorded in the country.[9,10,11,12] The affected area encompasses the biggest remnants of the Atlantic Forest in Brazil, which are often close to cities with the highest human population densities in the country, low YFV vaccination coverage and high *Aedes aegypti* infestation raising concern about the risks of YFV re-urbanisation.[4,13,14]

In March 2017, RJ reported the first human YF cases (n = 8); all of them were detected in the municipality of Casimiro de Abreu.[15] In this first transmission season (July 2016 to June 2017), a total of 25 YFV human cases, including eight deaths, and 25 confirmed epizootics of NHPs were recorded in 17 municipalities in the northern and central regions of RJ (Fig. 1).[10] During the second transmission season (July 2017 to June 2018), and despite vaccination efforts, 282 human YFV cases (including 97 deaths) and 71 confirmed epizootics were registered in 45 municipalities distributed across the central and southern regions of RJ.[11] In the third cycle (July 2018 to June 2019), no human case has been recorded till date (February 2019) in RJ. Only one epizootic of NHPs in this season was confirmed by immunohistochemistry (IMH) in the southernmost municipality in RJ (Paraty), while seven other cases were diagnosed only by polymerase chain reaction (PCR) (n = 7), pending confirmation either by viral genome sequencing or IMH; some of these epizootics originated from areas without any epidemiological evidence of YFV circulation. Interestingly, all these epizootics occurred in marmosets (genus *Callithrix*) in July 2018,[16] suggest-

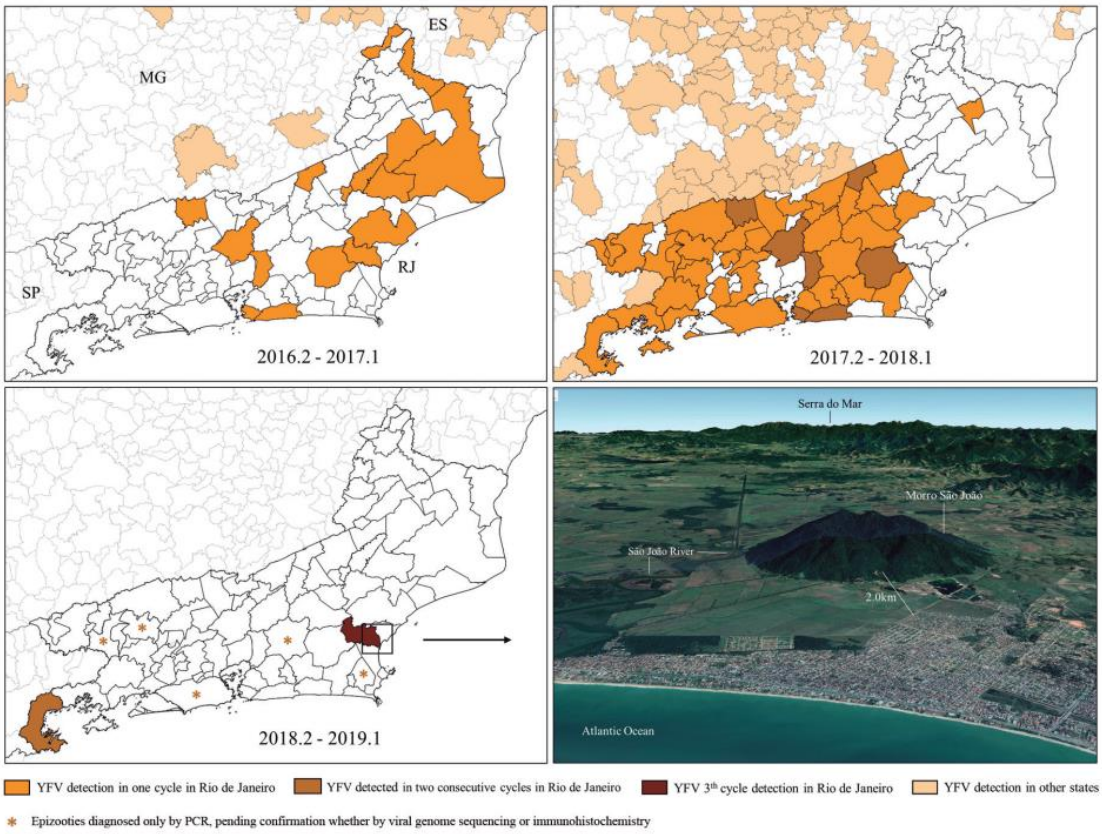

Fig. 1: Yellow Fever virus (YFV) detection in human and/or non-human primates, per county and seasonal transmission cycle, in Rio de Janeiro and its borders. The satellite image shows Morro São João, where the howler monkey was found, and its surroundings. This woody fragment is bordered by the São João River, whose gallery forest may serve as a corridor for virus dispersion between the more continuous forests on the steps of Serra do Mar. Frontal view of Morro do São João was obtained through the Google Earth Software, accessed on 22th Feb 2019.

ing that they were remnants of the previous transmission season. It is noteworthy that only one and four out of the 55 municipalities affected by the outbreak recorded confirmed YFV infections in humans and NHPs, respectively, for more than one transmission season.[17,18]

Epizootic surveillance combined with rapid laboratory diagnosis (molecular biology techniques and IMH) has been considered a potential tool for the early detection of the emergence of YFV and assures quick responses; it could also protect the human population by defining the affected areas and areas at risk and intensifying vaccination drives, communication, and appropriate control measures.[6,19,20] Simultaneous phylogeographical analyses of circulating viral genomes can also strengthen molecular epidemiology, detect the emergence of new lineages, and define transmission chains and virus dispersion routes, which in turn, may improve the timely establishment of prophylactic and control measures. Nevertheless, the efficient functioning of the epizootic surveillance depends on the support and alerts from a sensitised information network.[6,19,20]

By combining alerts from an information network constructed by us with the immediate use of the aforementioned laboratory tools, we promptly detected the re-emergence of YFV transmission in 2019 in an area where the outbreak had occurred two transmission seasons ago, and determined that virus transmission can be resilient in an Atlantic Forest zone, independent of new introductions.

MATERIALS AND METHODS

Sample collection - From 2015 onwards, we have built an information network comprising several kinds of agents (e.g. residents, environmental guards, health agents, conservation unit managers, and local guides) regularly visiting or living in target areas in RJ and consisting of contact chains of key institutions and inhabitants, to continuously monitor the epizootic events of NHPs in RJ using communication technologies such as message exchange applications (FVS Abreu, unpublished data).

On January 9th 2019, we received an alert from the information network reporting a dying howler monkey in Morro de São João (22°32'37.98"S 42°0'43.88"W), a mountain with a dense vegetation cover located in the coastal lowlands of Casimiro de Abreu, the municipality where the first human YF case was reported in RJ and whose last detected YFV circulation was recorded in early 2017. The animal was found only 2.0 km away from a considerably dense urban area (Fig. 1).

Our team immediately went to the area (166 km from the lab). On our arrival, the monkey had already died. We were attacked by numerous *Aedes albopictus* mosquitoes, which also tried to bite the recently dead animal, apparently without success, as engorged females were not observed (Fig. 2). We performed the necropsy and sample collection on the field, in accordance with the biosafety protocols and the current biodiversity laws.[20] The animal, a young adult female *Alouatta guariba clamitans* without any wound or external mark, was named RJ155. Samples of the animal's viscera (liver, spleen, heart, kidney, lung), whole blood, and serum were immediately frozen on dry ice. The carcass was kept refrigerated until delivery at the state health department of RJ, with the epizootic notification form filled out by the municipal health division, as officially recommended. One week later, we visited the site again for mosquito collection. Mosquitoes were collected and tested in pools from 1 to 10 individuals, according to the species, as previously described.[8,21]

RNA extraction, virus detection, and sequencing - On the same day, RNA was extracted from 140 µL of RJ155's serum and whole blood using the Qiagen RNA Viral Kit, following the manufacturer's recommendations. Real-time reverse transcription quantitative PCR (RT-qPCR) was performed in duplicate, as described previously.[8,21] The set of primers utilised for the PCR and viral genome sequencing procedures followed a previous report.[9] The nucleotide sequence was determined by capillary electrophoresis at the sequencing facility of Fiocruz-RJ (RPT01A — Sequenciamento de DNA - RJ). The sequences were assembled using SeqMan Pro version 8.1.5 (DNASTAR, Madison, WI, USA). The amino acid differences, as well as nucleotide and amino acid distances, were calculated using the molecular evolutionary genetics analysis (MEGA) 7.0 program.

YFV dataset assembly - The complete polyprotein open reading frame [complete coding sequence (CDS), 10,239 nt in length] of the genome of the newly generated 2019 YFV from the Casimiro de Abreu municipality was combined with all American YFV sequences available in GenBank on January 2019 (www.ncbi.nlm.nih.gov/genbank/) with known dates and countries of collection, covering at least 99% of the viral CDS, and with an associated publication. The sequences were aligned using MAFFT[22] and classified into South American genotypes I and II by reconstructing a maximum likelihood (ML) phylogenetic tree with PhyML[23], under the best substitution model selected by jModelTest v1.6.[24] Branch support was estimated with the approximate likelihood-ratio test (aLRT). Only YFV sequences classified as South American I genotype (SA-I) were retained for further analyses.

Evolutionary and phylogeographic analysis - The spatiotemporal viral diffusions were reconstructed by Bayesian inference with Markov chain Monte Carlo (MCMC) sampling, as implemented in the BEAST v1.8.4 package,[25] using BEAGLE[26] to improve the running time. The GTR+I nucleotide substitution model selected by jModelTest v1.6,[24] a relaxed lognormal molecular clock model calibrated with a normal prior based on previous estimates,[9] and the non-parametric Bayesian skyline coalescent model were used in case of all Bayesian phylogeographic inferences. The best discrete (symmetric or asymmetric)[27] and continuous (homogenous or heterogeneous)[28] phylogeographic models were selected using a marginal likelihood estimator (MLE), employing the path sampling (PS) and stepping-stone sampling (SS) approaches.[29] The analyses were run for 10^8 MCMC iterations, and the convergence (effective sample size > 200) was assessed using TRACER v1.7 (beast.community/tracer) after discarding a 10% burn-in. The maximum clade credibility (MCC) trees were summarised

using TreeAnnotator v.1.8.4 and visualised with FigTree v.1.4.4 (tree.bio.ed.ac.uk). The viral spatiotemporal diffusion was analysed and visualised in SPREAD,[30] and further projected in maps generated using the QGIS software (qgis.org) including cartographic information provided by the Brazilian Institute of Geography and Statistics (https://mapas.ibge.gov.br/) and information about the Atlantic Forest remnants (2016 estimates) from the Brazilian National Institute for Space Research and SOS Mata Atlântica Foundation (mapas.sosma.org.br/).

Genetic distance and selection analysis - The ancestral CDS of the node encompassing all YFV sequences from RJ closely related to the 2019 YFV strain was reconstructed by Bayesian inference as described above, and computed using the Geneious 9.1.4 program (https://www.geneious.com). Genetic differences relative to the inferred ancestral CDS were calculated based on the global (d_{nt}) (TN model), synonymous (*dS*) and nonsynonymous (*dN*)[31,32] nucleotide distances, which were obtained using MEGA 7. The analysis of selection was performed on the Datamonkey web server (www.datamonkey.org) using the HyPhy package[33] with the fixed-effect likelihood (FEL)[33] and mixed effect model evolution (MEME)[34] methods, and incorporating the best nucleotide substitution model.

Ethics - Our methods and protocols were previously approved by the institutional Ethics Committee for Animal Experimentation (protocol CEUA/IOC-029/2016, license L-037/2016), the Brazilian Ministry of the Environment (SISBIO 41837-3 and 54707-4), and RJ's Environment agency (INEA 012/2016 and 019/2018).

RESULTS

RT-qPCR analysis detected YFV RNA in the serum and whole blood collected from RJ155 (mean of CT value = 19.7); this diagnosis was further confirmed by sequencing the complete genome of YFV (GenBank accession number: MK533792). However, the 278 mosquitoes (one *Haemagogus janthinomys*, two *Hg. leucocelaenus*, five *Sabethes albiprivus*, 15 *Ae. albopictus*, and 255 *Ae. scapularis*) collected at the Morro de São João a small-time interval after the notification alert for the dying monkey tested negative for YFV.

The identity between the RJ155 YFV and the YFV sequenced in the 2016-2017 transmission season from the neighbouring counties is high, from 99.8 to 99.9% and from 99.7 to 100%, which are the nucleotide and amino acid identity values, respectively [Supplementary data (Table I)]. The number of nucleotide changes indicates the evolution of the viral genome, most probably due to a bottleneck effect, promoted by the dissemination of the virus into a small number of hosts while circulating silently in the zone [Supplementary data (Table II)]. However, we noticed six amino acid alterations not only in the YFV samples from bordering municipalities, but also in case of all YFV genomes described so far in the ongoing outbreak (Table). The amino acid variations mainly map at the following non-structural YFV proteins: C 103 (R); NS1 51 (D); NS3 (G); NS4A (I); NS5 391 (K), NS5 622 (M), and NS5 645 (I).

The ML phylogenetic analysis revealed that the RJ155 YFV 2019 genome showed the highest support values (aLRT = 1) when clustered with other YFV strains from the ongoing outbreak in SE Brazil [Supplementary data (Figure)]. In order to better understand the geographic origin of the RJ155 YFV, we performed a discrete Bayesian phylogeographic analysis of all YFVs from the current SE Brazilian outbreak. As there was no significant evidence favouring one of the discrete phylogeographic models [Supplementary data (Table III)], we have presented the results of the symmetric one. The inferred Bayesian MCC tree supports at least four independent introductions of YFV into RJ from ES and confirmed at least two viral transmission chains in RJ[9] (Fig. 3). The RJ155 YFV clustered within the RJ transmission chain here called $YFV_{RJ\text{-}I}$ that likely originated in RJ [posterior state probability (PSP) = 0.95] around December 2016 [95% Bayesian credible interval (BCI) July 2016-March 2017]. The $YFV_{RJ\text{-}I}$ clade also comprises an older YFV sample from Casimiro de Abreu (March 2017) obtained from a human case and other YFVs obtained from humans and NHPs in neighbouring municipalities (Macaé, Silva Jardim and Guapimirim) from April to June 2017.

To assess the spatial spread of the $YFV_{RJ\text{-}I}$ lineage with more precision, we employed a relaxed random walk phylogeographic model with a lognormal distribution, selected as the fittest continuous phylogeographic model [Supplementary data (Table IV)]. The continuous diffusion inference placed the origin of the $YFV_{RJ\text{-}I}$ lineage in the municipality of Macaé in January 2017 (BCI September 2016-February 2017), from where it rapidly spread throughout the coastal steps of the Serra do Mar mountain chain and the lowlands shared with the neigh-

TABLE

Polyprotein polymorphisms present in the 2019 Yellow Fever virus (YFV) from Morro de São João in comparison with previous circulating YFV in Casimiro de Abreu, and in the bordering municipalities (Macaé and Silva Jardim) in the Rio de Janeiro state

YFV sample / polyprotein position	103	829	1744	2897	3128	3151
MK533792/2019/RJ155/MorroSJoão/M	R	D	G	K	M	I
MF423375/2017/RJ87/MacaéAtalaia/M	Q	E	E	N	I	T
MF538786/2017/RJ104/Guapimirim/M	Q	E	E	N	I	T
MF423376/ 2017/RJ94/MacaéSana/M	Q	E	E	N	I	T
MF434851/ 2017/H199/SilvaJardim/H	Q	E	E	N	I	T

bouring municipalities of Casimiro de Abreu and Silva Jardim, and the more distant municipality of Guapimirim (Fig. 4). The continuous diffusion model supports that the dissemination range of the YFV_{RJ-I} lineage covers an extensive area of Atlantic Forest remains situated along the coastal side of Serra do Mar, shared by the municipalities of Macaé, Rio das Ostras, Casimiro de Abreu, Nova Friburgo, and Silva Jardim. This zone is very close to urbanised areas, including the place where the RJ155 was sampled, which is located only two kilometres away from the contiguous urban perimeter of Casimiro de Abreu and Rio das Ostras (Fig. 1). These results clearly support that the YFV has persisted in the Atlantic Forest area of RJ for three consecutive YFV transmission seasons (2016-2017, 2017-2018, and 2018-2019).

To examine the genetic divergence of the RJ155 YFV relative to previous virus samples, we plotted the root-to-tip divergence from the ML tree as a function of sampling time (Fig. 5A). We found a strong correlation between the divergence and time of sampling ($R^2 = 0.94$). The RJ155 YFV showed a low deviation from the mean regression line, with residuals comparable with other YFV genomes sampled previously. The RJ155 showed a low mean global nucleotide genetic divergence ($d_{nt} >$ 0.2%) from the ancestral CDS of the YFV_{RJ-I} lineage. The mean nucleotide synonymous (*dS*) distance was much higher than the mean nonsynonymous (*dN*) distance in both the structural and non-structural genomic regions (Fig. 5B). The analysis of selective pressure (normalised *dN-dS* along the CDS) showed an overrepresentation of sites accumulating synonymous substitutions. No positively selected codon positions were identified at the entire CDS sequence by the MEME or REL methods, and two sites in the NS5 gene (positions 3017 and 3406) displayed a trend (p-values 0.081 and 0.072, respectively) towards negative selection (Fig. 5C). These observations support the claim that the evolution of the YFV_{RJ-I} lineage was mainly driven by genetic drift.

Fig. 2: *Aedes albopictus* mosquitoes trying to bite one hand of the recently dead *Alouatta guariba clamitans* found in Morro de São João (22°32'37.98"S 42°0'43.88"W) on January 10th, 2019.

DISCUSSION

The present work describes the first record of YFV circulation in RJ in 2019, which essentially consists of the primary sign of virus re-emergence in the state since July 2018, when the epidemiological transmission season had started. Epizootics reported in marmosets in the central and southern zones of RJ nearly six months ago were remnants of the previous transmission season. In turn, we report a YFV infection in a howler monkey from the north-coastal zone, where the YF outbreaks and epizootic records had peaked in early 2017 and then moved southward. This suggests that viral circulation may be soundless in the coastal SE Brazil. Moreover, our phylogeographic and evolutionary analyses showed that YFV may persist in this zone for at least three consecutive transmission seasons, without the need for new introduction. These data unprecedentedly demonstrate that YFV can be locally maintained in the Atlantic Forest for more than two transmission seasons.

When investigating YF sylvatic epidemics occurring in SE Brazil during the 1930s and 1940s, the authors concluded that in this region, YFV spread in the form of an intermittent wave from infected areas to unaffected neighbouring sites, involving a series of annual outbreaks coinciding with the rainy season (summer), and essentially stopping during the winter, only to retake its course and spread in the next rainy season into sites that were close to those affected during the previous summer.[5,35,36] Despite describing the same phenomenon in the current outbreak, Rezende et al.[37] named this spreading as persistence. Here, we demonstrated that besides spreading to new areas, YFV was able to persist in the same zone for at least three transmission seasons in RJ. The mosaic pattern of the Atlantic Forest may have contributed to the maintenance of YFV circulation in such a zone for at least three seasons, where some isolated fragments may not be affected by the expanding wave. Such "virgin islands," where the YFV has not circulated, may serve as new focal points for viral re-emergence and amplification.

Besides the fragmentation of the Atlantic Forest, the increase of NHP diversity and abundance may have also contributed to YFV persistence in RJ. Previous studies have concluded that YFV typically remains during only one, but not for more than two transmission seasons in the same area in the Atlantic Forest of the SE region.

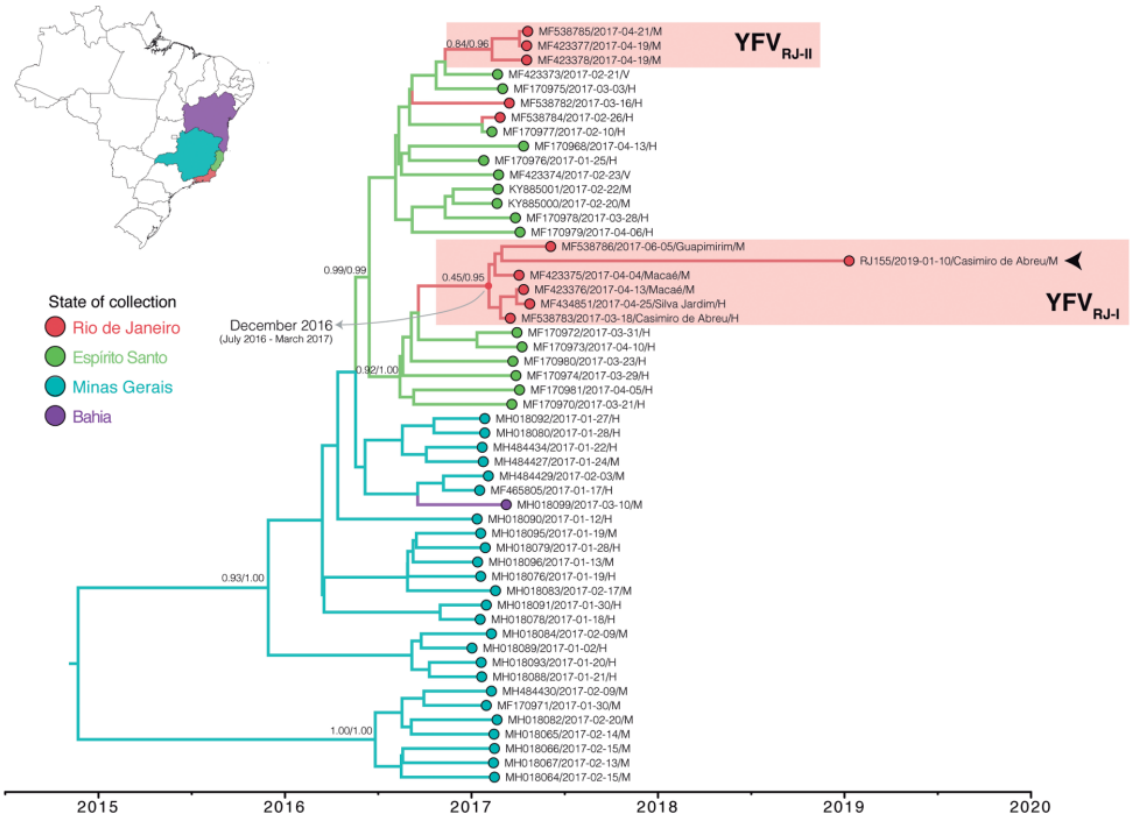

Fig. 3: maximum clade credibility phylogeographic tree of the Yellow Fever virus (YFV) strains involved in the ongoing outbreak. The colours' branches represent the most probable location of their descendent nodes, as indicated in the legend and the map. The posterior and posterior state probability (PP/PSP, respectively) of key nodes are indicated above the branches. All horizontal branch lengths are drawn to a scale of years. The two lineages found in Rio de Janeiro state (YFV_{RJ-I} and YFV_{RJ-II}) are indicated by red shaded boxes, and the RJ155 samples are indicated by arrows.

[5,35,38,39,40] Taylor and Fonseca-Cunha,[36] while describing the 1934-1936 sylvatic YFV epidemic in the SE, claimed that "the virus appeared to "burn itself out" as it progressed and rarely lasted longer than one season in any given locality". The main reason for this pattern would be the reduction in the number of local susceptible hosts due to the rapid and intense transmission and spatial spread of YFV[39,40] across the fragmented Atlantic Forest in the SE region, the most populated and developed Brazilian region. On the other hand, in the Amazon Region, the dense and continuous forests, together with the great diversity of NHPs, enable an almost perennial circulation of the virus. In fact, YFV had disappeared spontaneously from the coastal Atlantic Forest for almost 80 years. However, during the last decades, environmental and biodiversity protection policies have achieved considerable success. Significant augmentation of conservation units and numerous reforestation initiatives, and the recovery of ecological corridors in the Atlantic Forest have been observed; this may have expanded the zones with suitable environmental conditions to support higher mosquito and NHP species diversity and abundance, especially compared to the case for when the Brazilian developmental policies were put into practice after the second war.[1,41] Interestingly, the zone where we detected the virus during the third transmission season (2018-2019) coincides with the RJ Atlantic Forest region which has one of the greatest diversities of native NHP genera, including species of *Alouatta*, *Sapajus* (capuchins), *Callicebus* (titis), *Callithrix* and *Leontopithecus* (lion-tamarins); the endangered species *L. rosalia* (golden-lion-tamarin), which was affected during the 2017-2018 season,[34] is endemic to this zone of RJ, and the major conservation unit established for the protection of this species is in the same zone. Two recent studies that modelled the epidemiology of YF found that the distribution and diversity of NHP genera are highly associated with the risk of YFV outbreak.[42,43]

YFV was also likely maintained in RJ through vertical transmission in the vectors between seasonal peaks of disease spread,[44] which in Brazil occurs during the summer, when higher mean temperatures and abundant rainfall favour the reproduction of mosquitoes like *Haemagogus sp.*, a tree-hole breeding species.[45,46] Although we have not found infected mosquitoes in our collections made where we sampled the RJ155 YFV, primary (*Hg. leucocelaenus*, *Hg. janthinomys*) and secondary or potential (*Sa. albiprivus*, *Ae. scapularis*, *Ae. albopictus*) vectors[8,47,48] were detected. The most abundant mosquito species, i.e. *Ae. scapularis* and *Ae. albopictus*, usually

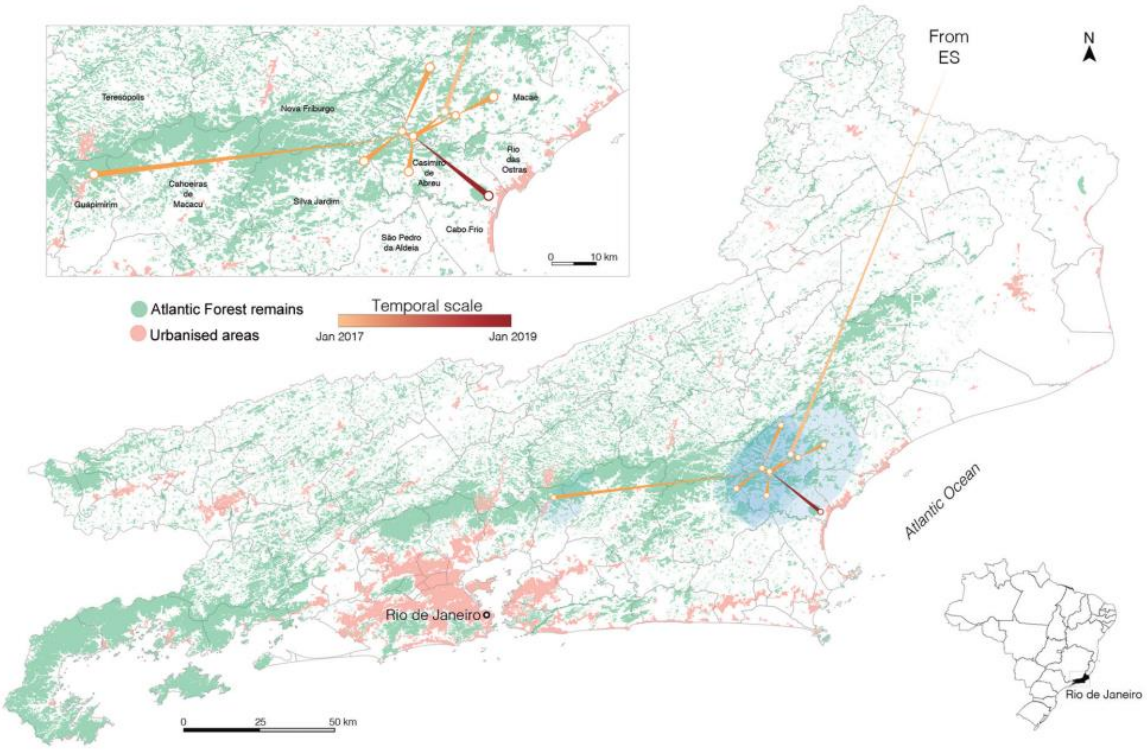

Fig. 4: reconstructed spatiotemporal diffusion of the Yellow Fever virus $(YFV)_{RJ\text{-}I}$ lineage. The branches of the $YFV_{RJ\text{-}I}$ lineage phylogeny were arranged in space (map of Rio de Janeiro state with municipalities borders) according the locations of known (external) and inferred (internal) nodes (circles). The blue shaded regions represent the 95% credible regions of the inferred internal nodes. The branch thickness represents the spread direction (thin to thick) between locations. The inset shows a close view of viral dissemination, with the municipalities' names. ES: Espírito Santo state.

bite at the ground level, which is a behaviour limiting their chance of getting infected by feeding on viraemic forest canopy-inhabiting NHPs such as howler monkeys. However, many sick monkeys get down from the canopy and agonise for several hours on the ground; this is when they may be easily bitten by such species of mosquitoes.[8] *Ae. albopictus* is considered a probable bridge vector for YFV at the urban area-forest interface in Brazil;[47,49] its contact with viraemic howler monkeys inhabiting regions that are in close proximity to an urban area registered in our study (Figs 1-2) has been highlighted as an important risk factor for YF re-urbanisation in the SE.[1]

YFV evolution in fragmented habitats through vertical transmission in the vectors might impose strong bottlenecks to viral diversity. The divergence accumulated in the RJ155 YFV, however, was compatible with the expected mean divergence for YFV, indicating that the substitutions were not accumulated in this strain at a faster rate compared with that in other viral strains. The overrepresentation of negative dN-dS values found in the YFVRJ-I lineage indicates that the vast majority of mutations in these viruses are synonymous and fixed by purifying the selection and/or genetic drift, as previously observed for YFV.[50] Arthropod-borne viruses like YFV are characterised by higher levels of negative selection pressure than RNA viruses transmitted by other routes, which is probably a consequence of a life cycle involving phylogenetically divergent hosts.[51]

Our study clearly supports that YFV circulation may be soundless in the coastal SE and that the transmission of this virus may gain force, and cause its re-emergence in the form of epizootics when conditions are suitable. Silent viral circulation or absence of notifications can lead to the demobilisation and deintensification of prophylactic and communication actions, which can have serious consequences. The local persistence of YFV and the possibility of seasonal re-emergences reinforce the need for maintaining continuous surveillance and high vaccination coverage in the SE, particularly in RJ, a state that receives a large influx of (vaccinated and unvaccinated) tourists during the summer who could become infected and export the virus to other states and countries.[52] Effective epizootic surveillance, combined with fast diagnostic and phylogenetic analyses is critical in raising timely awareness and establishing control measures. Using this strategy, we could alert the state and municipal health systems in RJ about YFV re-emergence within 48 hours allowing the authorities to take appropriate preventive actions immediately.

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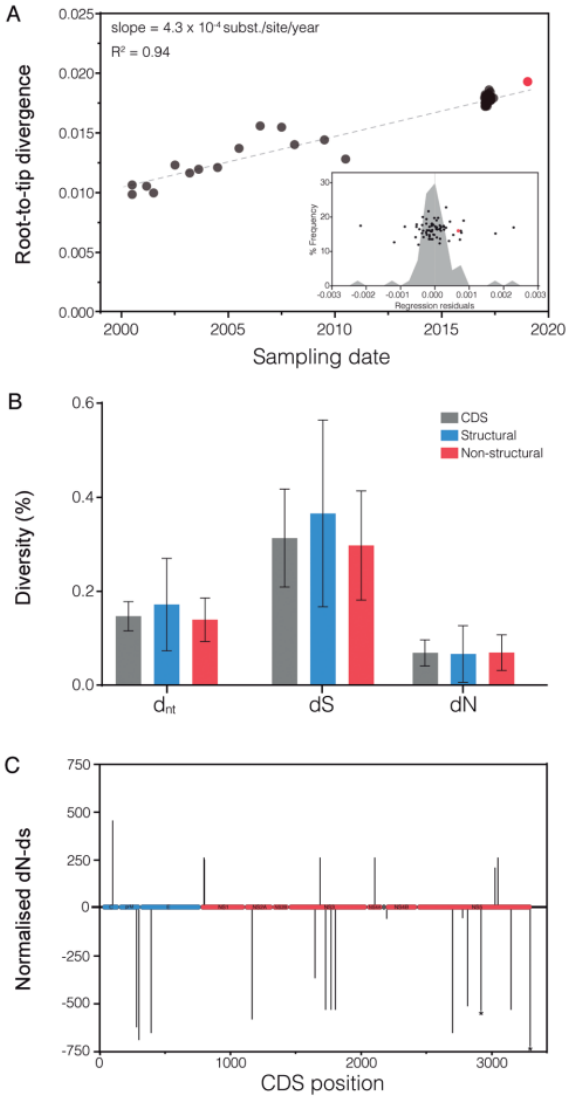

Fig. 5: Yellow Fever virus $(YFV)_{RJ-I}$ divergence and selection analysis. (A) Root-to-tip regression of sequence sampling date against genetic divergence from the root of the South American I genotype. The inset panel contains a histogram and scatterplot of the residuals of the linear regression. The RJ155 sequence is represented using red colour. (B) Mean divergence of RJ155 at the nucleotide (d_{nt}), synonymous (dS), and non-synonymous (dN) levels in the complete coding sequence (CDS, grey), and the structural (blue) and non-structural (red) genes. The columns represent mean distances, and error bars represent ± standard errors of the mean obtained by bootstrap. (C) Normalised dN-dS values by codon position across the complete YFV CDS (structural and non-structural genes are coloured blue and red, respectively). The asterisks indicate positions with p-values < 0.1 in the REL analysis.

AUTHORS' CONTRIBUTION

ED, FVSA, GB, MCB, and RLO conceived the study; FVSA and WPV monitored and collected the biological specimens; FVSA performed the identification of mosquito species; AFB, FVSA, and MGC carried out viral RNA extraction from the biological specimens and the diagnosis by RT-PCR; MGC inoculated the biological specimens in the cell culture; AACS, IPR, and NDF performed the genome sequencing; AACS, IPR, MCB, and NDF analysed the genome sequence; ED and GB performed the phylogenetic/phylogeographic analyses; ED, FVSA, GB, IPR, MCB, NDF, and RLO prepared the figures, tables, and/or supplementary material; FVSA, MSR, PM, and WPV gathered, systematised, and illustrated the epidemiological records; ED, FVSA, GB, IPR, MCB, and RLO prepared the manuscript. All authors have critically read and approved the final version of the manuscript. The authors declare that there is no conflict of interest.

REFERENCES

1. Possas C, Lourenço-de-oliveira R, Tauil PL, Pinheiro FDP, Pissinatti A, da Cunha RV, et al. Yellow fever outbreak in Brazil : the puzzle of rapid viral spread and challenges for immunisation. Mem Inst Oswaldo Cruz. 2018; 113(10): e180278.

2. Nunes MRT, Palacios G, Cardoso JF, Martins LC, Sousa EC, de Lima CPS, et al. Genomic and phylogenetic characterization of Brazilian yellow fever virus strains. J Virol [Internet]. 2012 [cited 2019 Feb 13]; 86(24): 13263-71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23015713.

3. Mir D, Delatorre E, Bonaldo M, Lourenço-de-Oliveira R, Vicente AC, Bello G. Phylodynamics of Yellow Fever virus in the Americas: new insights into the origin of the 2017 Brazilian outbreak. Sci Rep [Internet]. 2017 [cited 2018 Aug 12]; 7(1): 7385. Available from: http://www.nature.com/articles/s41598-017-07873-7.

4. Vasconcelos PFC. Yellow Fever in Brazil: thoughts and hypotheses on the emergence in previously free areas. Rev Saude Publica. 2010; 44(6): 1144-9.

5. Strode GK. Yellow Fever. New York-London: McGraw-Hill; 1951. 710 pp.

6. Romano APM, Costa ZGA, Ramos DG, Andrade MA, de Jayme VS, de Almeida MAB, et al. Yellow Fever outbreaks in unvaccinated populations, Brazil, 2008-2009. PLoS Negl Trop Dis. 2014; 8(3): 18-21.

7. Mascheretti M, Tengan CH, Sato HK, Suzuki A, de Souza RP, Maeda M, et al. Yellow Fever: reemerging in the state of São Paulo, Brazil, 2009. Rev Saude Publica. 2013; 47(5): 881-9.

8. Abreu FVS, Ribeiro IP, Ferreira-de-Brito A, dos Santos AAC, de Miranda RM, Bonelly IS, et al. *Haemagogus leucocelaenus* and *Haemagogus janthinomys* are the primary vectors in the major Yellow Fever outbreak in Brazil, 2016-2018. Emerg Microbes Infect. 2019; 8(1): 218-31. Available from: https://www.tandfonline.com/doi/full/10.1080/22221751.2019.1568180.

9. Gómez MM, de Abreu FVS, dos Santos AAC, de Mello IS, Santos MP, Ribeiro IP, et al. Genomic and structural features of the Yellow Fever virus from the 2016-2017 Brazilian outbreak. J Gen Virol. 2018; 99(4): 536-48.

10. MS/SVS - Ministério da Saúde/Secretaria de Vigilância em Saúde. Monitoramento dos casos e óbitos de Febre Amarela no Brasil — Informe No 43/2017 [Internet]. 2017 [cited 2019 Feb 7]. Available from: http://portalarquivos.saude.gov.br/images/pdf/2017/junho/02/COES-FEBRE-AMARELA---INFORME-43---Atualiza---o-em-31maio2017.pdf.

11. MS/SVS - Ministério da Saúde/Secretaria de Vigilância em Saúde. Monitoramento do período sazonal da Febre Amarela [Internet]. 2018 [cited 2019 Feb 7]. Available from: http://portalarquivos2.saude.gov.br/images/pdf/2018/fevereiro/21/Informe-n14-FA-20fev18-c.pdf.

12. Faria NR, Kraemer MUG, Hill SC, de Jesus JG, Aguiar RS, Iani FCM, et al. Genomic and epidemiological monitoring of Yellow Fever virus transmission potential. Science [Internet]. 2018 [cited 2019 Feb 9]; 361(6405): 894-9. Available from: http://science.sciencemag.org/content/361/6405/894.long.

13. Bonaldo MC, Gómez MM, dos Santos AA, de Abreu FVS, Ferreira-de-Brito A, de Miranda RM, et al. Genome analysis of Yellow Fever virus of the ongoing outbreak in Brazil reveals polymorphisms. Mem Inst Oswaldo Cruz [Internet]. 2017; 112(6): 447-51. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762017000600447&lng=en&tlng=en.

14. Massad E, Amaku M, Antonio F, Coutinho B, Struchiner CJ, Lopez LF, et al. The risk of urban Yellow Fever resurgence in *Aedes*-infested American cities. Epidemiol Infect. 2018; 146(10): 1219-25.

15. Secretaria de Saúde de Estado do Rio de Janeiro. Vigiliância em Saúde. Informe Epidemiológico 001/2017 [Internet]. 2017 [cited 2019 Feb 5]. Available from: http://www.riocomsaude.rj.gov.br/Publico/MostrarArquivo.aspx?C=xjekgJZZmu4%3D.

16. MS/SVS - Ministério da Saúde/Secretaria de Vigilância em Saúde. Monitoramento do período sazonal da Febre Amarela Brasil — 2018/2019 [Internet]. 2019 [cited 2019 Feb 7]. Available from: http://portalarquivos2.saude.gov.br/images/pdf/2019/janeiro/28/informe-FA-n.3-21jan19.pdf.

17. Secretaria de Estado de Saúde do Rio de Janeiro. Vigilância em Saúde. Informe Epidemiológico 05/07/2018 [Internet]. 2018 [cited 2019 Feb 7]. Available from: http://www.febreamarelarj.com.br/comum/code/MostrarArquivo.php?C=NjE%2C.

18. Secretaria de Saúde de Estado do Rio de Janeiro. Vigilância em Saúde. Informe Epidemiológico 071/2017 [Internet]. 2017 [cited 2019 Feb 7]. Available from: http://www.riocomsaude.rj.gov.br/Publico/MostrarArquivo.aspx?C=hBeIh5wX1BE%3D.

19. Almeida MAB, Cardoso JC, dos Santos E, da Fonseca DF, Cruz LL, Faraco FJC, et al. Surveillance for Yellow Fever virus in non-human primates in Southern Brazil, 2001-2011: a tool for prioritizing human populations for vaccination. PLoS Negl Trop Dis. 2014; 8(3): e2741.

20. MS/SVS – Ministério da Saúde/Secretaria de Vigilância em Saúde/Departamento de Vigilância das Doenças Transmissíveis. Guia de vigilância de epizootias em primatas não humanos e entomologia aplicada à vigilância da Febre Amarela. 2a. ed. Brasília: Ministério da Saúde; 2017 [cited 2019 Feb 2]. 99 pp. Available from: http://portalarquivos.saude.gov.br/images/pdf/2017/marco/24/Guia_Epizootias_Febre_Amarela_2a_ed_atualizada_2017.pdf.

21. Domingo C, Patel P, Yillah J, Weidmann M, Mendez JA, Nakoune ER, et al. Advanced Yellow Fever virus genome detection in point-of-care facilities and reference laboratories. J Clin Microbiol [Internet]. 2012 [cited 2019 Mar 12]; 50(12): 4054-60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23052311.

22. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol [Internet]. 2013 [cited 2019 Feb 21]; 30(4): 772-80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23329690.

23. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol [Internet]. 2010 [cited 2019 Feb 21]; 59(3): 307-21. Available from: https://academic.oup.com/sysbio/article/59/3/307/1702850.

24. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods [Internet]. 2012 [cited 2019 Feb 21]; 9(8): 772. Available from: http://www.nature.com/articles/nmeth.2109

25. Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol [Internet]. 2012 [cited 2019 Feb 21]; 29(8): 1969-73. Available from: https://academic.oup.com/mbe/article-lookup/doi/10.1093/molbev/mss075.

26. Ayres DL, Darling A, Zwickl DJ, Beerli P, Holder MT, Lewis PO, et al. BEAGLE: an application programming interface and high-performance computing library for statistical phylogenetics. Syst Biol [Internet]. 2012 [cited 2019 Feb 21]; 61(1): 170-3. Available from: https://academic.oup.com/sysbio/article/61/1/170/1680634.

27. Lemey P, Rambaut A, Drummond AJ, Suchard MA. Bayesian phylogeography finds its roots. PLoS Comput Biol [Internet]. 2009 [cited 2019 Feb 21]; 5(9): e1000520. Available from: https://dx.plos.org/10.1371/journal.pcbi.1000520.

28. Lemey P, Rambaut A, Welch JJ, Suchard MA. Phylogeography takes a relaxed tandom walk in continuous space and time. Mol Biol Evol [Internet]. 2010 [cited 2019 Feb 21]; 27(8): 1877-85. Available from: https://academic.oup.com/mbe/article-lookup/doi/10.1093/molbev/msq067.

29. Baele G, Lemey P, Bedford T, Rambaut A, Suchard MA, Alekseyenko AV. Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. Mol Biol Evol [Internet]. 2012 [cited 2019 Feb 21]; 29(9): 2157-67. Available from: https://academic.oup.com/mbe/article-lookup/doi/10.1093/molbev/mss084.

30. Bielejec F, Rambaut A, Suchard MA, Lemey P. SPREAD: spatial phylogenetic reconstruction of evolutionary dynamics. Bioinformatics [Internet]. 2011 [cited 2019 Feb 21]; 27(20): 2910-2. Available from: https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btr481.

31. Li WH. Unbiased estimation of the rates of synonymous and nonsynonymous substitution. J Mol Evol [Internet]. 1993 [cited 2019 Feb 21]; 36(1): 96-9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8433381.

32. Pamilo P, Bianchi NO. Evolution of the *Zfx* and *Zfy* genes: rates and interdependence between the genes. Mol Biol Evol [Internet]. 1993 [cited 2019 Feb 21]; 10(2): 271-81. Available from: https://academic.oup.com/mbe/article/10/2/271/1140324/Evolution-of-the-Zfx-and-Zfy-genes-rates-and.

33. Pond SLK, Frost SDW, Muse SV. HyPhy: hypothesis testing using phylogenies. Bioinformatics [Internet]. 2005 [cited 2019 Feb 21]; 21(5): 676-9. Available from: https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/bti079.

34. Murrell B, Wertheim JO, Moola S, Weighill T, Scheffler K, Pond SLK. Detecting individual sites subject to episodic diversifying selection. PLoS Genet [Internet]. 2012 [cited 2019 Feb 21]; 8(7): e1002764. Available from: https://dx.plos.org/10.1371/journal.pgen.1002764.

35. Soper FL. Yellow Fever: the present situation (October 1938) with special reference to South America. Trans R Soc Trop Med Hyg [Internet]. 1938 [cited 2019 Jan 4]; 32(3): 297-322. Available from: https://academic.oup.com/trstmh/article-lookup/doi/10.1016/S0035-9203(38)90045-8.

36. Taylor RM, Da Cunha JF. Part I — Epidemiology of human infections. Am J Trop Med Hyg [Internet]. 1946 [cited 2019 Jan 26]; s1-26(6_Suppl_2): 1-21. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1946.s1-26.1.

37. de Rezende IM, Sacchetto L, Mello ÉM, Alves PA, Iani FCM, Adelino TÉR, et al. Persistence of Yellow Fever virus outside the Amazon Basin, causing epidemics in Southeast Brazil, from 2016 to 2018. PLoS Negl Trop Dis. 2018; 12(6): 1-12.

38. Causey OR, Hughes TP, Laemmert HW. The invasion of small forests by Yellow Fever virus as indicated by immunity in *Cebus* monkeys 1. Am J Trop Med Hyg [Internet]. 1949 [cited 2019 Jan 26]; s1-29(4): 555-65. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1949.s1-29.555.

39. Laemmert HW, Ferreira LDC, Taylor RM. Part II — Investigations of vertebrate hosts and arthropod vectors. Am J Trop Med Hyg [Internet]. 1946 [cited 2019 Jan 26]; s1-26(6_Suppl_2): 23-69. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1946.s1-26.23.

40. Bugher JC, Boshell-Manrique J, Roca-García M, Osorno-Mesa E. Epidemiology of jungle Yellow Fever in Eastern Colombia. Am J Epidemiol [Internet]. 1944 [cited 2019 Fev 02]; 39(1): 16-51. Available from: https://academic.oup.com/aje/article-lookup/doi/10.1093/oxfordjournals.aje.a118895.

41. Lafer C. JK e o programa de metas (1956-1961) processo de planejamento e sistema político no Brasil. Rio de Janeiro: FGV; 2002. 256 pp.

42. de Almeida MAB, dos Santos E, Cardoso JC, da Silva LG, Rabelo RM, Bicca-Marques JC. Predicting Yellow Fever through species distribution modeling of virus, vector, and monkeys. Ecohealth [Internet]. 2018 [cited 2019 Feb 9]; 1–14. Available from: http://link.springer.com/10.1007/s10393-018-1388-4.

43. Hamrick PN, Aldighieri S, Machado G, Leonel DG, Vilca LM, Uriona S, et al. Geographic patterns and environmental factors associated with human Yellow Fever presence in the Americas. PLoS Negl Trop Dis. 2017; 11(9): 1-27.

44. Mondet B, Vasconcelos PFC, Travassos da Rosa APA, Travassos da Rosa ES, Rodrigues SG, Travassos da Rosa JFS, et al. Isolation of yellow fever virus from Nulliparous *Haemagogus* (*Haemagogus*) *janthinomys* in Eastern Amazonia. Vector-Borne Zoonotic Dis [Internet]. 2002 [cited 2019 Feb 9]; 2(1): 47-50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12656130.

45. Galindo P, Carpenter SJ, Trapido H. A contribution to the ecology and biology of tree hole breeding mosquitoes of Panama. Ann Entomol Soc Am [Internet]. 1955 [cited 2019 Feb 9]; 48(3): 158-64. Available from: https://academic.oup.com/aesa/article-lookup/doi/10.1093/aesa/48.3.158.

46. Alencar J, de Almeida HM, Marcondes CB, Guimarães AE. Effect of multiple immersions on eggs and development of immature forms of *Haemagogus janthinomys* from South-Eastern Brazil (Diptera: Culicidae). [Internet]. 2008 [cited 2019 Feb 9]; 119(3): 239-45. Available from: https://bioone.org/journals/Entomological-News/volume-119/issue-3/0013-872X(2008)119[239:EOMIOE]2.0.CO;2/Effect-Of-Multiple-Immersions-On-Eggs-And-Development-Of-Immature/10.3157/0013-872X(2008)119[239:EOMIOE]2.0.CO;2.full.

47. dos Santos TP, Roiz D, de Abreu FVS, Luz SLB, Santalucia M, Jiolle D, et al. Potential of *Aedes albopictus* as a bridge vector for enzootic pathogens at the urban-forest interface in Brazil. Emerg Microbes Infect [Internet]. 2018 [cited 2018 Dec 2]; 7(1): 1-8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30482898.

48. Goenaga S, Fabbri C, Dueñas JCR, Gardenal CN, Rossi GC, Calderon G, et al. Isolation of Yellow Fever virus from mosquitoes in Misiones province, Argentina. Vector-Borne Zoonotic Dis. 2012; 12(11): 986-93.

49. Honório NA, Silva WC, Leite PJ, Gonçalves JM, Lounibos LP, Lourenço-de-Oliveira R. Dispersal of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in an urban endemic dengue area in the state of Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz [Internet]. 2003 [cited 2019 Feb 9]; 98(2): 191-8. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762003000200005&lng=en&tlng=en.

50. Sall AA, Faye O, Diallo M, Firth C, Kitchen A, Holmes EC. Yellow fever virus exhibits slower evolutionary dynamics than dengue virus. J Virol [Internet]. 2010 [cited 2019 Feb 21]; 84(2): 765-72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19889759.

51. Woelk CH, Holmes EC. Reduced positive selection in vector-borne RNA viruses. Mol Biol Evol [Internet]. 2002 [cited 2019 Feb 21]; 19(12): 2333-6. Available from: http://academic.oup.com/mbe/article/19/12/2333/997669.

52. Hamer DH, Angelo K, Caumes E, van Genderen PJJ, Florescu SA, Popescu CP, et al. Fatal Yellow Fever in travelers to Brazil, 2018. MMWR Morb Mortal Wkly Rep. 2018; 67(11): 340-1.

4.8 Artigo 8: Survey on non-human primates and mosquitoes does not provide evidences of virus spillover/spillback between the urban and sylvatic cycles of Yellow Fever and Zika viruses following severe outbreaks in southeast Brazil

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Survey on Non-Human Primates and Mosquitoes Does not Provide Evidences of Spillover/Spillback between the Urban and Sylvatic Cycles of Yellow Fever and Zika Viruses Following Severe Outbreaks in Southeast Brazil

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Abstract: In the last decade, Flaviviruses such as yellow fever (YFV) and Zika (ZIKV) have expanded their transmission areas. These viruses originated in Africa, where they exhibit both sylvatic and interhuman transmission cycles. In Brazil, the risk of YFV urbanization has grown, with the sylvatic

transmission approaching the most densely populated metropolis, while concern about ZIKV spillback to a sylvatic cycle has risen. To investigate these health threats, we carried out extensive collections and arbovirus screening of 144 free-living, non-human primates (NHPs) and 5219 mosquitoes before, during, and after ZIKV and YFV outbreaks (2015–2018) in southeast Brazil. ZIKV infection was not detected in any NHP collected at any time. In contrast, current and previous YFV infections were detected in NHPs sampled between 2017 and 2018, but not before the onset of the YFV outbreak. Mosquito pools screened by high-throughput PCR were positive for YFV when captured in the wild and during the YFV outbreak, but were negative for 94 other arboviruses, including ZIKV, regardless of the time of collection. In conclusion, there was no evidence of YFV transmission in coastal southeast Brazil before the current outbreak, nor the spread or establishment of an independent sylvatic cycle of ZIKV or urban *Aedes aegypti* transmission of YFV in the region. In view of the region's receptivity and vulnerability to arbovirus transmission, surveillance of NHPs and mosquitoes should be strengthened and continuous.

Keywords: arboviruses; *Flavivirus*; serology; PRNT; high throughput real time PCR

1. Introduction

In the last decade, several arboviruses of medical importance have caused outbreaks of global or national dimensions, among which are Zika (ZIKV) and Yellow Fever (YFV) viruses [1–3]. Both are *Flavivirus* originating from Africa, where they exhibit at least two ecologically distinct transmission cycles: a sylvatic cycle, in which arboreal mosquito species transmit them among non-human primates (NHPs), with humans accidentally infected; and an interhuman cycle, where the virus is transmitted among humans by *Aedes* mosquitoes, including the anthropophilic mosquito *Aedes aegypti* in the domicile or peridomicile [4–7]. The worldwide spread of the competent vector *Ae. aegypti* favored the dissemination of YFV and ZIKV out of Africa, triggering severe urban outbreaks in several continents, but at different times.

Urban Yellow Fever has been identified in the Americas since the 17th century, causing great outbreaks, especially in port cities [8,9]. Until 1930, only the YFV urban transmission cycle was described [7,9] The establishment of a sylvatic cycle in the New World was documented after confirmation of human cases occurred in an *Ae. aegypti*-free rural area in southeast Brazil in 1932 [10]. It was subsequently shown that YFV had adapted to neotropical NHPs and sylvatic mosquitoes such as *Haemagogus spp*. [10–12]. The existence of a sylvatic cycle in Africa was thereafter described [13]. This spillback from the urban to the sylvatic cycle in the Americas prevented the eradication of YFV in the continent, even after a continental *Ae. aegypti* eradication campaign and the availability of an efficient human vaccine [9–11]. Epizooties waves of YFV initiated in the north of South America, especially in the Amazon, have frequently caused outbreaks in Brazil and neighboring countries [14,15]. Accordingly, since 2014, a YFV spread from the Amazon toward the south and southeast Brazil was detected, which culminated in the largest and most severe sylvatic outbreak ever recorded in the country [16]. Therefore, as of 2016, YFV spread into the most populous Brazilian areas in the Southeast, having a low vaccination coverage, resulting in thousands of epizootics of NHPs and 2170 confirmed human cases and 932 deaths, mainly in 2017–2018 (Figure 1) [2,17]. Human cases had not been reported in most of the affected areas (the coastal Southeast) for almost 80 years, but the assessment of non-detected local enzootic sylvatic transmission in recent decades was lacking. In addition, the YFV sylvatic outbreak reached areas recording high *Ae. aegypti* house infestation indices, increasing the risk of urban transmission.

ZIKV was first isolated in 1947 from Rhesus monkeys used as sentinels in studies of sylvatic yellow fever in the Zika Forest in Uganda, Africa [6,18]. The virus has spread to several continents causing outbreaks in recent decades [18]. The first confirmation of the circulation of ZIKV in the Americas

was made in northeast Brazil in 2015 [19] (Figure 1). In the same year, ZIKV reached the five Brazilian regions and other American countries. The outbreaks had dramatic consequences, such as association with cases of congenital microcephaly and other neurological disorders, and reports of non-vector transmission. The global dimension of the outbreaks led the World Health Organization (WHO) to declare Zika as a global sanitary emergency in 2016 [20]. Despite the problems in recognizing and notifying a new etiological agent, some authors have estimated 400.000 to 1.000.000 human Zika cases in 2015 [21]. Between 2016 and 2019, Brazil confirmed 253,221 Zika cases, peaking in 2015–2016 in the Southeast (Figure 1), as well as in most Brazilian regions. All Zika cases were of urban origin, where the mosquito *Ae. aegypti* was determined to be responsible for the vector transmissions [22].

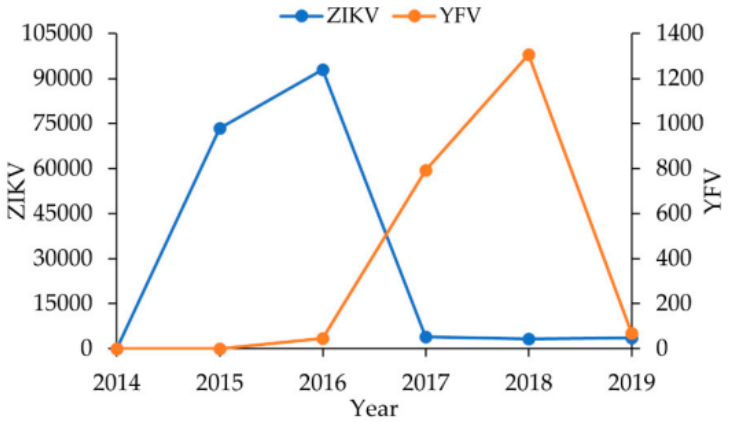

Figure 1. Number of human cases of Zika virus (ZIKV) and Yellow Fever virus (YFV) registered in Brazilian Southeast between 2014 and 2019 (https://www.saude.gov.br/boletins-epidemiologicos). In 2015, the year of the emergence of ZIKV in Brazil, there were no specific diagnostic and notification protocols. Therefore, the number of ZIKV cases showed in 2015 is an estimate for Brazilian Southeast, based in the conservative number of 440,000 cases in Brazil, in 2015, predicted by Helkeubach et al. (2016) [21].

Due to similarities in natural history between YFV and ZIKV, concerns have grown about the possibility of ZIKV establishing a sylvatic cycle in Brazil, which would prevent its eradication, as happened with YFV [4]. Although ZIKV has never been isolated from any other vertebrate besides humans in the Americas, and sylvatic NHP-biting mosquitoes have never been detected to be naturally infected with ZIKV, the findings of RNA fragments compatible with ZIKV and antibodies against this virus in synanthropic marmosets and capuchin monkeys captured in peri urban areas in Brazil, and the demonstration that marmosets can experimentally sustain viremia [23–25] have led to the hypothesis that NHPs would play a role in sustaining the urban transmission cycle or in establishing an sylvatic transmission cycle in the Americas.

However, as far as we know, there are no published data on the investigations of ZIKV natural infection in wild free-living NHPs and mosquitoes. Dozens of other arboviruses have already been identified in Brazil, mainly in the Amazon forest [26] but, except for a few areas [27–30], little is known about the circulation of arboviruses in mosquitoes from the Atlantic forest of southeastern Brazil.

In this context, we carried out extensive collections of free-living NHPs and mosquitoes before, during and after the recent ZIKV and YFV outbreaks (2015–2018) in both sylvatic and periurban areas in the state of Rio de Janeiro (RJ) and its borders in southeast Brazil, to explore the spatiotemporal circulation of these two viruses and test the hypothesis of their spillover/spillback between the urban and sylvatic cycles. As well as this, for the first time, we screened southeastern Atlantic forest mosquitoes for 35 other arboviruses species belonging to 94 different genotypes/serotypes.

2. Materials and Methods

2.1. Ethic Issues

Capture, biosafety and the handling of NHPs and mosquitoes were approved by the institutional Ethics Committee for Animal Experimentation (protocol CEUA/IOC-004/2015 - 10/04/2015, license L-037/2016 – 24/08/2016) and Brazilian Ministry of Environment (SISBIO 41837-3 - 20/05/2015, 52472-2 – 26/01/2016 and 54707-5 – 25/08/2016) and Rio de Janeiro's Environment Agency (INEA 012/2016 - 07/02/2016 and 019/2018 - 19/04/2018).

2.2. Field Expeditions

Fieldwork was carried out from May 2015 to June 2018, comprising distinct epidemiological situations concerning ZIKV and YFV transmission in the southeast, that is before, during and after the ZIKV outbreak and before and during the YFV outbreak. We conducted short (1–5 days) and long (15 days) expeditions to catch mosquitoes and NHPs in the wild and periurban sites in RJ and its bordering states of Minas Gerais (MG), São Paulo (SP) and Espírito Santo (ES) (Figure 2).

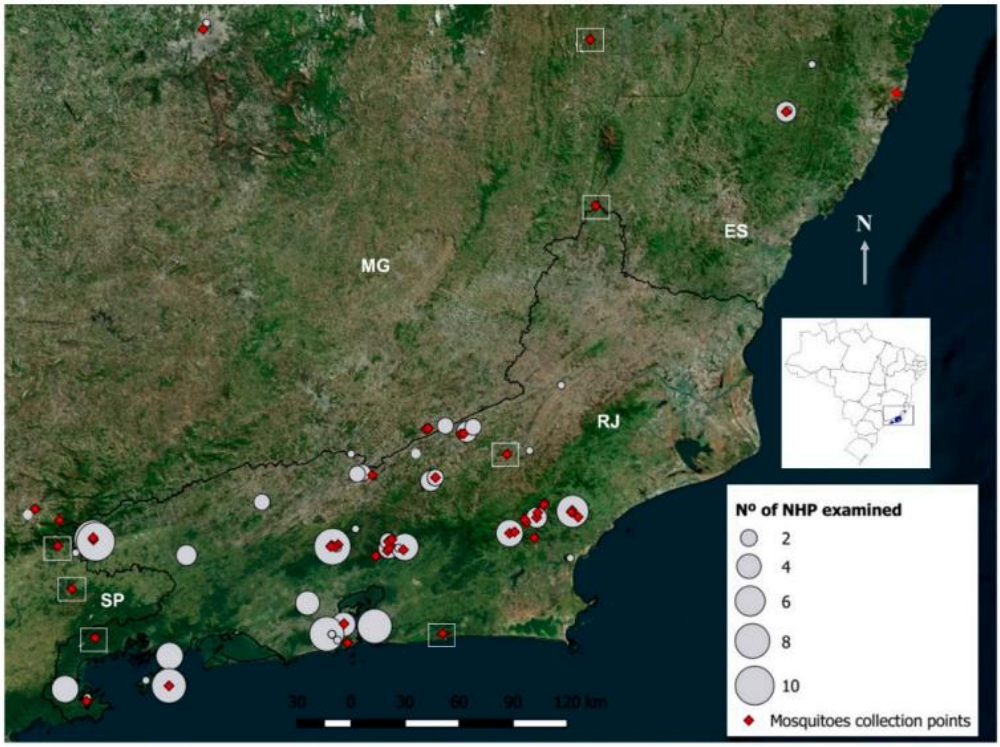

Figure 2. Satellite image showing mosquito and non-human primate (NHP) collection points. The number of NHPs captured is proportional to the size of the circle. Empty squares indicate areas where we attempt to capture NHPs without success.

2.3. Non-Human Primate Samplings

NHP captures were performed using baited traps for small species, and mostly using anesthetic darts for larger ones [31,32]. A blood sample of 3–6 mL was collected from anesthetized animals. A subsample of ~500 µL of whole blood was immediately frozen in dry-ice or N_2, and the remaining blood was left to coagulate. Following centrifugation (2000*g* × 10 min), sera were aliquoted and stored at −80 °C until use. Animals were released in the same capturing site after complete recovery from the anesthetic's effects. Liver and/or blood samples were also collected from dying or recently dead NHPs, found by alerts from an information network we constructed [32].

2.4. Mosquito Collections

Mosquitoes were collected with entomological nets and manual aspirators indoors and outdoors, or with BG sentinel traps baited with CO_2 installed along 300 m transects from the modified environment and forest edge to deep into the woods, as described in detail by Santos et al. (2018) [33]. In rural and forest areas, the collections were preferably done in the same areas the NHPs were captured (Figure 2). Caught mosquitoes were transported and identified at low temperature, pooled according to species, and stored at −80 °C until RNA extraction for viral diagnosis, as previously described [34,35].

2.5. Molecular Analyses

RNA was extracted from 140 µL of NHP serum, liver and/or whole blood using the Qiagen RNA Viral Kit, and from 140 µL of supernatant of mosquito pool homogenates using MACHEREY-NAGEL NucleoSpin 96 RNA extraction kits, following the manufacturer's recommendations. For NHP samples, real-time RT-qPCR was performed in duplicate and in separate assays for YFV and ZIKV detection. For confirmation of any diagnosis, the amplicons obtained were directly sequenced without molecular cloning. The set of primers utilized in RT-qPCR and viral genome sequencing procedures followed previous reports [17,36].

Mosquito RNA were submitted to high-throughput, real-time PCR, developed by Moutailler et al. [35], to screen for 35 arboviruses of 94 different genotype/serotype (Table S1), including YFV and ZIKV. Briefly, specific primers and probes for the above-cited 35 mosquito-borne viruses, including 94 genotypes/serotypes targeted, were designed and validated on reference RNAs. Then, we used the BioMark Dynamic Arrays technology (Fluidigm Corporation, South San Francisco, CA, USA) for high-throughput, microfluidic, real-time PCR in plates of 96 x 96 which automatically cross, up to 96 samples with the 96 primers/probes sets, allowing the simultaneous screening as previously described [35].

2.6. Immunological Assays

Plaque reduction neutralization test (PRNT): PRNTs were carried out on Vero cells in 24-well and 96-well plates for antibody neutralizing detections in all NHP serum samples against ZIKV and/or YFV, respectively [37]. Samples with PRNTs suspected to be positive for ZIKV or, exceptionally, against both YFV and ZIKV, were also tested by PRNT against DENV1-4 to check for cross-reaction among these flaviviruses. Briefly, serum samples were serially diluted from 1:5 to 640 (dilution factor = 2, to PRNT of YFV) and from 1:10 to 1:31,250 (dilution factor = 5, to PRNT of DENV and ZIKV), followed by the addition (volume/volume) of 120, 60 or 50 µL of DENV (1, 2, 3 or 4), ZIKV or YFV, corresponding to approximately 60 PFU / 200 µL (DENV), 100 PFU / 100 µL (ZIKV), 40 PFU / 50 µL (YFV), respectively. Then, plates were incubated at 37 °C in 5% CO_2 for 1 h (neutralization step). For the adsorption step, 200 µL (PRNT of DENV) or 100 µL (PRNT of ZIKV) of mixture (serum + virus) were transferred to cell monolayers of 6-well or 24-well plates, previously prepared with 45 x 10^4 cells/well or 20 x 10^4 cells/well, respectively and incubated at 37 °C for 1 h. PRNT YFV was prepared in 96-well plates, then suspension of Vero cells (8 × 10^4 cells/50 µL/well) was added into the mix (serum + virus) and plates were incubated at 37 °C for 3 h. After the adsorption step, media were discarded, and cell monolayers were overlaid with E199 medium, and incubated for 6–8 days at 37 °C in 5% CO_2, until fixed with 10% formalin, and stained with crystal violet for plaques count. Neutralizing antibody titers were expressed by 90% of plaque reduction ($PRNT_{90}$), and samples with titers > 10 were considered positive.

Enzyme Immunoassay (ELISA): ELISA was used for the determination of total IgG antibodies against the YFV, ZIKV and DENV of NHP samples with positive PRNT against one or more of these flaviviruses. The objective was to confirm diagnosis, as well as discriminate antiviral immune responses from the potential physical or chemical interference of any molecules and/or virucide factors in blood samples that may have affected the virus and/or reduced its adsorption to cellular monolayer in PRNT, as previously described [38], especially in some wild animal serum [27]. Briefly, the detection of the

dengue IgG antibody was performed by a modification of Miagostovich et al. (1999) method [39]. For this, 100 µL of hyper-immune ascitic fluid, diluted in 0.1 M sodium carbonate buffer, pH 9.6, was added to each 96-well and plates were incubated overnight at 4 °C. After washing with PBS pH 7.4, wells were blocked by filling with standard diluent (PBS pH 7.4, 0.05% Tween, 3% normal goat serum) and incubated for 1 h at 37 °C. An antigen mix (DENV-1, 2, 3 and 4 samples) was applied to each well, following incubation for 1 h at 37 °C. Plates were washed again with PBS, and 100 µL of serum diluted 1:40 in Non-Fat Dry Milk (NFDM) diluent (PBS pH 7.4, 0.05% Tween, 3% non-fat dry milk) were added to each well. Plates were incubated for 1 h at 37 °C and washed. In total, 40 microliters of anti-human IgG conjugated to horseradish peroxidase diluted in NFDM diluent was added. After incubating for 1 h, plates were washed with PBS and 100 µL of substrate ABTS (2,2'-azino-di [3-etil-benthlazoline sulfonate]) and hydrogen peroxide were added to each well. Color development was continued at room temperature (RT) at an optical density of 405 nm, and the optical density of each dilution was subtracted from the corresponding dilution of each test sample. Index values > 0.150 were considered positive.

For the detection of YFV IgG antibodies, 96-well plates were coated with 2.5 µg/mL of whole yellow fever virus particle diluted in coating buffer (carbonate–bicarbonate buffer, pH 9.6) and incubated overnight at 4 °C. In all washing steps, plates were rinsed mechanically five times with washing buffer (PBS pH 7.4 with 0.05% (v/v) of Tween-20 –PBS/T). Then, plates were blocked for 1 h at 37 °C with a blocking/diluent solution (BDS) (PBS/T, 0.05% (v/v) of BSA, 3% (w/v) of fetal bovine serum (FBS) and 5% (w/v) of skimmed milk). Serum samples were submitted to serial dilutions. An anti-YFV Serum, Monkey (YF - NIBSC) antibody was employed to derive a standard curve, in the range of 1 to 0.015 IU/mL. After 1 h at RT, plates were washed and incubated with the Anti-Monkey IgG peroxidase conjugated (A2054-Sigma), diluted 1:5000 in BDS, following incubation for 1 h at RT. After washing, 100 µL/well of substrate solution (TMB Plus^{TM} kem-en-tec) were added and after 15 min the reaction was stopped by adding stop-solution (2M H_2SO_4). The endpoint measurements were done at 450 nm and the absorbances of the serum sample dilutions were plotted on the standard curve. The results obtained by absorbance values >0.150 were calculated using the software SoftMax Pro® by regression logistic for four parameters and the antibody titers were expressed in IU/mL.

The Zika-Euroimmun commercial kit was used for ZIKV IgG antibody detection, following the manufacturer's recommendations [40].

For all assays, serum of ZIKV-experimentally infected monkey, DENV-positive human, goat and healthy monkeys from different families (Callitrichidae, Cebidae and Atelidae), besides of YFV 17DD vaccinated monkeys were used as sensibility and specificity controls.

3. Results

3.1. Molecular Findings

3.1.1. Non-Human Primates

In total, 144 primates belonging to six neotropical species were captured in 27 counties in RJ and bordering southeastern states and tested by RT-qPCR for ZIKV and YFV detection. Two NHPs were examined before, 71 during, and 71 after the ZIKV outbreak, while 73 NHPs were tested before and 71 during the YFV outbreak in the Brazilian Southeast.

All animals consistently tested negative for ZIKV, regardless of collection site or epidemiological situation, that is before, during or after the ZIKV outbreak (Figures 1–3; Table 1 and Table S2). On the other hand, 14 (9.7%) NHP samples were positive for YFV (CT values ranging from 10.4 to 16.8), all of which were collected during the YFV outbreak (from Jan/2017 to Jun/ 2018) and in counties where YFV circulation was suspected. Twelve out of these NHPs were howler monkeys—*Alouatta guariba clamitans*—and two were marmosets *Callithrix jacchus* x *Callithrix penicillata* hybrid. In total, 82% of howlers examined during the outbreak were positive. Eleven of the YFV-positive NHPs were collected in nine epizootic counties of RJ, while the other three were from a single county in ES.

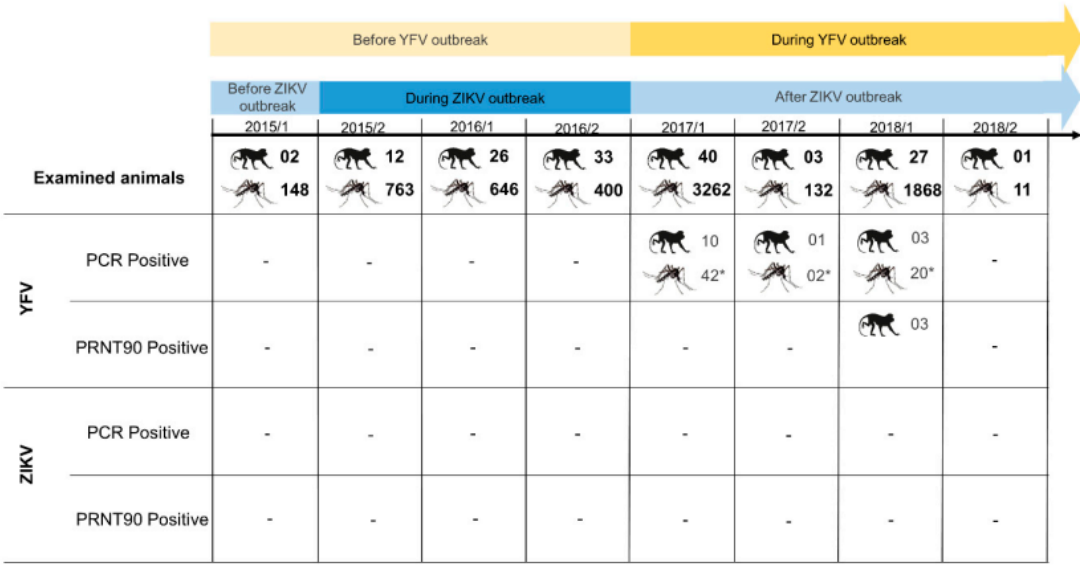

Figure 3. Number of examined and positive non-human primates and mosquitoes, according to epidemiological scenario, semester, year and diagnosis methods. Results of mosquito infections (*) are expressed in number of positive pools.

Table 1. Mosquito species collected and screened through 94 arbovirus genotypes in four Brazilian states: Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP).

Species Per Year / Semester	Number	Date	Counties (State)	Result PCR[a]
Aedes albopictus	4	2015/1	Macaé (RJ)	Neg.
Aedes scapularis	12	2015/1		Neg.
Culex sp.	1	2015/1		Neg.
Haemagogus leucocelaenus	17	2015/1		Neg.
Limatus durhamii	2	2015/1		Neg.
Psorophora ferox	10	2015/1		Neg.
Psorophora sp.	5	2015/1		Neg.
Runchomyia humboldti	1	2015/1		Neg.
Runchomyia reversa / theobaldi	1	2015/1		Neg.
Sabethes albiprivus	1	2015/1		Neg.
Wyeomyia aporonoma/staminifera	1	2015/1		Neg.
Wyeomyia (Pho.) sp.	89	2015/1		Neg.
Wyeomyia sp.	4	2015/1		Neg.
2015/1	**148**			Neg.
Aedes albopictus	14	2015/2	Guapimirim, Macaé, Magé, Miguel Pereira, Teresópolis (RJ)	Neg.
Aedes fulvithorax	1	2015/2		Neg.
Aedes scapularis	94	2015/2		Neg.
Aedes serratus	1	2015/2		Neg.
Aedes terrens	25	2015/2		Neg.
Anopheles cruzii	1	2015/2		Neg.
Anopheles sp.	3	2015/2		Neg.
Culex sp.	9	2015/2		Neg.
Culex nigripalpus	10	2015/2		Neg.
Haemagogus janthinomys	60	2015/2		Neg.
Haemagogus leucocelaenus	87	2015/2		Neg.
Limatus durhamii	26	2015/2		Neg.
Limatus pseudomethisticus	2	2015/2		Neg.
Onirion personatum	41	2015/2		Neg.
Psorophora ferox	21	2015/2		Neg.
Psorophora sp.	3	2015/2		Neg.

Table 1. *Cont.*

Species Per Year / Semester	Number	Date	Counties (State)	Result PCR[a]
Runchomyia cerqueirai	15	2015/2		Neg.
Runchomyia frontosa	8	2015/2		Neg.
Runchomyia humboldti	48	2015/2		Neg.
Runchomyia reversa / theobaldi	1	2015/2		Neg.
Runchomyia sp.	20	2015/2		Neg.
Sabethes albiprivus	2	2015/2		Neg.
Sabethes aurescens	19	2015/2		Neg.
Sabethes chloropterus	1	2015/2		Neg.
Sabethes chloropterus'	5	2015/2		Neg.
Sabethes fabricii'	2	2015/2		Neg.
Sabethes identicus	1	2015/2		Neg.
Sabethes intermedius	2	2015/2		Neg.
Sabethes melanonymphe	7	2015/2		Neg.
Sabethes xyphides	2	2015/2		Neg.
Sabethes sp.	8	2015/2		Neg.
Sh. fluviatilis	49	2015/2		Neg.
Shanonniana sp	28	2015/2		Neg.
Trichoprosopon digitatum	24	2015/2		Neg.
Trichoprosopon pallidiventer	5	2015/2		Neg.
Trichoprosopon sp.	1	2015/2		Neg.
Wyeomyia aporonoma/staminifera	5	2015/2		Neg.
Wyeomyia bonnei/deanei	1	2015/2		Neg.
Wyeomyia davisi	11	2015/2		Neg.
Wyeomyia mystes	5	2015/2		Neg.
Wyeomyia pilicauda	42	2015/2		Neg.
Wyeomyia theobaldi	4	2015/2		Neg.
Wyeomyia (Pho.) sp.	43	2015/2		Neg.
Wyeomyia sp.	6	2015/2		Neg.
2015/2	**763**			Neg.
Aedes albopictus	1	2016/1	**Guapimirim, Macaé, Miguel Pereira, Nova Friburgo (RJ)**	Neg.
Aedes scapularis	56	2016/1		Neg.
Aedes serratus	2	2016/1		Neg.
Aedes terrens	44	2016/1		Neg.
Anopheles bellator	2	2016/1		Neg.
Anopheles cruzii	12	2016/1		Neg.
An. hominales	1	2016/1		Neg.
Anopheles lutzi	1	2016/1		Neg.
Anopheles sp.	14	2016/1		Neg.
Culex sp.	44	2016/1		Neg.
Haemagogus janthinomys	7	2016/1		Neg.
Haemagogus leucocelaenus	10	2016/1		Neg.
Limatus durhamii	31	2016/1		Neg.
Limatus pseudomethisticus	12	2016/1		Neg.
Onirion personatum	30	2016/1		Neg.
Psorophora ferox	3	2016/1		Neg.
Runchomyia cerqueirai	1	2016/1		Neg.
Runchomyia frontosa	22	2016/1		Neg.
Runchomyia humboldti	25	2016/1		Neg.
Runchomyia sp.	29	2016/1		Neg.
Sabethes aurescens	4	2016/1		Neg.
Sabethes identicus	3	2016/1		Neg.
Sabethes melanonymphe	6	2016/1		Neg.
Sabethes sp.	19	2016/1		Neg.
Sabethini	5	2016/1		Neg.
Shannoniana fluviatilis	84	2016/1		Neg.
Trichoprosopon digitatum	4	2016/1		Neg.

Table 1. *Cont.*

Species Per Year / Semester	Number	Date	Counties (State)	Result PCR[a]
Trichoprosopon pallidiventer	18	2016/1		Neg.
Wyeomyia aporonoma/staminifera	5	2016/1		Neg.
Wyeomyia bonnei/deanei	4	2016/1		Neg.
Wyeomyia cerqueirai	1	2016/1		Neg.
Wyeomyia confusa	1	2016/1		Neg.
Wyeomyia davisi	1	2016/1		Neg.
Wyeomyia pallidoventer	6	2016/1		Neg.
Wyeomyia palmata/galvoi	3	2016/1		Neg.
Wyeomyia pilicauda	8	2016/1		Neg.
Wyeomyia (Pho.) sp.	118	2016/1		Neg.
Wyeomyia sp.	9	2016/1		Neg.
2016/1	**646**			Neg.
Aedes aegypti	9	2016/2		Neg.
Aedes fluviatilis	1	2016/2		Neg.
Aedes scapularis	11	2016/2		Neg.
Aedes terrens	4	2016/2		Neg.
Anopheles cruzii	4	2016/2		Neg.
Anopheles sp.	13	2016/2		Neg.
Culex sp.	1	2016/2		Neg.
Haemagogus janthinomys	8	2016/2		Neg.
Haemagogus leucocelaenus	22	2016/2		Neg.
Limatus durhamii	34	2016/2		Neg.
Limatus pseudomethisticus	42	2016/2		Neg.
Onirion personatum	8	2016/2		Neg.
Psorophora ferox	1	2016/2		Neg.
Runchomyia frontosa	7	2016/2		Neg.
Runchomyia humboldti	3	2016/2		Neg.
Runchomyia sp.	13	2016/2		Neg.
Sabethes albiprivus	4	2016/2		Neg.
Sabethes aurescens	3	2016/2		Neg.
Sabethes auresces	2	2016/2	**Itamonte (MG); Queluz (SP); Itatiaia, Miguel Pereira, Rio de Janeiro, Sumidouro, Teresópolis (RJ)**	Neg.
Sabethes intermedius	3	2016/2		Neg.
Sabethes melanonymphe	3	2016/2		Neg.
Sabethes sp.	26	2016/2		Neg.
Shannoniana fluviatilis	18	2016/2		Neg.
Trichoprosopon castroi/similis	9	2016/2		Neg.
Trichoprosopon digitatum	6	2016/2		Neg.
Trichoprosopon pallidiventer	18	2016/2		Neg.
Wyeomyia antunesi	3	2016/2		Neg.
Wyeomyia aporonoma/staminifera	1	2016/2		Neg.
Wyeomyia confusa	60	2016/2		Neg.
Wyeomyia davisi	5	2016/2		Neg.
Wyeomyia exallos	1	2016/2		Neg.
Wyeomyia incaudata	2	2016/2		Neg.
Wyeomyia longirostris	2	2016/2		Neg.
Wyeomyia lutzi	4	2016/2		Neg.
Wyeomyia palmata/galvoi	6	2016/2		Neg.
Wyeomyia pilicauda	11	2016/2		Neg.
Wyeomyia (Pho.) sp.	23	2016/2		Neg.
Wyeomyia sp.	9	2016/2		Neg.
2016/2	**400**			Neg.
Aedes aegypti	94	2017/1		Neg
Aedes albopictus	1	2017/1		Neg.
Aedes argyrothorax	2	2017/1		Neg.
Aedes fulvithorax	2	2017/1		Neg.
Aedes rhyacophilus	1	2017/1		Neg.
Aedes scapularis	876	2017/1		YFV

Table 1. *Cont.*

Species Per Year / Semester	Number	Date	Counties (State)	Result PCR[a]
Aedes serratus	9	2017/1	**Belo Horizonte, Simonésia (MG); Domingos Martins, Serra (ES); Angra dos Reis, Casimiro de Abreu, Macaé, Maricá, Petrópolis, Rio de Janeiro (RJ)**	Neg.
Aedes taeniorhynchus	892	2017/1		YFV
Aedes terrens	7	2017/1		Neg.
Aedes sp.	22	2017/1		Neg.
Anopheles sp.	4	2017/1		Neg.
Coquillettidia sp.	72	2017/1		Neg.
Coquillettidia albicosta	1	2017/1		Neg.
Coquillettidia hermanoi	1	2017/1		Neg.
Coquillettidia justamansonia	16	2017/1		Neg.
Coquillettidia nigricans	6	2017/1		Neg.
Coquillettidia shannoni	1	2017/1		Neg.
Coquillettidia venezuelensis	2	2017/1		Neg.
Culex grupo Coronata	1	2017/1		Neg.
Culex sp.	56	2017/1		Neg.
Culex declarator	4	2017/1		Neg.
Culex nigripalpus	103	2017/1		Neg.
Haemagogus janthinomys	8	2017/1		YFV
Haemagogus leucocelaenus	199	2017/1		YFV
Limatus durhamii	189	2017/1		Neg.
Limatus pseudomethisticus	2	2017/1		Neg.
Limatus sp.	12	2017/1		Neg.
Mansonia indubitans	75	2017/1		Neg.
Mansonia titillans	14	2017/1		Neg.
Mansonia sp.	10	2017/1		Neg.
Psorophora ferox	21	2017/1		Neg.
Psorophora lutzii/amazonica	4	2017/1		Neg.
Psorophora sp.	10	2017/1		Neg.
Runchomyia frontosa	13	2017/1		Neg.
Runchomyia humboldti	12	2017/1		Neg.
Runchomyia sp.	10	2017/1		Neg.
Sabethes petrocchiae	60	2017/1		Neg.
Sabethes albiprivus	194	2017/1		Neg.
Sabethes aurescens	2	2017/1		Neg.
Sabethes chloropterus	4	2017/1		YFV
Sabethes fabricii'	3	2017/1		Neg.
Sabethes quasicyaneus	1	2017/1		Neg.
Sabethes whitmani	1	2017/1		Neg.
Sabethes sp.	47	2017/1		Neg.
Shannoniana fluviatilis	2	2017/1		Neg.
Trichoprosopon digitatum	1	2017/1		Neg.
Trichoprosopon pallidiventer	7	2017/1		Neg.
Trichoprosopon sp.	3	2017/1		Neg.
Wyeomyia aporonoma/staminifera	7	2017/1		Neg.
Wyeomyia bourrouli/ forcipenis	11	2017/1		Neg.
Wyeomyia confusa	40	2017/1		Neg.
Wyeomyia edwardsi	9	2017/1		Neg.
Wyeomyia incaudata	7	2017/1		Neg.
Wyeomyia medioalbipes	14	2017/1		Neg.
Wyeomyia melanocephala'	1	2017/1		Neg.
Wyeomyia mystes	9	2017/1		Neg.
Wyeomyia palmata/galvoi	17	2017/1		Neg.
Wyeomyia pilicauda	4	2017/1		Neg.

Table 1. *Cont.*

Species Per Year / Semester	Number	Date	Counties (State)	Result PCR[a]
Wyeomyia (Mia.) sp.	1	2017/1		Neg.
Wyeomyia (Pho.) sp.	20	2017/1		Neg.
Wyeomyia sp.	45	2017/1		Neg.
2017/1	**3262**			
TOTAL	**5219**	**2015–2017**	**19**	**YFV**

a: The YFV mosquito infections revealed by the high throughput PCR were confirmed by RNA sequencing and RT-qPCR using distinct set of primers, whose results were published elsewhere [34,35]. Mosquitoes captured before ZIKV and YFV outbreaks. Mosquitoes examined during the ZIKV and before YFV outbreaks. Mosquitoes examined after ZIKV and during YFV outbreaks

3.1.2. Mosquitoes

A total of 5219 female mosquitoes belonging to 69 species were collected from the modified environment to deep into the forest of 19 counties, 13 in RJ, three in MG, two in ES and one in SP, undergoing a distinct epidemiological situation cornering ZIKV and YFV transmission (Figure 2; Table 1). Mosquitoes were grouped into 1298 pools and screened for the 94 genotypes and/or serotypes of arbovirus cited above, including YFV and ZIKV.

All mosquitoes were negative for ZIKV, regardless of whether they originated from the wild of periurban and rural sites or were captured before, during or after the ZIKV outbreaks in the region (Figures 2 and 3; Table 1). No other screened arbovirus was found in mosquitoes, except for YFV. The YFV mosquito infections revealed by the high-throughput PCR were confirmed by RNA sequencing and matched with those obtained when the homogenates of the same mosquito pools were screened by RT-qPCR using a distinct set of primers whose results were published elsewhere [34]. Infections by YFV were mostly found in the traditional vector *Haemagogus janthinomys*, as well as in *Haemagogus leucocelaenus*, while only one pool of *Sabethes chloropterus*, *Aedes scapularis* and *Aedes taeniorhynchus* were positives (Table 1). Viruses were successfully isolated in C6/36 cell culture from six pools of *Hg. janthinomys* and four of *Hg. leucocelaenus*.

The complete genome sequencing of YFV detected in NHPs and mosquitoes is available at GenBank (accession numbers MF423373, MF423374, MK333800, KY885000, KY885001, MF423375, MF423376, MF423377, MF423378, MF538785, MF538786) and confirmed the existence of a unique molecular signature of fixed amino acid mutations in highly conserved positions at NS3 and NS5 proteins in YFV, causing the current southeastern Brazilian outbreak [17,41].

3.2. Immunological Findings

In total, the sera of 118 out of 144 collected NHPs could be screened for neutralizing antibodies against YFV and ZIKV. The sera of 26 NHPs were not available, either because they were already found dead or consisted of small animals with low volaemia, such as young marmosets, preventing the collection of ideal amounts of blood.

None of the 73 NHPs captured before the YFV outbreak had neutralizing antibodies against YFV, while 4.2% (three out of 71 animals) captured during the outbreak were positive, all being capuchins (*Sapajus nigritus*) from the Itatiaia National Park, southern RJ (Figure 3, Table S2). We examined 12 capuchins from this park before the YFV outbreak and nine during the outbreak—of which eight were captured for the first time and one recaptured. A total of 37.5% (three of eight) were seropositive, and the recaptured one showed no seroconversion.

Finally, we captured 14 marmosets in the edge of small forest fragments and modified environments inside the cities of Rio de Janeiro, Niterói and Belo Horizonte, where there was suspicion of YFV circulation, due to only one dead marmoset preliminarily diagnosed as due for YFV by the state surveillance system using PCR, but not confirmed by immunohistochemistry or RNA sequencing. Although captured in exactly the same site as where the epizootics were recorded, and belonging to the same family/group of dead animals, all 14 tested marmosets were negative for YFV neutralizing antibodies, showing no evidence of viral circulation in the suspected periurban and urban areas.

After testing NHP sera by PRNT and checking the results by ELISA, we concluded that none of them had specific neutralizing antibodies against ZIKV (Table S2, Table 2). Although the results of $PRNT_{90}$ of 15 samples preliminarily suggested a protective response against ZIKV (Table 2), the combination of their results in PRNT-DENV, PRNT-YFV and ELISA for the detection of IgG against DENV, YFV, ZIKV confirmed cross-reactions and/or unspecific reactions.

Table 2. List of samples that showed unspecific response during the $PRNT_{90}$ assays for tested viruses.

Non-Human Primate Data				Molecular Results		PRNT Results							ELISA Results			Conclusions	
Code	Species	State	Health state	rt-PCR	CT	YFV 90%	ZIKV 90%	DENV1 90%	DENV2 90%	DENV3 90%	DENV 4 90%	Serum Quality	YFV	ZIKV	DENV	Imunological	Molecular
RJ10	*Callithrix jacchus**	RJ	Healthy	Neg.	_	< 5	168.3	28.9	23.9	48.0	< 10	Hemolyzed++	Neg.	Neg.	Neg.	Negative	Negative
RJ18	*Callithrix jacchus**	RJ	Healthy	Neg.	_	< 5	17.2	<20	< 10	< 10	< 10	Hemolyzed++	Neg.	Neg.	Neg.	Negative	Negative
RJ46	*Alouatta g. clamitans*	MG	Dying	Neg.	_	29.9	341.8	180.0	<10	< 10	< 10	Hemolyzed+++	Neg.	Neg.	Neg.	Negative	Negative
RJ60	*Leontopithecı rosalia*	RJ	Healthy	Neg.	_	<10	10.8	-	< 10	< 10	< 10	Hemolyzed++	Neg.	Neg.	Neg.	Negative	Negative
RJ62	*Leontopithecı rosalia*	RJ	Healthy	Neg.	_	< 5	41.6	<100	< 10	< 10	< 10	Hemolyzed++	-	-	-	Negative	Negative
RJ64	*Leontopithecı rosalia*	RJ	Healthy	Neg.	_	< 5	13.1	<100	14.4	< 10	< 10	Hemolyzed++	-	-	-	Negative	Negative
RJ87	*Alouatta g. clamitans*	RJ	Dead	YFV	Conv.	<20	87.1	<500	< 10	29.5	< 10	Hemolyzed+++	Neg.	Neg.	Neg.	Negative	YFV
RJ91B	*Callithrix jacchus**	RJ	Healthy	Neg.	_	< 5	31.26	<20	-	< 10	< 10	Hemolyzed++	Neg.	Neg.	Neg.	Negative	Negative
RJ95	*Alouatta g. clamitans*	RJ	Dead	YFV	11.7	< 5	65.9	30.0	10.3	< 10	< 10	Hemolyzed+++	Neg.	Neg.	Neg.	Negative	YFV
RJ96	*Alouatta g. clamitans*	RJ	Dead	YFV	Conv.	<40	420.3	-	>250	< 10	< 10	Hemolyzed+++	Neg.	Neg.	Neg.	Negative	YFV
RJ104	*Callithrix jacchus**	RJ	Dead	YFV	13.7	< 5	11.6	-	-	-	-	Hemolyzed+	Neg.	Neg.	Neg.	Negative	YFV
AR03	*Alouatta g. clamitans*	RJ	Healthy	Neg.	_	< 5	12.7	-	-	-	-	Hemolyzed++	Neg.	Neg.	Neg.	Negative	Negative
RJ118	*Callithrix jacchus**	RJ	Healthy	Neg.	_	< 5	39.1	-	-	-	-	Hemolyzed+++	Neg.	Neg.	Neg.	Negative	Negative
ES01	*Brachyteles arachnoides*	ES	Healthy	Neg.	_	< 5	14.5	<10	< 10	17.17	< 10	Normal	Neg.	Neg.	Neg.	Negative	Negative
ES04	*Alouatta g. clamitans*	ES	Dying	YFV	Conv.	6.9	85.4	-	24.51	< 10	< 10	Hemolyzed+++	Neg.	Neg.	Neg.	Negative	YFV

"-" means that it was not possible to perform the test due to sample exhaustion; + : indicate the degree of hemolysis; conv: conventional RT-PCR; *: hybrids of *C. jacchus* and *C. penicillata*.

NHPs examined during Zika (ZIKV) and before Yellow Fever (YFV) outbreaks. NHPs examined after ZIKV and during YFV outbreaks.

4. Discussion

YFV and ZIKV caused large outbreaks in Brazil from 2015 onwards, raising concern about the possibility of spillover from the sylvatic to the urban transmission cycle (in the case of the YFV) and spillback from the urban to an independent sylvatic cycle (for ZIKV). In the present work, after extensive sampling of both mosquito vectors and NHPs, we did not find evidence of spillover/spillback between urban and sylvatic cycles for these viruses.

Concerning the suspicion of YFV reurbanization, all urban or periurban mosquitoes and NHPs tested negative for YFV. Indeed, all vectors found infected herein and for previous authors were sylvatic species [34,42]. This finding, together with epidemiological, genetic and entomological records obtained during the outbreak [34,42–46], reinforced the sylvatic nature of the outbreak and the absence of spillover. Briefly, the demographic characteristics of the infected humans [43,46] and the spread rates of YFV over time and space and the genetic clades found in phylogeographic analyses were consistent with an NHP—sylvatic mosquitoes—human transmission [3,44,45,47,48]. Furthermore, all investigated cases also shared ecological conditions indicating their sylvatic origin, such as contact with forested areas, including people that entered into the jungle or live in the interface between cities and the natural environment [3,49,50].

YFV was not detected in any mosquito or NHPs captured prior to the outbreak. Moreover, the absence of protective antibodies against YFV in all NHPs examined before the outbreak suggests the absence of recent YFV circulation in southeastern brazil. Our results evidenced susceptible vertebrate hosts (NHP) of at least six species spread throughout RJ and its bordering states. This result also indicates that the territory was receptive to YFV transmission. In fact, the entire Atlantic coastal forest was considered a YFV-free area, without vaccination recommendation until the diagnosis of the first cases in early 2017 [15,16]. The large number of non-immune human and NHPs contributed to the rapid spread of the virus after its reintroduction. The occurrence and high frequency of competent vectors throughout the region [34] reinforced the receptivity and vulnerability of the region to YFV transmission. It seemed to be a matter of time. This scenario, combined with changes in the human behavioral patterns [3,49] and the potential role of distinct genetic characteristics of the circulating virus [17,41,47], may help to explain the magnitude of the outbreak.

Howler monkeys (*Alouatta*) were the most affected genus by YFV in our sampling, as previously reported in Brazil and Argentina [51–55]. Even so, we did not find antibodies against YFV in the captured howlers, even in those animals tested during the outbreak. Probably, the majority of the exposed *Alouatta* were not able to produce enough protective antibodies and were rapidly killed by virus effects, as observed by Kumm and Laemmert, 1944 [56]. The same authors concluded that the difficulty of capturing these animals and the almost always fatal effect of some YFV strains on this NHP genus reduce the chances of finding immunized howler monkeys. In fact, Almeida et al. (2019) did not find any *Alouatta caraya* immunized in Rio Grande do Sul after the largest outbreak of YFV reached the region [51,57]. The encounter of non-immune howlers after the outbreak is worrisome because, although the size of the remaining populations is unknown, it could indicate that the Atlantic forest is still vulnerable to the circulation of YFV, as demonstrated by the recent finding of an infected *Alouatta g. clamitans* in the same area as the virus was detected in 2017 [58]. Continued surveillance and the vaccination campaigns in the region are imperative.

Unlike the howler monkeys, protective antibodies against YFV were detected in three *Sapajus nigritus* from the Itatiaia National Park, examined after confirmation of the YFV in the same reserve. Encountering immunized healthy animals against YFV with negative PCR, indicates that despite the susceptibility of some individuals, the immune response of this genus is more efficient in protection against the virus. Interestingly, immune capuchins were found after more than 10 years from confirmed YFV in humans in several regions of Brazil [56,59]. Due to their apparent higher resistance, longevity (reaching 40 years in laboratory) [60], abundance in several Brazilian cities and the relative ease of capture in some regions, we recommend that transverse immunological studies should be implemented in groups of *Sapajus sp*. with periodic recaptures for serological and molecular tests. These groups

could be useful as sentinels, aiming at the early detection of viral circulation and human protection through the identification of priority areas for vaccination [61], mainly in areas with scant populations of howlers.

Marmosets are susceptible to YFV infections but seem to exhibit intermediate resistance to death when compared to capuchin and howler monkeys [62]. We found two individuals killed by the virus and many epizootics have been YFV-confirmed in the recent and previous outbreaks [55,63,64]. These findings raise concerns as many marmoset groups transit between forest fragments and cities, which could facilitate their role as a "bridge host" due to their behaviour, sometimes establishing close contact with humans [55,62]. Although we did not find marmosets with protective antibodies against YFV, longitudinal examinations of *Callithrix* groups may also be useful for YFV surveillance because they provide a shorter time cut than capuchin monkeys due to their shorter life cycle [31,56].

In relation to ZIKV, the combination of our analyzes (entomological, molecular and immunological) did not reveal evidence that an independent sylvatic cycle of this virus could have been established in RJ and the surroundings. Similar results were pointed out by Moreira-Soto et al. (2018), after analyzing 207 NHPs from two regions and three Brazilian states between 2012 and 2017 [38]. The authors did not detect ZIKV through RT-qPCR and reported to have found specific antibodies in six NHPs, with low titers, even though they were collected in urban and periurban areas in some of the regions most affected by the outbreak [38]. On the other hand, after performing NHP captures in a peridomestic environment contiguous to houses whose inhabitants had Zika, Favoretto et al. (2019) reported to have detected ZIKV genome in nine out of 132 marmosets or capuchin monkeys, captured mainly in 2015 [24]. A genome compatible to ZIKV was also detected in the viscera of urban and periurban marmosets and capuchins under suspicion of yellow fever infection, found dead in SP and MG [23]. Despite the generally high CTs and the discordant results obtained from different viscera of the same animal, the authors claimed to have detected the presence of the virus in 32 (39%) of the 82 tested NHPs [23]. The same authors also detected ZIKV in the domestic mosquitoes *Ae. aegypti*, the species implicated in the urban transmission in Brazil [22]. Although the evidence of ZIKV circulation among urban or periurban NHPs and mosquitoes is a concern, it does not directly imply the establishment of an independent sylvatic cycle, maintained between wild mosquitoes and NHPs.

Although we found 15 suggestive responses in ZIKV-PRNT$_{90}$, almost all were detected in hemolyzed serum and cross-reacted with other viruses when screened by PRNT-YFV, PRNT-DENV1-4 and/or ELISA for DENV, YFV and ZIKV. Notably, samples from monkeys that died of yellow fever [six out of the 15 (RJ87, RJ95, RJ96, RJ104, ES04, ES05)] whose blood was obtained from postmortem cardiac puncture, had a viscous, greenish appearance. The PRNT of serum samples with these aspects was suggested to have high antibody titles against all tested viruses (Table 2). The same was observed with the serum of a howler (RJ46) that was attacked by bees at the time of capture and died of anaphylaxis; its blood was collected before death. Therefore, we suspected that the PRNT results of these specific animals were consequences of non-specific virucidal agents, inactivating or reducing viruses' adsorption capacity in the cellular monolayer. In addition to the already known cross-reaction between *Flavivirus* [24,57,61], this non-specific agent explains the high degree of cross-reactivity with the other virus tested, especially in the hemolyzed samples. This hypothesis was confirmed by ELISA assays, which did not recognize specific immunoglobulins in any of the 13 available sera against any of the tested viruses (ZIKV, DENV, YFV) (Table 2). It is known that serum quality, especially the degree of hemolysis, directly influences serological tests [65,66]. Nevertheless, we decided to test all the samples, including the hemolyzed, because they were the only available material obtained from wild animals that rarely can be examined. In this way, the ELISA test is a useful tool when aiming to resolve doubts about specific or nonspecific PRNT responses, as demonstrated in few of our results.

From an entomological point of view, the evidence of ZIKV-sylvatic transmission was null. All captured mosquitoes tested negative for this and other tested arboviruses, except YFV. Noteworthy, during a ZIKV outbreak in Guadeloupe and French Guiana, we succeed in detecting ZIKV in urban mosquitoes with the same high throughput system [35]. Sylvatic Neotropical mosquitoes belonging to

six species and three genera that have been experimentally challenged with three ZIKV isolates thus far were refractory or exhibited low infection rates [67,68]. Therefore, considering the low vectorial competence of sylvatic mosquitoes tested thus far, coupled with the short, low and asymptomatic viremia of experimentally injected NHPs [69,70], and the fact that recent evidence has concentrated their findings in urban or periurban environments, we assume that there are still no reasons to suppose that ZIKV has established an independent sylvatic cycle in the forests of the RJ and surroundings in Southeast Brazil. Further studies, especially experimental infections of NHP and systematic collections of NHPs for molecular and serological surveillance should be conducted in order to shed more light on this issue.

Finally, it is important to emphasize the importance of combining arbovirus diagnosis techniques, especially during outbreaks when surveillance becomes more sensitive and a very large number of samples arrive at the laboratories. The combination of serological, immunohistochemical and molecular tests is extremely useful for retelling the history and/or confirming the infection of a pathogen. For example, we provided evidence that the marmosets (*Callithrix sp.*) whose cause mortis was diagnosed as YFV by RT-PCR in three large Brazilian cities may have been false positives, since other finds, such as serology of the other members of the group, allied to the entomological, histopathological and epidemiological results, did not support the molecular diagnosis. The same occurred with some suspicions of human urban yellow fever cases, which were later proven to have been of sylvatic origin [50].

In summary, the present study showed that there is no evidence that the ZIKV established an independent sylvatic cycle in RJ, and provided new evidence that there was no urban transmission of YFV in southeast Brazil during the current outbreak [34,44]. However, in view of the receptivity of the state, verified by the low prevalence of antibodies in NHPs examined during the outbreak, we recommend the strengthening of surveillance. Immunological and molecular techniques associated with the monitoring of NHP (mainly *Sapajus sp.*) and wild mosquitoes' populations should be implemented in order to detect the early circulation of arboviruses that could threaten humans.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/4/364/s1, Table S1: List of viruses and serotypes tested by high throughput real time PCR, Table S2: Summary of the characteristics of non-human primates examined.

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References

1. de Oliveira Garcia, M.H. Zika: The continuing threat. *Bull. World Health Organ.* **2019**, *97*, 6–7.
2. Brasil Monitoramento do Período Sazonal da Febre Amarela. Available online: http://portalarquivos2.saude.gov.br/images/pdf/2018/fevereiro/21/Informe-n14-FA-20fev18-c.pdf (accessed on 14 October 2019).
3. Possas, C.; Lourenço-de-oliveira, R.; Tauil, P.L.; Pinheiro, F.D.P.; Pissinatti, A.; Venâncio, R.; Freire, M.; Martins, R.M.; Homma, A. Yellow fever outbreak in Brazil: The puzzle of rapid viral spread and challenges for immunisation. *Mem. Inst. Oswaldo Cruz* **2018**, *113*, e180278. [CrossRef] [PubMed]
4. Althouse, B.M.; Vasilakis, N.; Sall, A.A.; Diallo, M.; Weaver, S.C.; Hanley, K.A. Potential for Zika Virus to Establish a Sylvatic Transmission Cycle in the Americas. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0005055. [CrossRef] [PubMed]
5. Couto-Lima, D.; Madec, Y.; Bersot, M.I.; Campos, S.S.; Motta, M.; de Albuquerque Motta, M.; Dos Santos, F.B.; Vazeille, M.; da Costa Vasconcelos, P.F.; Lourenço-de-Oliveira, R.; et al. Potential risk of re-emergence of urban transmission of Yellow Fever virus in Brazil facilitated by competent Aedes populations. *Sci. Rep.* **2017**, *7*, 1–12. [CrossRef] [PubMed]
6. Weaver, S.C. Urbanization and geographic expansion of zoonotic arboviral diseases: Mechanisms and potential strategies for prevention. *Trends Microbiol.* **2013**, *21*, 360–363. [CrossRef]
7. Klitting, R.; Gould, E.A.; Paupy, C. What does the future hold for yellow fever virus? *Genes* **2018**, *9*, 291. [CrossRef]
8. Franco, O. História da Febre Amarela no Brasil. *Ministério Saúde* **1969**, *1*, 210.
9. Bryant, J.E.; Holmes, E.C.; Barrett, A.D.T. Out of Africa: A Molecular Perspective on the Introduction of Yellow Fever Virus into the Americas. *PLoS Pathog.* **2007**, *3*, e75. [CrossRef]
10. Soper, F.L.; Penna, H.A.; Cardoso, E.; Serafim, J., Jr.; Frobisher, M., Jr.; Pinheiro, J. Yellow Fever without *Aedes aegypti*. Study of a rural epidemic in the Valle do Chanaan, Espirito Santo, Brazil, 1932. *Am. J. Hyg.* **1933**, *18*, 555–587. [CrossRef]
11. Soper, F.L. Jungle Yellow Fever. A New Epidemiological Entity in South America. *Rev. Hig. Saude Publica* **1937**, *10*, 104–144.
12. Davis, N.C. Susceptibility of Capuchin (Cebus) monkeys to yellow fever virus. *Am. J. Epidemiol.* **1930**, *11*, 321–334. [CrossRef]
13. Haddow, A.J.; Smithburn, K.C.; Dick, G.W.A.; Kitchen, S.F.; Lumsden, W.H.R. Implication of the Mosquito *aëdes (Stegomyia) Africanus* Theobald in the Forest Cycle of Yellow Fever in Uganda. *Ann. Trop. Med. Parasitol.* **1948**, *42*, 218–223. [CrossRef] [PubMed]
14. Vasconcelos, P.F.D.C. Yellow fever in Brazil: Thoughts and hypotheses on the emergence in previously free areas. *Rev. Saude Publica* **2010**, *44*, 1144–1149. [CrossRef] [PubMed]
15. Monath, T.P.; Vasconcelos, P.F.C. Yellow fever. *J. Clin. Virol.* **2015**, *64*, 160–173. [CrossRef]
16. Brasil Aspectos Epidemiológicos da Febre amarela Silvestre e a Vigilância Intensificada Durante Período de Monitoramento, Brasil, 2012/2013. Available online: https://portalarquivos2.saude.gov.br/images/pdf/2014/maio/27/BE-V45-n---07-FebreAmarela.pdf (accessed on 10 December 2019).
17. Bonaldo, M.C.; Gómez, M.M.; dos Santos, A.A.; Abreu, F.V.S.; Ferreira-de-Brito, A.; Miranda, R.M.; de Castro, M.G.; Lourenço-de-Oliveira, R. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. *Mem. Inst. Oswaldo Cruz* **2017**, *112*, 447–451. [CrossRef]
18. Mayer, S.V.; Tesh, R.B.; Vasilakis, N. The emergence of arthropod-borne viral diseases: A global prospective on dengue, chikungunya and zika fevers. *Acta Trop.* **2017**, *166*, 155–163. [CrossRef]
19. Zanluca, C.; de Melo, V.C.A.; Mosimann, A.L.P.; dos Santos, G.I.V.; dos Santos, C.N.D.; Luz, K. First report of autochthonous transmission of Zika virus in Brazil. *Mem. Inst. Oswaldo Cruz* **2015**, *110*, 569–572. [CrossRef]

20. de Araújo, T.V.B.; Rodrigues, L.C.; de Alencar Ximenes, R.A.; de Barros Miranda-Filho, D.; Montarroyos, U.R.; de Melo, A.P.L.; Valongueiro, S.; de Albuquerque, M.; Souza, W.V.; Braga, C.; et al. Association between Zika virus infection and microcephaly in Brazil, January to May, 2016: Preliminary report of a case-control study. *Lancet Infect. Dis.* **2016**, *16*, 1356–1363. [CrossRef]
21. Heukelbach, J.; Alencar, C.H.; Kelvin, A.A.; de Oliveira, W.K.; de Góes Cavalcanti, L.P. Zika virus outbreak in Brazil. *J. Infect. Dev. Ctries* **2016**, *10*, 116–120. [CrossRef]
22. Ferreira-de-Brito, A.; Ribeiro, I.P.; de Miranda, R.M.; Fernandes, R.S.; Campos, S.S.; da Silva, K.A.B.; de Castro, M.G.; Bonaldo, M.C.; Brasil, P.; Lourenço-de-Oliveira, R. First detection of natural infection of Aedes aegypti with Zika virus in Brazil and throughout South America. *Mem. Inst. Oswaldo Cruz* **2016**, *111*, 655–658. [CrossRef]
23. Terzian, A.C.B.; Zini, N.; Sacchetto, L.; Rocha, R.F.; Parra, M.C.P.; Del Sarto, J.L.; Dias, A.C.F.; Coutinho, F.; Rayra, J.; da Silva, R.A.; et al. Evidence of natural Zika virus infection in neotropical non-human primates in Brazil. *Sci. Rep.* **2018**, *8*, 16034. [CrossRef]
24. Favoretto, S.R.; Araujo, D.B.; Duarte, N.F.H.; Oliveira, D.B.L.; da Crus, N.G.; Mesquita, F.; Leal, F.; Machado, R.R.G.; Gaio, F.; Oliveira, W.F.; et al. Zika Virus in Peridomestic Neotropical Primates, Northeast Brazil. *Ecohealth* **2019**, *16*, 61–69. [CrossRef] [PubMed]
25. Berry, N.; Ferguson, D.; Ham, C.; Hall, J.; Jenkins, A.; Giles, E.; Devshi, D.; Kempster, S.; Rose, N.; Dowall, S.; et al. High susceptibility, viral dynamics and persistence of South American Zika virus in New World monkey species. *Sci. Rep.* **2019**, *9*, 1–12. [CrossRef] [PubMed]
26. Travassos-da-Rosa, A.P.D.A. The history of Arbovirology at Instituto Evandro Chagas, Belém, Pará, Brazil, from 1954 to 1998. *Rev. Pan-Amaz. Saúde* **2016**, *7*, 61–70. [CrossRef]
27. Laemmert, H.W.; Ferreira, L.D.C.; Taylor, R.M. Part II—Investigations of vertebrate hosts and arthropod vectors. *Am. J. Trop. Med. Hyg.* **1946**, *1*, 23–69. [CrossRef]
28. Catenacci, L.S.; Ferreira, M.; Martins, L.C.; De Vleeschouwer, K.M.; Cassano, C.R.; Oliveira, L.C.; Canale, G.; Deem, S.L.; Tello, J.S.; Parker, P.; et al. Surveillance of Arboviruses in Primates and Sloths in the Atlantic Forest, Bahia, Brazil. *Ecohealth* **2018**, *4*, 777–791. [CrossRef] [PubMed]
29. Lopes, O.D.S.; Coimbra, T.L.M.; Sachetta, L.D.A.; Calisher, C.H. Emergence of a new arbovirus disease in Brazil. *Am. J. Epidemiol.* **1978**, *107*, 444–449. [CrossRef]
30. Iversson, L.B.; Coimbra, T.L.M. Encefalite na região do Vale do Ribeira, São Paulo, Brasil, no período pós-epidêmico de 1978 a 1983: Situação do diagnóstico etiológico e características epidemiológicas. *Rev. Saude Publica* **1984**, *18*, 323–332. [CrossRef]
31. *Brasil Guia de Vigilância de Epizootias em Primatas não Humanos e Entomologia Aplicada à Vigilância da Febre Amarela*, 2nd ed.; Ministério da Saúde: Brasília, Brazil, 2017; ISBN 9788533421028.
32. Abreu, F.V.S.; dos Santos, E.; Gomes, M.Q.; Vargas, W.P.; Oliveira Passos, P.H.; Nunes e Silva, C.; Araújo, P.C.; Pires, J.R.; Romano, A.P.M.; Teixeira, D.S.; et al. Capture of *Alouatta guariba clamitans* for the surveillance of sylvatic yellow fever and zoonotic malaria: Which is the best strategy in the tropical Atlantic Forest? *Am. J. Primatol.* **2019**, *81*, e23000. [CrossRef]
33. Santos, T.P. Potential of Aedes albopictus as a bridge vector for enzootic pathogens at the urban-forest interfacein Brazil—Approved with minor corrections. *Emerg. Microbes Infect.* **2018**, *7*, 1–8. [CrossRef]
34. Abreu, F.V.S.D.; Ribeiro, I.P.; Ferreira-de-Brito, A.; Santos, A.A.C.D.; Miranda, R.M.D.; Bonelly, I.D.S.; Neves, M.S.A.S.; Bersot, M.I.; Santos, T.P.D.; Gomes, M.Q.; et al. *Haemagogus leucocelaenus* and *Haemagogus janthinomys* are the primary vectors in the major yellow fever outbreak in Brazil, 2016–2018. *Emerg. Microbes Infect.* **2019**, *8*, 218–231. [CrossRef] [PubMed]
35. Moutailler, S.; Yousfi, L.; Mousson, L.; Devillers, E.; Vazeille, M.; Vega-Rúa, A.; Perrin, Y.; Jourdain, F.; Chandre, F.; Cannet, A.; et al. A New High-Throughput Tool to Screen Mosquito-Borne Viruses in Zika Virus Endemic/Epidemic Areas. *Viruses* **2019**, *11*, 904. [CrossRef] [PubMed]
36. Bonaldo, M.C.; Ribeiro, I.P.; Lima, N.S.; dos Santos, A.A.C.; Menezes, L.S.R.; da Cruz, S.O.D.; de Mello, I.S.; Furtado, N.D.; de Moura, E.E.; Damasceno, L.; et al. Isolation of Infective Zika Virus from Urine and Saliva of Patients in Brazil. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0004816. [CrossRef]
37. *WHO Guidelines for Plaque Reduction Neutralization Testing of Human Antibodies to Dengue Viruses*; WHO: Geneva, Switzerland, 2003.

38. Moreira-Soto, A.; Carneiro, I.D.O.; Fischer, C.; Feldmann, M.; Kümmerer, B.M.; Silva, N.S.; Santos, U.G.; Souza, B.F.D.C.D.; Liborio, F.D.A.; Valença-Montenegro, M.M.; et al. Limited Evidence for Infection of Urban and Peri-urban Nonhuman Primates with Zika and Chikungunya Viruses in Brazil. *mSphere* **2018**, *3*, e00523-17. [CrossRef]
39. Miagostovich, M.P.; Nogueira, R.M.; dos Santos, F.B.; Schatzmayr, H.G.; Araújo, E.S.; Vorndam, V. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. *J. Clin. Virol.* **1999**, *14*, 183–189. [CrossRef]
40. Steinhagen, K.; Probst, C.; Radzimski, C.; Schmidt-Chanasit, J.; Emmerich, P.; van Esbroeck, M.; Schinkel, J.; Grobusch, M.P.; Goorhuis, A.; Warnecke, J.M.; et al. Serodiagnosis of Zika virus (ZIKV) infections by a novel NS1-based ELISA devoid of cross-reactivity with dengue virus antibodies: A multicohort study of assay performance, 2015 to 2016. *Euro Surveill.* **2016**, *21*, 30426. [CrossRef] [PubMed]
41. Gómez, M.M.; de Abreu, F.V.S.; Dos Santos, A.A.C.; de Mello, I.S.; Santos, M.P.; Ribeiro, I.P.; Ferreira-de-Brito, A.; de Miranda, R.M.; de Castro, M.G.; Ribeiro, M.S.; et al. Genomic and structural features of the yellow fever virus from the 2016-2017 Brazilian outbreak. *J. Gen. Virol.* **2018**, *99*, 536–548. [CrossRef]
42. Pinheiro, G.G.; Rocha, M.N.; de Oliveira, M.A.; Moreira, L.A.; Andrade Filho, J.D. Detection of Yellow Fever Virus in Sylvatic Mosquitoes during Disease Outbreaks of 2017–2018 in Minas Gerais State, Brazil. *Insects* **2019**, *10*, 136. [CrossRef]
43. Faria, N.R.; Kraemer, M.U.G.; Hill, S.C.; Jesus, J.G.D.; Aguiar, R.S.; Iani, F.C.M.; Xavier, J.; Quick, J.; Plessis, L.D.; Dellicour, S.; et al. Genomic and epidemiological monitoring of yellow fever virus transmission potential. *Science* **2018**, *361*, 894–899. [CrossRef]
44. Moreira-Soto, A.; Torres, M.C.; Lima de Mendonça, M.C.; Mares-Guia, M.A.; dos Santos Rodrigues, C.D.; Fabri, A.A.; dos Santos, C.C.; Machado Araújo, E.S.; Fischer, C.; Ribeiro Nogueira, R.M.; et al. Evidence for multiple sylvatic transmission cycles during the 2016–2017 yellow fever virus outbreak, Brazil. *Clin. Microbiol. Infect.* **2018**, *24*, 1019.e1–1019.e4. [CrossRef]
45. Delatorre, E.; Abreu, F.V.S.D.; Ribeiro, I.P.; Gómez, M.M.; dos Santos, A.A.C.; Ferreira-de-Brito, A.; Neves, M.S.A.S.; Bonelly, I.D.S.; Miranda, R.M.D.; Furtado, N.D.; et al. Distinct YFV Lineages Co-circulated in the Central-Western and Southeastern Brazilian Regions from 2015 to 2018. *Front. Microbiol.* **2019**, *10*, 1079. [CrossRef] [PubMed]
46. Moussallem, T.M.; Gava, C.; Ardisson, K.S.; Marques, C.S.; Graceli, G.C.; Valadares Koski, A.D.P.; Almada, G.L.; Silva, A.R.D.; Jesus, F.A.A.D.; Rodrigues, G.A.P.; et al. Yellow fever outbreak in a rural-urban mixed community of Espírito Santo, Brazil: Epidemiological aspects. *Rev. Panam. Salud Pública* **2019**, *43*, e29. [CrossRef] [PubMed]
47. Brasil, P.; Zalis, M.G.; de Pina-Costa, A.; Siqueira, A.M.; Júnior, C.B.; Silva, S.; Areas, A.L.L.; Pelajo-Machado, M.; de Alvarenga, D.A.M.; da Silva Santelli, A.C.F.; et al. Outbreak of human malaria caused by Plasmodium simium in the Atlantic Forest in Rio de Janeiro: A molecular epidemiological investigation. *Lancet Glob. Health* **2017**, *5*, e1038–e1046. [CrossRef]
48. Cunha, M.D.P.; Duarte-Neto, A.N.; Pour, S.Z.; Ortiz-Baez, A.S.; Černý, J.; Pereira, B.B.D.S.; Braconi, C.T.; Ho, Y.L.; Perondi, B.; Sztajnbok, J.; et al. Origin of the São Paulo Yellow Fever epidemic of 2017–2018 revealed through molecular epidemiological analysis of fatal cases. *Sci. Rep.* **2019**, *9*, 1–10. [CrossRef]
49. Ávila-Pires, F.D.D. The spread of jungle Yellow fever. *Rev. Patol. Trop. J. Trop. Pathol.* **2018**, *47*, 1. [CrossRef]
50. Brasil Não há Registro Confirmado de Febre Amarela Urbana no Brasil. Available online: http://www.saude.gov.br/noticias/agencia-saude/42486-nao-ha-registro-confirmado-de-febre-amarela-urbana-no-brasil (accessed on 6 June 2019).
51. Almeida, M.; Dos Santos, E.; Da Cruz Cardoso, J.; Da Fonseca, D.F.; Noll, C.A.; Silveira, V.R.; Maeda, A.Y.; De Souza, R.P.; Kanamura, C.; Brasil, R.A. Yellow fever outbreak affecting Alouatta populations in southern Brazil (Rio Grande do Sul State), 2008–2009. *Am. J. Primatol.* **2012**, *74*, 68–76. [CrossRef]
52. Moreno, E.S.; Agostini, I.; Holzmann, I.; Di Bitetti, M.S.; Oklander, L.I.; Kowalewski, M.M.; Beldomenico, P.M.; Goenaga, S.; Martínez, M.; Lestani, E.; et al. Yellow fever impact on brown howler monkeys (Alouatta guariba clamitans) in Argentina: A metamodelling approach based on population viability analysis and epidemiological dynamics. *Mem. Inst. Oswaldo Cruz* **2015**, *110*, 865–876. [CrossRef]

53. de Souza, R.P.; Petrella, S.; Coimbra, T.L.M.; Maeda, A.Y.; Rocco, I.M.; Bisordi, I.; Silveira, V.R.; Pereira, L.E.; Suzuki, A.; Santos Silva, S.J.D.; et al. Isolation of Yellow Fever virus (YFV) from naturally infected Haemagogus (Conopostegus) leucocelaenus (Diptera, Culicidae) in São Paulo state, Brazil, 2009. *Rev. Inst. Med. Trop. Sao Paulo* **2011**, *53*, 133–139. [CrossRef]
54. Bicca-marques, J.C.; Calegaro-Marques, C.; Rylands, A.B.; Karen, B.; Mittermeier, R.A.; Almeida, M.A.B.; Castro, P.H.G.; Chaves, Ó.M.; Ferraz, L.P.; Fortes, V.B.; et al. Yellow fever threatens Atlantic Forest primates Júlio. *Sci. Adv.* **2017**, *1600946*, 18–20.
55. Cunha, M.S.; da Costa, A.C.; de Azevedo Fernandes, N.C.C.; Guerra, J.M.; dos Santos, F.C.P.; Nogueira, J.S.; D'Agostino, L.G.; Komninakis, S.V.; Witkin, S.S.; Ressio, R.A.; et al. Epizootics due to Yellow Fever Virus in São Paulo State, Brazil: Viral dissemination to new areas (2016–2017). *Sci. Rep.* **2019**, *9*, 5474. [CrossRef]
56. Kumm, H.W.; Laemmert, H.W. The geographical distribution of immunity to yellow fever among the primates of Brazil. *Am. J. Trop. Med. Hyg.* **1950**, *30*, 733–748. [CrossRef] [PubMed]
57. Almeida, M.A.B.D.; Santos, E.D.; Cardoso, J.D.C.; Noll, C.A.; Lima, M.D.M.; Silva, F.D.A.E.; Ferreira, M.S.; Martins, L.C.; Vasconcelos, P.F.D.C.; Bicca-Marques, J.C. Detection of antibodies against Icoaraci, Ilhéus, and Saint Louis Encephalitis arboviruses during yellow fever monitoring surveillance in non-human primates (*Alouatta caraya*) in southern Brazil. *J. Med. Primatol.* **2019**, *48*, 211–217. [CrossRef] [PubMed]
58. Abreu, F.V.S.D.; Delatorre, E.; dos Santos, A.A.C.; Ferreira-de-Brito, A.; de Castro, M.G.; Ribeiro, I.P.; Furtado, N.D.; Vargas, W.P.; Ribeiro, M.S.; Meneguete, P.; et al. Combination of surveillance tools reveals that Yellow Fever virus can remain in the same Atlantic Forest area at least for three transmission seasons. *Mem. Inst. Oswaldo Cruz* **2019**, *114*, e190076. [CrossRef] [PubMed]
59. Causey, O.R.; Hughes, T.P.; Laemmert, H.W. The Invasion of Small Forests by Yellow Fever Virus as Indicated by Immunity in Cebus Monkeys 1. *Am. J. Trop. Med. Hyg.* **1949**, *1*, 555–565.
60. Fedigan, L.M. Capuchin Monkeys (*Sapajus* and *Cebus*). In *The International Encyclopedia of Primatology*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2017; pp. 1–2.
61. de Oliveira-Filho, E.F.; Oliveira, R.A.S.; Ferreira, D.R.A.; Laroque, P.O.; Pena, L.J.; Valença-Montenegro, M.M.; Mota, R.A.; Gil, L.H.V.G. Seroprevalence of selected flaviviruses in free-living and captive capuchin monkeys in the state of Pernambuco, Brazil. *Transbound. Emerg. Dis.* **2018**, *65*, 1094–1097. [CrossRef]
62. Davis, N.C. The susceptibility of Marmosets to Yellow fever virus. *J. Exp. Med.* **1930**, *52*, 405–416. [CrossRef]
63. Araújo, F.A.A.; Ramos, D.G.; Santos, A.L.; Passos, P.H.D.O.; Elkhoury, A.N.S.M.; Costa, Z.G.A.; Leal, S.G.; Romano, A.P.M. Epizootias em primatas não humanos durante reemergência do vírus da febre amarela no Brasil, 2007 a 2009. *Epidemiol. Serviços Saúde* **2011**, *20*, 527–536. [CrossRef]
64. Fernandes, N.C.C.D.A.; Cunha, M.S.; Guerra, J.M.; Réssio, R.A.; Cirqueira, C.D.S.; Iglezias, S.D.; de Carvalho, J.; Araujo, E.L.L.; Catão-Dias, J.L.; Díaz-Delgado, J. Outbreak of Yellow Fever among Nonhuman Primates, Espirito Santo, Brazil, 2017. *Emerg. Infect. Dis.* **2017**, *23*, 2038–2041. [CrossRef]
65. Kuno, G.; Chang, G.J.; Tsuchiya, K.R.; Karabatsos, N.; Cropp, C.B. Phylogeny of the genus Flavivirus. *J. Virol.* **1998**, *72*, 73–83. [CrossRef]
66. Shan, C.; Ortiz, D.A.; Yang, Y.; Wong, S.J.; Kramer, L.D.; Shi, P.-Y.; Loeffelholz, M.J.; Ren, P. Evaluation of a Novel Reporter Virus Neutralization Test for Serological Diagnosis of Zika and Dengue Virus Infection. *J. Clin. Microbiol.* **2017**, *55*, 3028–3036. [CrossRef]
67. Karna, A.K.; Azar, S.R.; Plante, J.A.; Yun, R.; Vasilakis, N.; Weaver, S.C.; Hansen, I.A.; Hanley, A.A. Colonized Sabethes cyaneus, a Sylvatic New World Mosquito Species, Shows a Low Vector Competence for Zika Virus Relative to Aedes aegypti. *Viruses* **2018**, *10*, 434. [CrossRef] [PubMed]
68. Fernandes, R.S.; Bersot, M.I.; Castro, M.G.; Telleria, E.L.; Ferreira-de-Brito, A.; Raphael, L.M.; Bonaldo, M.C.; Lourenço-de-Oliveira, R. Low vector competence in sylvatic mosquitoes limits Zika virus to initiate an enzootic cycle in South America. *Sci. Rep.* **2019**, *9*, 20151. [CrossRef] [PubMed]

69. Chiu, C.Y.; Sánchez-San Martín, C.; Bouquet, J.; Li, T.; Yagi, S.; Tamhankar, M.; Hodara, V.L.; Parodi, L.M.; Somasekar, S.; Yu, G.; et al. Experimental Zika Virus Inoculation in a New World Monkey Model Reproduces Key Features of the Human Infection. *Sci. Rep.* **2017**, *7*, 17126. [CrossRef] [PubMed]
70. Vanchiere, J.A.; Ruiz, J.C.; Brady, A.G.; Kuehl, T.J.; Williams, L.E.; Baze, W.B.; Wilkerson, G.K.; Nehete, P.N.; Mcclure, G.B.; Rogers, D.L.; et al. Experimental Zika Virus Infection of Neotropical Primates. *J. Trop. Med. Hyg.* **2017**, *98*, 173–177. [CrossRef]

5 DISCUSSÃO

As investigações das zoonoses de origem silvestre geralmente necessitam de expedições em campo para captura da fauna de vertebrados e/ou invertebrados suspeitos de participarem dos ciclos de transmissão (Brasil 2017a). Estas atividades têm ficado em segundo plano em virtude do avanço dos campos da biologia molecular, genética e bioinformática que atraem jovens pesquisadores e priorizam atividades laboratoriais. A despeito disso, o presente trabalho realizou um amplo esforço amostral na investigação de dois agravos com potencial zoonótico – malária das bromélias e febre amarela silvestre (FA) – cujos agentes recentemente causaram surtos na Mata Atlântica do estado do Rio de Janeiro e áreas contíguas. O ciclo de ambas pode ser mantido em ambientes florestais, onde PNHs e mosquitos silvestres atuam como hospedeiros (Soper 1937; Deane et al. 1966; Deane 1992), levando-nos à necessidade de capturá-los e examiná-los (Imagens das atividades de campo se encontram no Anexo VI).

Dentre os PNHs, a amostragem de indivíduos do gênero *Alouatta* é de especial importância devido à susceptibilidade ao YFV (Moreno et al. 2015) e às elevadas taxas de infecção por *Plasmodium* reportadas em outras regiões do país, tendo sido o principal gênero encontrado infectado com *P. simium* (Deane 1992). No entanto, devido às dificuldades de captura, pouquíssimos *Alouatta* tinham sido examinados com esse propósito no RJ até 2019 (Deane 1992; Brasil et al. 2017). A captura de PNHs é realizada levando-se em conta hábitos, habitats na floresta e porte das espécies alvo. Animais de pequeno a médio porte, de alimentação predominantemente frutífera e que vivem em estratos florestais mais baixos, como os *Callithrix* e *Sapajus,* presentes na Mata Atlântica, podem ser capturados facilmente com armadilhas (Watsa et al. 2015). Animais de médio a grande porte com alimentação predominantemente folívora e/ou hábitos acrodendrófilos (ex: *Alouatta*, *Callicebus* e *Brachyteles*) geralmente são capturados através de dardos anestésicos devido ao alto índice de insucesso das armadilhas (Scott et al. 1976). Apesar disso, grupos de *Alouatta caraya* foram capturados com sucesso em grandes armadilhas instaladas em dossel, após oferta regular e abundante de frutas durante dois meses, na região Sul do Brasil (Aguiar et al. 2007). No entanto, devido ao tempo gasto para cevar os grupos, a busca ativa na mata seguida da projeção de dardos anestésicos é o método mais utilizado para captura de bugios. Com este método, cerca de 230 bugios (*A. caraya* e *A. g. clamitans)* foram amostrados no Rio Grande do Sul para vigilância de FA após o surto de 2009 (Almeida et al. 2012; Brasil 2017a) e há relatos

de captura de centenas de bugios (*A. palliata, A. villosa*) para estudos ecológicos na América Central (Clarke et al. 1986; Milton et al. 2009). Porém, as duas estratégias (armadilhas em dossel e busca ativa com dardos anestésicos) se mostraram muito ineficientes no RJ, resultado que atribuímos às condições ecológicas e físicas da Mata Atlântica fluminense e à pressão de caça. A grande oferta e diversidade vegetal, a competição de outros animais pela ceva e o comportamento alimentar mais restrito de *A. g. clamitans* contribuíram para o insucesso das armadilhas. Por outro lado, a dificuldade de encontrar os animais no terreno íngreme das encostas da Serra do Mar, aliado à altura do dossel com diversos estratos vegetais intermediários que limitam a visão, e à pressão de caça, que torna os animais mais arredios, reduziram enormemente a eficiência de captura através de busca ativa com uso de dardos anestésicos, a despeito dos esforços para construção de armadilhas e busca ativa dos animais, como ilustrado no anexo VI.

A solução encontrada para contornar estas dificuldades foi a criação da rede de informações multidisciplinar, englobando os principais agentes e instituições de cada localidade alvo e mantida principalmente através de aplicativos de troca de mensagens. Através dela, populações de *Alouatta* de diferentes áreas foram constantemente monitoradas e recebíamos alertas da presença dos animais em tempo real, o que aumentou enormemente a eficiência de captura e reduziu os gastos. Na primeira expedição, ainda sem apoio da rede, gastamos 15 dias, com uma equipe de oito pessoas e nenhum bugio foi capturado. Na segunda, com a mesma configuração, apenas dois. Caso a média fosse mantida, finalizaríamos as sete coletas previstas tendo examinado apenas sete animais. No entanto, a rede permitiu examinar muitos animais gastando menos tempo, e contribuiu para o encontro e notificações de epizootias durante o surto de YFV, como mostra a figura 7.

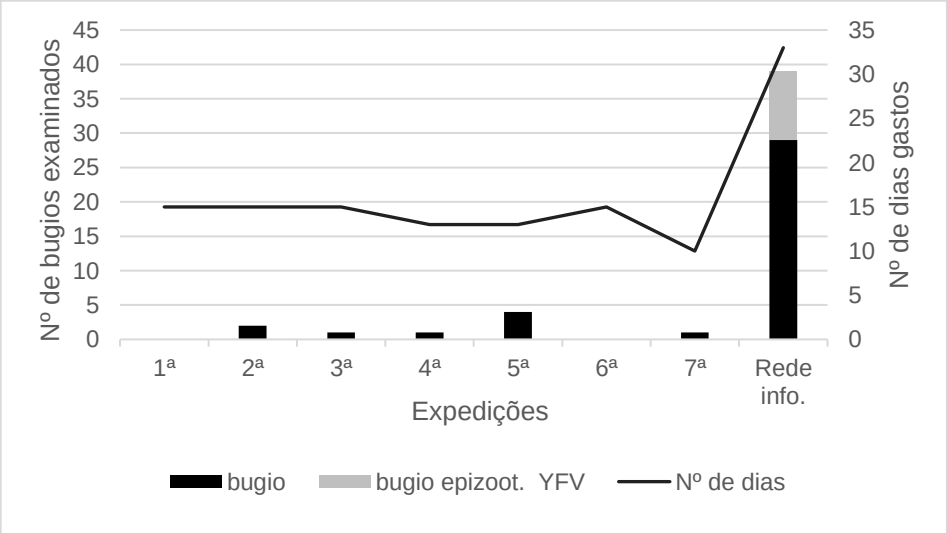

Figura 7: Número de dias gastos para examinar os bugios sem (1ª a 7ª expedições) e com a rede de informações. Barra cinza mostra as epizootias em bugios examinadas durante o surto de YF.

Apesar do investimento inicial para criação da rede, com visitas e palestras nas áreas alvo, trata-se de uma estratégia simples e de baixo custo que pode contribuir para o monitoramento da fauna e para a vigilância ambiental das referidas zoonoses. Também recomendamos que a busca ativa com dardos anestésicos deve ser priorizada em fragmentos de até 100ha e/ou sob domínio de floresta estacional semidecidual, onde a eficiência de captura foi maior do que em grandes fragmentos de floresta ombrófila densa. De fato, o sucesso de captura com dardos nos demais locais da América foi obtido em regiões com estas características ecológicas, que facilitam o encontro dos grupos e a projeção dos dardos (Clarke et al. 1986).

Outro ponto importante durante estudos de vigilância eco-epidemiológica é o aproveitamento das amostras. Os protocolos de coleta para o diagnóstico virológico ou sorológico de febre amarela e outras arboviroses, preconizam o armazenamento do soro e descarte do coágulo (Brasil 2017a). Por outro lado, o material mais utilizado para diagnóstico de *Plasmodium* é a “papa de hemácias”, separada do plasma após coleta em tubo contendo anticoagulante (Brasil et al. 2017). No entanto, a volemia de animais silvestres pode ser baixa, sobretudo naqueles de pequeno porte, como os saguis por exemplo, dos quais raramente se obtém mais do que 2-3 ml de sangue sem prejuízo para a saúde do animal. Além disso, tendo em vista a raridade dos eventos de captura, esforço demandado e grau de estresse a que os animais são submetidos, é importante que se aproveite a oportunidade de coleta para contemplar o máximo de estudos possível. Por isso a padronização de técnicas para otimizar o diagnóstico a partir de amostras pouco invasivas, como fezes ou outros materiais tradicionalmente descartados, como os coágulos, é extremamente importante (de Assis et al. 2016). Nesse sentido, a padronização da técnica para uso do coágulo na detecção das duas espécies de *Plasmodium* simiano existentes no Brasil (*P. simium* e *P. brasilianum / malariae*), tanto em PNHs quanto em humanos (no caso de *P. simium*), mesmo em baixas parasitemias, como descrito no presente trabalho, foi de extrema importância. Além do diagnóstico de *Plasmodium*, o material genético extraído de coágulo pode ser utilizado para diagnóstico de outros parasitos (ex: *Trypanosoma sp*. que pretendemos realizar em breve) e/ou para estudos genéticos e ecológicos do próprio hospedeiro (Se Fum Wong et al. 2007; McCulloch et al. 2009; Bank et al. 2013; Rodrigues et al. 2019). Obtivemos sucesso na amplificação de genes

de DNA mitocondrial extraído a partir de coágulo de todos os bugios, com vistas a estudar a taxonomia desta espécie em colaboração com outro grupo de pesquisa, o que reforça a utilidade do material. Por conta de nossos resultados, recomendamos que o protocolo de coleta de amostras para febre amarela preconizado pelo Ministério da Saúde passe a contemplar o armazenamento dos coágulos e que se crie um banco desse material em laboratórios de referência, pois trata-se de uma fonte de informação genética preciosa.

A partir das técnicas relatadas para captura, coleta e processamento das amostras foi possível, de maneira inédita, estudar a distribuição espacial e biológica de plasmódios, nas espécies de PNHs encontradas na Mata Atlântica. Desta forma, definimos *A. g. clamitans* como único hospedeiro de vida livre de *P. simium e P. brasilianum* confirmado na região. A infecção por *Plasmodium* em bugios é descrita desde a década de 50, mas a dimensão de sua importância só foi reconhecida após os trabalhos pioneiros e abrangentes de Leônidas Deane conduzidos a partir da década de 60 (Fonseca 1951; Deane 1992).

Os resultados descritos no presente estudo são produtos do primeiro levantamento deste tipo, que incluindo a captura de *A. g. clamitans*, realizado no Rio de Janeiro, estado no qual a malária foi erradicada há quatro décadas, depois de longa história de elevada endemicidade. A sobreposição geográfica entre as ocorrências dos bugios e os humanos parasitados, aliada à identidade molecular revelada pela presença dos SNPs específicos de *P. simium* em 100% das infecções, contribuem para confirmação da transmissão zoonótica e do papel de reservatório dos *Alouatta* (Brasil et al. 2017). Além do RJ, outros estados recobertos pela Mata Atlântica (ES, SP) também relatam evidências de transmissão zoonótica de *P. simium* (Cerutti et al. 2007; de Castro Duarte et al. 2008; de Alencar et al. 2017). A epidemiologia, os sinais clínicos, a baixa parasitemia e o histórico de contato com ambientes florestais é a mesma em todas as descrições (Deane 1992; Curado et al. 2006; Cerutti et al. 2007; Brasil et al. 2017). É importante que se esclareça a real dimensão da transmissão zoonótica na Mata Atlântica e a implicação desta transmissão nas campanhas mundiais de erradicação da malária.

Plasmodium brasilianum/malariae parece ter menos especificidade de hospedeiros vertebrados, tendo sido encontrado em todas as famílias de PNHs neotropicais. No entanto, no Sudeste, os exames de *Sapajus* e *Callithrix* em busca deste protozoário têm sido sistematicamente negativos, o que reforça a importância dos *Alouatta* para a região (Deane 1992; de Castro Duarte et al. 2008). Recentemente,

reportou-se o encontro de DNA compatível com o de *P. brasilianum/malariae* em macacos dos gêneros *Callithrix, Leontopithecus, Mico* e *Saguinus*, (Família Callitrichidae) criados em um cativeiro localizado dentro da Mata Atlântica fluminense (Alvarenga et al. 2017). Mas, não foram vistos parasitos nas preparações sanguíneas desses animais examinadas ao microscópio. A região é considerada enzoótica para essa malária e especula-se que as condições de cativeiro tenham favorecido a infecção (Alvarenga et al. 2017). Portanto, é provável que a relevância destes animais na manutenção do ciclo do *P. brasilianum* / *malariae* seja baixa ou nula em ambientes naturais. Ao contrário, a parasitemia foi detectável em lâminas de cinco dos seis *Alouatta* que diagnosticamos com malária quartã. Além disso, foi demonstrado pela primeira vez, que as taxas de infecção e a distribuição geográfica de *P. brasilianum/malariae* são semelhantes às de *P. simium* no RJ, e que a coinfecção é um evento relativamente comum, assim como relatado por Deane, em 1992 (Deane 1992).

Tendo em vista a frequência e a distribuição de *P. brasilianum* / *malariae* nos PNH do RJ, a capacidade deste protozoário de infectar o homem experimentalmente e a presença do vetor (*An. cruzii*), é intrigante que relatos de casos humanos de malária causadas por este parasito sejam escassos. Seriam as infecções humanas por este parasito assintomáticas, paucissintomáticas ou com conjunto de sintomas pouco definidos e de curso rápido na maioria dos casos e, por conseguinte, não ainda detectadas pela vigilância? Em Guapimirim, em área de intensa influência da Mata Atlântica, *P. brasilianum* / *malariae* foi diagnosticado por PCR e humanos residentes, sem parasitemia ou sintomas, e 30% de uma população da área tinha anticorpos contra o parasito. Também existem evidências imunológicas de infecção de humanos por esse parasito na Amazônia (Coatney 1971; de Arruda et al. 1989; Lourenço-de-Oliveira and Luz 1996; Curado et al. 1997; Lalremruata et al. 2015; Miguel et al. 2019). É imprescindível que se intensifiquem os esforços de coleta, com foco nos locais onde macacos foram diagnosticados com malária quartã, para se investigar o potencial zoonótico deste parasito na região.

A concentração dos registros (tanto de casos e infecções humanas quanto de infecção de macacos) na vertente oceânica da Serra do Mar é interessante. Algumas hipóteses para esse fenômeno foram discutidas no artigo 4.3, dentre elas a influência direta da umidade oceânica nos parâmetros determinantes da capacidade vetorial das populações locais do mosquito *An.* (*Kerteszia*) *cruzii* (Rachou 1952; Coutinho and Rachou 1966; Deane 1992; Machado et al. 2010), além de evidências de diferenças

e estruturação genética entre os *An. cruzii* coletados na vertente costeira, comparados com os coletados na vertente continental, o que também pode influenciar na competência vetorial (de Rezende Dias et al. 2018) e, por conseguinte, na capacidade vetorial.

Não conseguimos, ainda, confirmar a espécie vetora desta malária zoonótica no RJ. No momento da redação desta tese, infelizmente não tinha, ainda, sido possível finalizar a análise das coletas e exames de anofelinos realizados tanto anteriormente quanto concomitantemente aos de PNHs nos focos de malária humana. No entanto, qualitativamente, foi possível perceber a predominância de *An. cruzii*, que correspondeu a mais de 90% dos anofelinos coletados, ainda que as capturas só tenham sido realizadas ao nível do solo. Essa predominância ao nível do solo, onde transitam os humanos, já credencia, por si só, esta espécie de anofelino como o provável vetor da malária zoonótica no RJ, enquanto os resultados das PCR para a pesquisa da infecção natural por plasmódio não estão disponíveis. *An. cruzii* apresenta marcada acrodendrofilia em algumas regiões do Sudeste, como São Paulo, mas apresentava movimento vertical (entre a copa e o solo) em outras áreas, como em Joinville, Santa Catarina (Deane et al. 1984). Importantemente, casos humanos de malária só eram registrados nas localidades nas quais estes anofelinos eram encontrados picando também no nível do solo. Especula-se que o relevo de declive acentuado nas proximidades da Serra dos Órgãos faça com que as copas de determinadas árvores estejam ao mesmo nível das raízes de outras localizadas mais acima na montanha, o que pode favorecer a presença dos *An. cruzii* em virtualmente todo o gradiente (Pina-Costa et al. 2014). O mesmo provavelmente acontece para *Hg. janthinomys*, vetor primário da febre amarela, como será discutido posteriormente.

À parte do potencial papel do clima, ambiente e entomológico já comentados acima na diferente frequência de infecções plasmodiais em humanos e PNHs na vertente oceânica da Serra do Mar, um tema interessante a ser melhor investigado estaria no campo da geografia humana. Aparentemente, comparado à vertente continental, é na vertente oceânica que se concentram mais atrativos naturais em ambiente de mata procurados pela população urbana, na qual se registram a enorme maioria dos casos clínicos detectados pela vigilância. Campanhas turísticas e loteamentos de fragmentos florestais de fazendas são aparentemente mais comuns na vertente oceânica. Cachoeiras e parques ecológicos, além de chácaras de veraneio são numerosos ali. “Promessas” de dias aprazíveis em contato estreito com a natureza representada pela mata e sua fauna silvestre em contraponto à vivência

na metrópole que se divulga doente física e mentalmente tem orientado a escolha de uma grande porção das populações no mundo. O sonho de ter uma residência secundária ou mesmo primária na floresta para repouso e distanciamento do ambiente urbano tornou-se uma moda, não só dentre os habitantes das metrópoles brasileiras, mas mundialmente difundida. Há muitos movimentos, seitas, práticas, filosofias que surgiram ou ressurgiram no mundo contemporâneo que, direta ou indiretamente, aumentam o contato homem-ambientes naturais onde vivem os reservatórios (bugios) e os vetores (*An. cruzii*), favorecendo o estabelecimento da infecção zoonótica (Pina-Costa et al. 2014). Coincidentemente, as mesmas práticas podem ter tido papel importante na magnitude da epidemia de febre amarela silvestre, cujos aspectos da transmissão também estudamos no presente trabalho, como discutidos por Possas et al (2018).

Durante o surto de febre amarela silvestre registrado um ano após o pico dos casos de malária, foram registradas centenas de epizootias nos estados de RJ, ES, MG e SP, número que deve estar enormemente subestimado pois a maioria dos PNHs morrem no interior das florestas, impedindo seu encontro e notificação (Brasil 2017b, 2018, 2019a). Além disso, a chegada abrupta da epidemia e epizootias na região e a falta de experiência pregressa por parte dos serviços de zoonose relativas à febre amarela reduziu bastante a sensibilidade da vigilância. *Alouatta* é o gênero de PNH mais susceptível ao vírus e extinções de populações locais já foram reportadas em outros surtos (Araújo et al. 2011; Almeida et al. 2012; Moreno et al. 2013, 2015; Bicca-marques et al. 2017). Este grupo de PNHs foi o mais afetado também na amostragem do presente trabalho, o que condiz com a maior resistência ao vírus relatada nos gêneros *Sapajus* e, possivelmente, em *Callithrix* (Causey et al. 1949; Laemmert and Kumm 1950). Tendo em vista o papel fundamental dos bugios na manutenção de *Plasmodium*, é plausível supor que a circulação deste protozoário e dos casos de malária zoonótica sofrerá mudanças em curto/médio prazo. É possível que a rápida diminuição das populações de *Alouatta* tenha reduzido também a fonte de infecção dos *An. cruzii*, o que levaria a uma menor circulação de *Plasmodium* nas regiões de Mata Atlântica afetadas pelo YFV, reforçando ainda mais a importância da transmissão zoonótica.

Os dois mosquitos considerados vetores primários no presente surto (*Hg. janthinomys* e *Hg. leucocelaenus*) são apontados como importantes transmissores de FA desde a década de 1930 (Shannon et al. 1938). Muito curiosamente, ambos também foram apontados como vetores em Bemposta, RJ, em 1938, no último surto

de FA silvestre registrado no estado antes do atual, 2016-2019 (Shannon et al. 1938). Isso mostra que mesmo com todas as mudanças ocorridas em 80 anos de história (ex: crescimento das cidades, desmatamento, mudanças climáticas) a região continua reunindo condições para sustentar populações perenes destas duas espécies. É interessante notar que a abundância de *Hg. leucocelaenus* é baixa no norte do país, onde *Hg. janthinomys* é o vetor mais importante (Dégallier et al. 1992) . Ao contrário, *Hg. leucocelaenus* é a espécie predominante no Sul do país, onde é considerada vetor primário (Cardoso et al. 2010). Na região Sudeste, as duas espécies parecem ocorrer em abundâncias semelhantes, com pequena vantagem para *Hg. leucocelaenus*. Por esta razão e por apresentarem altas taxas de infecção natural descritas nesta tese, somadas ao comportamento primatofílico e acrodendrófilo já descrito por outros autores, consideramos ambas como vetores primários do surto em curso. É difícil estimar se a co-ocorrência destas espécies influenciou a dimensão do surto, juntamente com outros aspectos que serão discutidos a seguir. Também é difícil explicar por que a febre amarela ficou quase 80 anos sem circular pelo litoral do Sudeste brasileiro. É provável que o aumento da conscientização acerca da conservação da natureza, que resultou em aumento das unidades de conservação e preservação da fauna tenha influenciado, assim como o movimento de "*back to nature*" (Possas et al. 2018) que pode ter aumentado a exposição aos vetores de malária e de FA, como mencionado acima e retomado ao final desta seção.

Os demais mosquitos que encontramos naturalmente infectados com o YFV parecem ter papel secundário ou muito local, devido às baixas taxas de infecção, baixa abundância e/ou distribuição geográfica restrita. Por outro lado, *Ae. scapularis* e *Ae. taeniorhynchus* geralmente realizam hematofagia no nível do solo o que reduz as chances de se infectarem em PNHs que vivem em estratos florestais mais elevados (Consoli and Oliveira 1994). No entanto, os macacos infectados geralmente descem ao chão, onde passam várias horas agonizando. Neste momento, eles poderiam servir de fonte de infecção para espécies que vivem ao nível do solo, como *Ae. scapularis* e *Ae. taeniorhynchus*, mas também por *Ae. albopictus*, considerado vetor-ponte em potencial, como registramos em nosso artigo 4.7. No local e nos momentos em que *Ae. scapularis* e *Ae. taeniorhynchus* foram detectados naturalmente infectados com YFV, encontrava-se elevada densidade populacional desses dois mosquitos e dos *Haemagogus,* vetores primários, exibindo elevados índices de infecção. Até onde sabemos, *Ae. taeniorhynchus* nunca foi encontrado naturalmente infectado com YFV e apresentou baixa competência vetorial em infecções experimentais (Whitman and

Antunes 1937). *Ae. scapularis* foi encontrado infectado com YFV durante surto na Bahia (Vasconcelos et al. 2001a), mas parece ter desempenhado apenas um papel secundário. *Sa. chloropterus* apresenta hábitos acrodendrófilos e já foi encontrado naturalmente infectado em outras ocasiões (Rawlins et al. 1990).

Destaca-se que a identificação taxonômica das espécies de mosquitos envolvidas na transmissão das duas zoonoses (malária e FA) requer o trabalho de recursos humanos capacitados para tal função. Durante o surto, notou-se uma grande deficiência de técnicos, agentes de saúde, e pesquisadores treinados em todo o território atingido, o que sobrecarrega os laboratórios de referência e atrasa o diagnóstico. Há que se fazer um esforço nacional com vistas a capacitar profissionais de todos os estados para a taxonomia dos vetores e metodologias de detecção da infecção natural nos invertebrados.

A partir das amostras de YFV obtidas nos PNHs e nos mosquitos coletados, somada às dos humanos, foi possível caracterizar o vírus circulante nos diferentes hospedeiros e estimar as rotas de dispersão ao longo do tempo. A clusterização do vírus junto ao genótipo Sulamericano I condiz com outros achados, pois este é o genótipo mais comum no Brasil e o subclado 1E inclui linhagens que circularam a partir de 2004 no país (Vasconcelos et al. 2004; Mir et al. 2017). Estima-se que este subclado tenha se originado na região norte da América Latina (Trinidad e Tobago, Venezuela), de onde teria iniciado ondas epizoóticas (Bonaldo et al. 2017; Fernandes et al. 2017; Mir et al. 2017; Faria et al. 2018; Gómez et al. 2018; Rezende et al. 2018), a princípio em direção ao centro-oeste. De lá, teria seguido duas rotas distintas de dispersão, uma em direção à bacia hidrográfica do atlântico sul, atingindo o leste de MG e posteriormente ES e RJ, e outra diretamente da bacia do Araguaia-Tocantins para a bacia do Rio Paraná, entre o Sudoeste de MG e Noroeste de SP(Rezende et al. 2018). No RJ, o vírus ainda percorreu duas rotas de transmissão, separadas pela cadeia de montanhas representada pela Serra do Mar, importante barreira geográfica que pode, de alguma maneira, limitar a dispersão de mosquitos e de PNHs. É interessante notar que esses achados condizem com as evidências epidemiológicas. Assim, o Ministério da Saúde reportava, entre 2014-2015, a ocorrência de uma onda de expansão do YFV, principalmente a partir da detecção de epizootias no centro-oeste (GO) e no oeste de MG (Brasil 2014). Em seguida, casos humanos e epizootias foram registrados concomitantemente nas duas rotas supracitadas, confirmando a separação das cadeias. Desde sua detecção no leste de MG, o vírus se dispersou no tempo e no espaço a velocidades e distâncias (0,8 a 4,0 km/dia) (Faria et al. 2018;

Fioravanti 2018; Gómez et al. 2018) que condizem com a dispersão dos mosquitos *Haemagogus leucocelaenus* e *Hg. janthinomys,* que já foram recapturados a 5,7 e 11,5Km, respectivamente, em poucos dias a partir de um ponto de soltura na floresta (Causey et al. 1950). Ainda que não efetivamente confirmado cientificamente, não se pode negligenciar o possível papel de humanos virêmicos na dispersão espacial do YFV, sobretudo entre o centro-oeste e o leste de MG, que parece ter um vazio de notificações entre 2015-2016, explicável também por baixa sensibilidade da vigilância.

Um dos aspectos mais instigantes da disseminação espacial do YFV no presente surto (2016-2019) é a incrível repetição das rotas traçadas pelo vírus nas décadas de 1930-1940 (Figura 8), por sua vez baseada nos registros de óbitos. Àquela época não haviam sido ainda padronizados métodos sorológicos para a detecção de infecções pregressas, assintomáticas ou não, e nem métodos moleculares e de filogenia viral. É intrigante que as condições propícias para o desencadeamento do surto continuem presentes após tanto tempo, o que novamente leva à reflexão de porque se passaram quase 80 anos até que estas rotas se repetissem. A região estava receptiva há, pelo menos, um ano antes do surto, com a presença de vetores competentes e hospedeiros vertebrados susceptíveis, descritos nos artigos 4.3 e 4.8. Suspeita-se que que as matas ciliares ao longo das grandes bacias sirvam de corredores de transmissão e, apesar de reduzidas pelo desmatamento, é provável que nunca tenham deixado de existir, o que dificulta a resposta a essa questão. Também desperta a curiosidade notar que no passado e atualmente existem grandes áreas no centro-oeste de MG onde praticamente não houve registros de casos humanos ou epizootias, talvez em decorrência de subnotificação ou de fatores ambientas ainda não revelados.

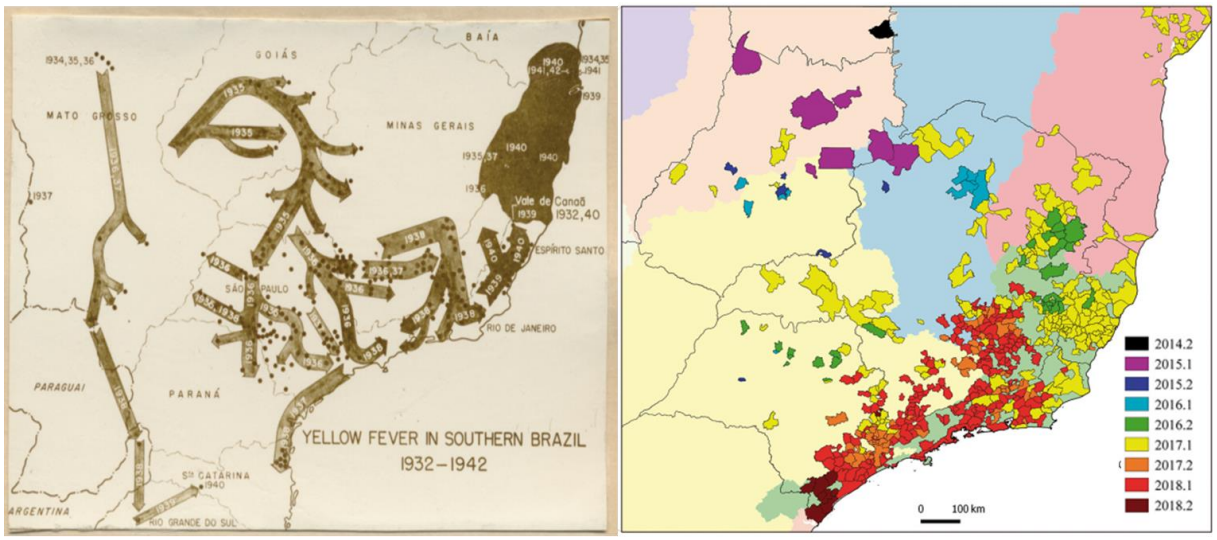

Figura 8: Esq. – Mapeamento das rotas traçadas pelo YFV durante as epidemias de 1932 a 1942. Esta teria sido a última vez que o vírus amarílico circulara no litoral do Sudeste, até o ano de 2015; Dir. – Casos humanos e epizootias confirmadas para YFV entre 2014-2019. (Fonte: esq. Acervo da Casa de Oswaldo Cruz, Departamento de Arquivo e Documentação - Foto FR (SFA-EC) 12-5 de A. Fialho; dir. Artigo 4.6 desta tese).

A reemergência da transmissão urbana do YFV é uma grande preocupação, desde que o último caso foi registrado na década de 1940, no Acre (Soper 1963). Nos anos seguintes logrou-se a erradicação do *Ae. aegypti,* que foi posteriormente reintroduzido. Atualmente, este mosquito é encontrado em virtualmente todas as cidades do país, tendo sido responsável pela transmissão nas epidemias urbanas de Dengue desde a década de 1980, e de Chikungunya e Zika, nos últimos anos (Braga and Valle 2007; Ferreira-de-Brito et al. 2016). A possibilidade de que um humano que tenha contraído a FA através da picada de mosquitos silvestres se desloque para o ambiente urbano durante o período virêmico e sirva de fonte de infecção para *Ae. aegypti* competentes não deve ser descartada. A transmissão urbana, sobretudo nas grandes cidades infestadas por este vetor e com baixa cobertura vacinal, poderia gerar consequências dramáticas e ameaçar os estoques vacinais (Vasconcelos 2002; Possas et al. 2017). No presente surto não houve evidências entomológicas, epidemiológicas ou moleculares de que tenha havido transmissão urbana (Faria et al. 2018). Todos os *Ae. albopictus*, espécie considerada vetor ponte, com relevante competência vetorial e grande abundância e presença nos municípios amostrados, foram negativos em nossos ensaios para busca de infecções naturais pelo YFV, assim como os *Ae. aegypti* capturados no interior de imóveis onde houve suspeita de transmissão urbana e onde residiam idosos infectados no ciclo silvestre, como descrito no artigo 4.3.

Além disso, devido à ampla distribuição de PNHs do gênero *Callithrix* em diversas cidades e a resultados moleculares apontando YFV em alguns destes animais, especulou-se também sobre o papel de “hospedeiro ponte” destes animais. No entanto, nossos exames sorológicos e moleculares não apontaram evidências de FA entre os saguis capturados em três grandes cidades (Niterói, Rio de Janeiro e Belo Horizonte). Importantemente, os animais examinados pertenciam aos mesmos grupos e foram capturados nas mesmas ruas onde as epizootias suspeitas ocorreram. Apesar de não ter havido evidências de transmissão urbana até o momento, as condições citadas (alta densidade de vetores urbanos e de PNHs nas cidades, baixa cobertura

vacinal) exigem que seja mantida a vigilância entomológica e primatológica com coletas constantes em populações alvo, sobretudo nos grandes parques urbanos (ex: Floresta da Tijuca, Parque da Cantareira e Parque das Mangabeiras no Rio de Janeiro, em São Paulo e Belo Horizonte, respectivamente) para monitoramento da presença e eventual detecção precoce do YFV. Tudo isso, simultâneo à intensificação da vacinação humana, obviamente. Projetos de pesquisa sobre a vacinação de PNHs, especialmente daqueles em risco de extinção ou albergados em parque e criatórios urbanos e periurbanos estão em curso. A redução de vertebrados susceptíveis pode representar um importante bloqueio da transmissão, especialmente nestes ambientes.

Outros dois achados refletem a necessidade de intensificação da vigilância e da cobertura vacinal na Mata Atlântica: (a) a maior parte dos PNHs sobreviventes que examinamos após a detecção do YFV no RJ continuava susceptível ao vírus, tal como detectamos antes do surto; e (b) a demonstração de que o vírus é capaz de permanecer na mesma área por pelo menos três estações de transmissão consecutivas, sem a necessidade de reintroduções. Juntos, estes achados ajudam a lançar dúvidas sobre a ideia corrente de que o YFV avança como um incêndio, sem permanecer na mesma localidade por mais de duas estações de transmissão em consequência da redução de hospedeiros susceptíveis. Em virtude da escassez de estudos de dinâmica populacional de macacos, não se sabe o tamanho da redução populacional e influência desta redução na manutenção do vírus (Araújo et al. 2008; Bicca-marques et al. 2017). De qualquer forma, é notório que o vírus tenha desaparecido espontaneamente da Mata Atlântica há mais de 70 anos e que observou-se em 2019 uma drástica redução de notificações, não só dos casos humanos (influenciados pela vacinação), mas também das epizootias na estação de transmissão 2018-2019 (Brasil 2019a). Isso poderia indicar que, de fato, o YFV pode desaparecer novamente da região. Não se sabe, no entanto, como as mudanças culturais / ecológicas supracitadas podem interferir na persistência do vírus (Possas et al. 2018). O YFV e outros arbovírus, também podem ser transmitidos entre mosquitos, por via horizontal ou venérea, fenômeno que pode auxiliar na persistência do vírus em dados focos, com o ressurgimento epizoótico quando o número de PNHs suscetíveis aumentar. Apenas uma vigilância transversal com amostragem de PNHs e mosquitos responderá se há capacidade de perenização do vírus na Mata Atlântica.

Após a introdução do ZIKV nas Américas, e devido às semelhanças apresentadas entre este arbovírus e o YFV, cresceu a preocupação com a possibilidade de estabelecimento de um ciclo silvestre no país, tal qual realizado pelo

YFV no início do século passado, impedindo sua erradicação. Relatos da susceptibilidade de primatas neotropicais e de encontro de RNA compatível com ZIKV em PNHs em pelo menos duas regiões do país (nordeste e sudeste) pareciam reforçar essa possibilidade (Nehete et al. 2018; Terzian et al. 2018; Favoretto et al. 2019). No entanto, em toda a nossa amostragem de PNHs e em uma sub amostragem de mosquitos, que incluía aqueles coletados no período mais próximo à epidemia de ZIKV, não obtivemos evidências nem moleculares nem sorológicas da circulação silvestre de ZIKV, resultados semelhantes aos encontrados por outros autores, que examinaram 207 PNHs em três estados brasileiros (Moreira-Soto et al. 2018). É possível que a amostragem dos PNHs, feita no peridomicílio das residências com registro de casos humanos de ZIKV, relatados por Favoretto et al. (2018), tenha favorecido a transmissão direta entre humanos e PNHs (contaminação pela saliva ou outro fluido infectado) e, talvez, humano-*Aedes*-PNH, mas achamos pouco provável a transmissão PNH-*Aedes*-PNH e a PNH-mosquito silvestre-PNH. Pouco se sabe sobre a competência vetorial de mosquitos silvestres ao ZIKV, e os únicos mosquitos testados até o momento, de uma colônia de laboratório antiga de *Sabethes cyaneus* (Karna et al. 2018), apresentaram resultados inferiores aos já descritos para *Ae. aegypti.* Além disso, PNHs infectados experimentalmente foram assintomáticos e apresentaram curto período de viremia, o que poderia reduzir as chances de infecção de mosquitos silvestres. O monitoramento transversal de populações de PNHs, proposto anteriormente, e o estudo de competência vetorial de diversas espécies silvestres seriam de extrema importância para esclarecer estas questões.

O presente trabalho utilizou de abordagem multidisciplinar ao alinhar conhecimentos de entomologia, (etno)primatologia, malariologia, virologia, biologia molecular, imunologia, evolução, filogeografia e epidemiologia, e contou com a colaboração de profissionais de formações diversas, como biólogos, veterinários, médicos, enfermeiros, designers, geógrafos, gestores e técnicos para investigar dois surtos zoonóticos (malária e febre amarela) detectados na Mata Atlântica do sudeste brasileiro, com foco no estado do Rio de Janeiro. Tal abordagem está em consonância com a ideia de *One Health,* que parte de uma perspectiva colaborativa por reconhecer que o estado de saúde de humanos, de animais e a qualidade dos ecossistemas estão intimamente relacionados entre si e com demais aspectos sócio-culturais (Zinsstag 2012). A partir da interdisciplinaridade foi possível alargar a compreensão de diversos pontos relacionados aos dois surtos, como a determinação dos principais hospedeiros e reservatórios, sua distribuição geográfica, a caracterização molecular dos parasitos,

as rotas de dispersão, além de estimar fatores que influenciam o risco de spillover. O conjunto de resultados e *insights*, incluindo a padronização de algumas técnicas de campo e laboratório, podem apoiar ações dos gestores de saúde, como por exemplo o estabelecimento do banco de coágulos, o fortalecimento a vigilância e incentivo a adoção de medidas de proteção individuais e coletivas.

Como preconiza a perspectiva *One health,* a epidemiologia dos dois surtos está intimamente interligada, mesmo tratando-se de parasitas tão diversos (protozoário e vírus). Para finalizar esta discussão, exemplificaremos alguns pontos que ilustram bem a interdependência destas zoonoses, que pode ser extrapolado para outros agravos. A malária de bromélia zoonótica e a Febre amarela silvestre apresentam ciclo análogo à medida em que ambas são transmitidas entre PNHs e/ou humanos através da picada de mosquitos silvestres. Do ponto de vista dos PNHs, destaca-se a importância do gênero *Alouatta,* que é o principal hospedeiro dos plasmódios simianos infectantes para humanos, e o mais afetado pelo surto de febre amarela. Ainda não se sabe os efeitos de uma possível coinfecção nestes animais e nem o impacto que a alta mortalidade registrada durante epizootias de YFV teve no passado e terá nos próximos anos na circulação de ambos, os Plasmódios e o YFV. O crescente aumento de registro de malária autóctone vinculado à Mata Atlântica nas últimas décadas pode ser explicado pela baixa vigilância no período pós-erradicação da transmissão endêmica no RJ, mas também pela potencial redução, no passado, da população de bugios no estado devido à epizootias pelo YFV, reduzindo as fontes de infecção para os mosquitos durante décadas.

Entre os mosquitos, diversos fatores populacionais e genéticos alteram a capacidade e a competência vetorial. Apesar de pertencerem a diferentes subfamílias (Aedinae x Anophelinae) dentro da família Culicidae (Consoli and Oliveira 1994), os vetores destas zoonoses compartilham o comportamento primatofílico e acrodendrófilo (Deane 1992; Marcondes and Alencar 2010). A capacidade de realizar movimentos verticais na floresta (voando da copa para o chão e vice versa) é determinante para a transmissão zoonótica dos dois agravos, pois possibilita a transmissão entre os hospedeiros arborícolas e terrestres (Deane et al. 1984; Alencar et al. 2016; Tátila-Ferreira et al. 2017a). Além disso, ambos se criam em pequenas coleções de água da chuva acumulada em fitotelmatas, principalmente em ocos de árvores (*Haemagogus*) e bromélias (*Anopheles cruzii*). Portanto, o regime de chuvas e a umidade influenciam diretamente a presença de criadouros e, consequentemente, a densidade populacional destes mosquitos, afetando a capacidade vetorial. O pico

de transmissão de ambas ocorre, portanto, na mesma época, que coincide com a estação mais quente e chuvosa (Costa et al. 2011; Brasil et al. 2017).

Finalmente, o padrão comportamental humano também influencia diretamente a ocorrência zoonótica. Nas últimas décadas vem crescendo a noção da importância da natureza, o que motivou dois movimentos: o primeiro é chamado “back to nature”, citado anteriormente, que se define pela tendência cada vez maior de ampliar o contato com a natureza através do ecoturismo ou da busca por propriedades (casas, chácaras) próximos à ambientes bucólicos e florestais. Este movimento acaba aproximando o homem dos demais hospedeiros, ampliando as chances de transmissão destes e de outros parasitos (Ávila-Pires 2018; Possas et al. 2018). De fato, ao contrário de outros surtos de febre amarela silvestre e/ou de casos de malária, que geralmente acometiam apenas trabalhadores rurais, indígenas ou pessoas que frequentavam a floresta como forma de sobrevivência, muitos dos casos registrados no surto 2016-2019 acometeram pessoas que visitavam as áreas florestais para veraneio, lazer ou moravam na borda das cidades, que cresceram em direção à mata devido à especulação imobiliária; o segundo é a crescente percepção da importância de preservação da natureza. Nas últimas décadas, em que pese a falta de fiscalização e a ampliação da fronteira agrícola, cresceu o número de unidades de conservação (parques, reservas, RPPNs) nas três esferas de organização do governo (Ávila-Pires 2018; Possas et al. 2018). Também houve redução da cultura da caça e esforços para preservação de espécies ameaçadas de extinção, muitas delas são PNHs (ex: Mico leão dourado e Muriquis)(Ávila-Pires 2018). Tudo isso pode ter levado a um crescimento (densidade, abundância, riqueza) da fauna de hospedeiros destas duas zoonoses e conectado fragmentos de matas para formação de corredores ecológicos. É possível que alguns destes corredores tenham facilitado, também, a dispersão do vírus juntamente com os hospedeiros. A associação destes fatores a partir de uma visão holística, alinhados aos outros já discutidos, nos ajuda a pensar as maneiras de compreender os novos desafios epidemiológicos e sanitários que nos são impostos, com vistas a proteger a sociedade e consequentemente a natureza.

5 CONCLUSÕES

A construção de uma rede de informações formada por instituições e moradores chave é a estratégia mais eficiente de captura de *Alouatta guariba clamitans* na Mata Atlântica.

A captura de bugios com dardos anestésicos deve ser priorizada em fragmentos pequenos (até 100ha) e de domínio estacional semidecidual. O uso de armadilhas é inútil para a captura destes animais nesse bioma.

A partir do coágulo sanguíneo é possível detectar as duas espécies de *Plasmodium* simiano existentes no Brasil – *P. simium* e *P. brasilianum*, mesmo quando o hospedeiro tem baixa parasitemia, sejam humanos ou PNHs. Seu aproveitamento otimiza o uso de amostras sanguíneas, restando o soro para outras análises.

Alouatta g. clamitans foi a única espécie de vida livre encontrada naturalmente infectada por *Plasmodium* na região amostrada. Ela alberga tanto *P. simium* quanto *P. brasilianum* em frequências similares.

Todos os plasmódios encontrados em bugios (*Alouatta g. clamitans*) e em humanos com malária terçã benigna na Mata Atlântica fluminense compartilham as mesmas características moleculares, especialmente a presença do SNPs descrito como exclusivo de *P. simium* na região.

Alouatta g. clamitans é o reservatório animal e única fonte provável das infecções humanas de malária terçã benigna causada por *Plasmodium simium* no RJ, caracterizando-as como infecções zoonóticas.

O Rio de Janeiro e suas fronteiras estavam altamente receptivos à circulação do YFV antes do início do surto, em 2016, devido à presença, abundância e distribuição de mosquitos considerados vetores tradicionais da febre amarela.

Não foram encontrados mosquitos infectados antes dos primeiros sinais de reemergência do vírus amarílico no Sudeste.

Hg. leucocelaenus e *Hg. janthinomys* foram os vetores primários do surto de YFV devido às altas taxas de infecção, ampla distribuição geográfica e grande abundância observadas nos focos, somados ao comportamento primatofílico e acrodendrófilo já conhecido.

Outras três espécies de mosquitos encontradas infectadas com o YFV (*Sa. chloropterus*, *Ae. scapularis* e *Ae. taeniorhinchus*) tiveram importância secundária ou local, por apresentarem baixas taxas de infecção e distribuição geográfica restrita.

O vírus circulante no surto corrente (2016-2019) pertence ao genótipo Sul Americano I, subclado 1E, e apresenta assinatura molecular representada por substituições de aminoácidos cuja consequência funcional ainda é desconhecida.

Duas sub-linhagens distintas de YFV circularam no centro-oeste do Brasil entre 2015-2017, uma delas alcançou o Sudeste dando origem ao surto a partir de duas rotas de disseminação: uma pela bacia litorânea do atlântico sudeste por onde atingiu MG, ES e RJ; e a outra pela bacia do Rio Paraná, que atingiu MG e SP.

No RJ o vírus seguiu duas rotas de transmissão distintas, uma costeira e uma continental, separadas pela cadeia de montanhas da Serra do Mar.

O vírus amarílico é capaz de persistir numa mesma área da Mata Atlântica por pelo menos três estações de transmissão consecutivas, sem a necessidade de novas introduções.

Todos os PNHs capturados antes do surto de febre amarela silvestre estavam susceptíveis ao vírus, confirmando a receptividade da região e a maior parte dos que foram capturados após a circulação viral, continuava susceptível. *Alouatta* foi o PNH mais afetado pelo surto.

Não houve evidência de transmissão urbana do YFV, uma vez que não foram encontrados *Ae. aegypti* e *Ae. albopictus* infectados, nem de circulação silvestre de ZIKV ou outros arbovírus na região amostrada, já que todos os testes realizados em mosquito (PCR) e em PNHs (PCR + sorologia) foram negativos para este vírus.

6 PERSPECTIVAS

a) Fazer análise espacial interpolando as relações entre características ambientais, fauna de mosquitos e transmissão de febre amarela objetivando compreender padrões e modelos de predição de risco.

b) Aplicar as estratégias de coleta padronizadas para realizar monitoramento transversal de populações de PNHs em áreas chave e com baixa cobertura vacinal.

c) Adicionar a recomendação de armazenamento de coágulos ao protocolo de coleta de PNHs padronizado no guia de vigilância de epizootias editado pelo Ministério da Saúde.

d) Estabelecer colaboração para sequenciamento genômico do *P. simium* e *P. brasilianum / malariae* com vistas a resolver problemas taxonômicos e filogeográficos.

e) Realizar ensaios sorológicos nos PNHs para determinar o histórico de infecção por *Plasmodium*.

f) Determinar os vetores naturais da malária autóctone zoonótica no Rio de Janeiro.

g) Avaliar comparativamente a competência vetorial de populações do *An. cruzii* das vertentes costeira e continental da Serra do Mar aos plasmódios simianos, em especial o *P. simium.*

h) Investigar o potencial da malária autóctone zoonótica no Rio de Janeiro se transformar em endêmica, através da avaliação da competência vetorial de anofelinos vetores tradicionais da malária humana no Sudeste (e.g. *An. aquasalis*, *An. darlingi*, *An. albitarsis*) aos plasmódios simianos *P. simium* e *P. brasilianum*.

i) Avaliar a potencial influência das mutações encontradas genoma do YFV circulante na epidemia em curso no Sudeste e Sul do Brasil na competência vetorial de mosquitos silvestres e domiciliares.

j) Esclarecer a relação taxonômica entre *Hg. janthinomys* e *Hg. capricornii* e o envolvimento na transmissão da febre amarela.

k) Investigar a presença e diversidade de outros parasitos (ex: *Trypanossoma*, helmintos, outros vírus) através de técnicas de parasitologia clássica e molecular.

l) Analisar a diversidade genética e evolução de subpopulações virais intra-hospedeiro e dentre diversos hospedeiros, vertebrado e invertebrado, através de deep sequencing dos YFV circulantes no corrente surto.

m) Colaborar com o estudo da taxonomia citogenética e molecular de *A. g. clamitans.*

n) Estender a linha de pesquisa à região Norte de Minas Gerais e vale do Jequitinhonha, uma região historicamente sub estudada.

7 REFERÊNCIAS BIBLIOGRÁFICAS

Aguiar LM, Ludwig G, Svoboda WK, Teixeira GM, Hilst CLS, Shiozawa MM, et al. Use of traps to capture black and gold howlers (Alouatta caraya) on the islands of the upper Paraná River, Southern Brazil. Am J Primatol. 2007;69(2):241–7.

de Alencar FEC, Malafronte RDS, Cerutti C, Natal Fernandes L, Buery JC, Fux B, et al. Reassessment of asymptomatic carriers of Plasmodium spp. in an endemic area with a very low incidence of malaria in extra-Amazonian Brazil. Malar J [Internet]. 2017;16(1):452. Available from: https://malariajournal.biomedcentral.com/articles/10.1186/s12936-017-2103-6

Alencar J, Almeida HM de, Marcondes CB, Guimarães AÉ. Effect Of Multiple Immersions On Eggs And Development Of Immature Forms Of Haemagogus janthinomys From South-Eastern Brazil (Diptera: Culicidae). Entomol News [Internet]. 2008 [cited 2019 Feb 9];119(3):239–45. Available from: https://bioone.org/journals/Entomological-News/volume-119/issue-3/0013-872X(2008)119[239:EOMIOE]2.0.CO;2/Effect-Of-Multiple-Immersions-On-Eggs-And-Development-Of-Immature/10.3157/0013-872X(2008)119[239:EOMIOE]2.0.CO;2.full

Alencar J, de Mello CF, Gil-Santana HR, Guimarï¿½es A ï¿½rico, de Almeida SAS, Gleiser RM. Vertical oviposition activity of mosquitoes in the Atlantic Forest of Brazil with emphasis on the sylvan vector, Haemagogus leucocelaenus (Diptera: Culicidae). J Vector Ecol. 2016;41(1):18–26.

Almeida M., Dos Santos E, Da Cruz Cardoso J, Da Fonseca DF, Noll CA, Silveira VR, et al. Yellow fever outbreak affecting Alouatta populations in southern Brazil (Rio Grande do Sul State), 2008-2009. Am J Primatol. 2012;74(1):68–76.

de Almeida MAB, dos Santos E, Cardoso J da C, da Silva LG, Rabelo RM, Bicca-Marques JC. Predicting Yellow Fever Through Species Distribution Modeling of Virus, Vector, and Monkeys. Ecohealth [Internet]. 2018 [cited 2019 Feb 9];1–14. Available from: http://link.springer.com/10.1007/s10393-018-1388-4

Althouse BM, Guerbois M, Cummings DAT, Diop OM, Faye O, Faye A, et al. Role of monkeys in the sylvatic cycle of chikungunya virus in Senegal. Nat Commun [Internet]. 2018 [cited 2019 Feb 18];9(1):1046. Available from: http://www.nature.com/articles/s41467-018-03332-7

Althouse BM, Vasilakis N, Sall AA, Diallo M, Weaver SC, Hanley KA. Potential for Zika Virus to Establish a Sylvatic Transmission Cycle in the Americas. PLoS Negl Trop

Dis [Internet]. 2016 [cited 2019 Mar 8];10(12):e0005055. Available from: https://dx.plos.org/10.1371/journal.pntd.0005055

Alvarenga DAM, de Pina-Costa A, de Sousa T, Pissinatti A, Zalis MG, Suaréz-Mutis MC, et al. Simian malaria in the Brazilian Atlantic forest: first description of natural infection of capuchin monkeys (Cebinae subfamily) by Plasmodium simium. Malar J. 2015;14(1):81.

Alvarenga DAM, Culleton R, de Pina-Costa A, Rodrigues DF, Bianco C, Silva S, et al. An assay for the identification of Plasmodium simium infection for diagnosis of zoonotic malaria in the Brazilian Atlantic Forest. Sci Rep [Internet]. 2018 [cited 2019 Mar 8] 8;8(1):86. Available from: http://www.nature.com/articles/s41598-017-18216-x

Alvarenga DAM, Pina-Costa A, Bianco C, Moreira SB, Brasil P, Pissinatti A, et al. New potential Plasmodium brasilianum hosts: tamarin and marmoset monkeys (family Callitrichidae). Malar J [Internet]. 2017 [cited 2019 Mar 8]; 10;16(1):71. Available from: http://malariajournal.biomedcentral.com/articles/10.1186/s12936-017-1724-0

Anderson CR, Wattley GH, Ahin NW, Downs WG, Reese AA. Mayaro Virus: A New Human Disease Agent. Am J Trop Med Hyg [Internet]. 1957 [cited 2019 Apr 23]; 6(6):1012–6. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1957.6.1012

Araújo FAA, Ramos DG, Santos AL, Passos PH de O, Elkhoury ANSM, Costa ZGA, et al. Epizootias em primatas não humanos durante reemergência do vírus da febre amarela no Brasil, 2007 a 2009. Epidemiol e Serviços Saúde [Internet].2011 [cited 2019 Mar 8]; 20(4):527–36. Available from: http://scielo.iec.pa.gov.br/scielo.php?script=sci_arttext&pid=S1679-49742011000400012&lng=en&nrm=iso&tlng=en

Araújo RM, Souza MB de, Ruiz-Miranda CR. Densidade e tamanho populacional de mamíferos cinegéticos em duas Unidades de Conservação do Estado do Rio de Janeiro, Brasil. Iheringia Série Zool. 2008;98(3):391–6.

de Araújo TVB, Rodrigues LC, de Alencar Ximenes RA, de Barros Miranda-Filho D, Montarroyos UR, de Melo APL, et al. Association between Zika virus infection and microcephaly in Brazil, January to May, 2016: preliminary report of a case-control study. Lancet Infect Dis [Internet]. 2016 [cited 2019 Apr 2];16(12):1356–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27641777

de Arruda M, Nardin EH, Nussenzweig RS, Cochrane AH. Sero-epidemiological

studies of malaria in Indian tribes and monkeys of the Amazon Basin of Brazil. Am J Trop Med Hyg [Internet]. 1989 [cited 2019 Apr 26];41(4):379–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2508499

Arruda ME de. Presença do Plasmodium brasilianum em macacos capturados na área de enchimento do reservatório da usina hidrelétrica de Tucuruí, Pará. Mem Inst Oswaldo Cruz. 2010;80(3):367–9.

de Assis GMP, de Alvarenga DAM, Costa DC, de Souza Junior JC, Hirano ZMB, Kano FS, et al. Detection of Plasmodium in faeces of the new world primate Alouatta clamitans. Mem Inst Oswaldo Cruz. 2016;111(9):570–6.

Ávila-Pires FD de. Ecologia da peste bubônica. Rev Soc Bras Med Trop [Internet]. 1976 [cited 2019 Apr 23]; 10(3):161–8. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037-86821976000300008&lng=pt&tlng=pt

Ávila-Pires FD De. Doenças humanas e doenças de outros animais: origem do conceito de zoonose. Rev Ecol Humanas [Internet]. 2015; [cited 2019 Mar 8]; 1(1):41–8. Available from: http://sabeh.org.br/?mbdb_book=revista-ecologias-humanas-no-1

Ávila-Pires FD De. The true nature of zoonoses: the identity of transmissible diseases in man and in other vertebrates. Rev Patol Trop / J Trop Pathol. 2017;46(3):215–20.

Ávila-Pires FD De. The "spread" of jungle Yellow fever. Rev Patol Trop / J Trop Pathol [Internet]. 2018 [cited 2018 Aug 11]; 47(1):1. Available from: https://www.revistas.ufg.br/iptsp/article/view/52274

Azevedo RSS, Silva EVP, Carvalho VL, Rodrigues SG, Nunes-Neto JP, Monteiro H, et al. Mayaro fever virus, Brazilian Amazon. Emerg Infect Dis [Internet]. 2009 Nov [cited 2019 Apr 23]; 15(11):1830–2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19891877

Bank S, Nexø BA, Andersen V, Vogel U, Andersen PS. High-Quality and -Quantity DNA Extraction from Frozen Archival Blood Clots for Genotyping of Single-Nucleotide Polymorphisms. Genet Test Mol Biomarkers [Internet]. 2013 [cited 2018 Jul 27]; 17(6):501–3. Available from: http://online.liebertpub.com/doi/abs/10.1089/gtmb.2012.0429

Barrett ADT. Epidemiology and ecology of yellow fever virus. Adv Virus Res [Internet]. 2003 Jan [cited 2018 Jul 27]; 61:291–315. Available from: https://www.sciencedirect.com/science/article/pii/S0065352703610079?via%3Di

hub

Bicca-marques JC, Calegaro-marques C, Rylands AB, Karen B, Mittermeier RA, Almeida MAB De, et al. Yellow fever threatens Atlantic Forest primates. Vol. 3, Science Advances. 2017.

Blitvich BJ. Transmission dynamics and changing epidemiology of West Nile virus. Anim Heal Res Rev [Internet]. 2008 [cited 2019 Apr 21]; 9(01):71–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18348742

Bonaldo MC, Gómez MM, dos Santos AA, Abreu FVS de, Ferreira-de-Brito A, Miranda RM de, et al. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. Mem Inst Oswaldo Cruz [Internet]. 2017 Jun; [cited 2019 Mar 5]; 112(6):447–51. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762017000600447&lng=en&tlng=en

Braga IA, Valle D. Aedes aegypti: histórico do controle no Brasil. Epidemiol e Serviços Saúde [Internet]. 2007 [cited 2019 May 26]; 16(2):113–8. Available from: http://scielo.iec.gov.br/scielo.php?lng=en&pid=S1679-49742007000200006&script=sci_abstract

Brasil. Aspectos epidemiológicos da febre amarela silvestre e a vigilância intensificada durante período de monitoramento, Brasil, 2012/2013 [Internet]. [cited 2019 Mar 8]; 45:1–10 ,. Available from: https://portalarquivos2.saude.gov.br/images/pdf/2014/maio/27/BE-V45-n---07-FebreAmarela.pdf

Brasil. Guia de vigilância de epizootias em primatas não humanos e entomologia aplicada à vigilância da febre amarela [Internet]. 2nd ed. Brasília: Ministério da Saúde; 2017a. Available from: http://portalarquivos.saude.gov.br/images/pdf/2017/marco/24/Guia_Epizootias_Febre_Amarela_2a_ed_atualizada_2017.pdf

Brasil. Monitoramento dos casos e óbitos de Febre Amarela no Brasil - Informe – Nº 43/2017. [Internet]. 2017b. [cited 2019 Mar 5]; 43:1–7 Available from: http://portalarquivos.saude.gov.br/images/pdf/2017/junho/02/COES-FEBRE-AMARELA---INFORME-43---Atualiza----o-em-31maio2017.pdf

Brasil. Monitoramento do Período Sazonal da Febre Amarela [Internet]. 2018. [cited 2019 Mar 5]; 14:1–15. Available from: http://portalarquivos2.saude.gov.br/images/pdf/2018/fevereiro/21/Informe-n14-FA-20fev18-c.pdf

Brasil. Monitoramento de Febre Amarela Brasil 2019. [Internet] 2019a [cited 2019 May 20]; 16:1–8. Available from: https://portalarquivos2.saude.gov.br/images/pdf/2019/maio/17/Informe-FA-n16.pdf

Brasil. Monitoramento do período sazonal da Febre Amarela Brasil – 2018 / 2019 [Internet]. 2019b [cited 2019 Mar 5]; 3:1–8 Available from: http://portalarquivos2.saude.gov.br/images/pdf/2019/janeiro/28/informe-FA-n.3-21jan19.pdf

Brasil. SIVEP MALÁRIA - Sistema de Informação de Vigilância epidemiológica - Notificação de Casos [Internet]. 2019c [cited 2019 Apr 24]. Available from: http://dw.saude.gov.br/gsid/servlet/mstrWeb;jsessionid=0E86D6F3C99241B5E32DC1105C685F8F?evt=2048001&hiddensections=header%2Cpath%2CdockTop%2CdockLeft%2Cfooter&documentID=90C3B2244FFA8421F335769827F2A96E&Server=srvbipdf03&Project=DMMalaria&

Brasil DP, de Carvalho FG, de Almeida CR, de Almeida AM. Natural infection by Yersinia pestis, in fleas from plague foci in the northeast of Brazil. Rev Soc Bras Med Trop [Internet]. 1989 [cited 2019 Apr 22]; 22(4):177–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2487774

Brasil P, Zalis MG, de Pina-Costa A, Siqueira AM, Júnior CB, Silva S, et al. Outbreak of human malaria caused by Plasmodium simium in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. Lancet Glob Heal [Internet]. 2017 Oct [cited 2018 Jul 27]; 5(10):e1038–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28867401

Bryant JE, Holmes EC, Barrett ADT. Out of Africa: A Molecular Perspective on the Introduction of Yellow Fever Virus into the Americas. PLoS Pathog [Internet]. 2007 [cited 2019 Apr 2]; 3(5):e75. Available from: https://dx.plos.org/10.1371/journal.ppat.0030075

Buery JC, Rodrigues PT, Natal L, Salla LC, Loss AC, Vicente CR, et al. Mitochondrial genome of Plasmodium vivax/simium detected in an endemic region for malaria in the Atlantic Forest of Espírito Santo state, Brazil: do mosquitoes, simians and humans harbour the same parasite? Malar J [Internet]. 2017 [cited 2018 Jul 27]; 16(1):437. Available from: http://malariajournal.biomedcentral.com/articles/10.1186/s12936-017-2080-9

Bugher JC, Boshell-Manrique J, Roca-García M, Osorno-Mesa E. Epidemiology of Jungle Yellow Fever in Eastern Colombia1. Am J Epidemiol [Internet]. 1944 [cited

2019 apr 27] 39(1):16–51. Available from: https://academic.oup.com/aje/article-lookup/doi/10.1093/oxfordjournals.aje.a118895

Bunning ML, Bowen RA, Cropp BC, Sullivan KG, Davis BS, Komar N, et al. Experimental Infection of Horses with West Nile virus. Emerg Infect Dis [Internet]. 2002 [cited 2019 Apr 21]; 8(4):380–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11971771

Burgos-Rodriguez AG. Zoonotic Diseases of Primates. Vet Clin North Am Exot Anim Pract [Internet]. 2011 [cited 2019 Apr 18]; 14(3):557–75. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1094919411000466

Campbell-Lendrum D, Dujardin J-P, Martinez E, Feliciangeli MD, Perez JE, Silans LNMP de, et al. Domestic and peridomestic transmission of American cutaneous leishmaniasis: changing epidemiological patterns present new control opportunities. Mem Inst Oswaldo Cruz [Internet]. 2001 [cited 2019 Apr 23]; 96(2):159–62. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762001000200004&lng=en&tlng=en

Cardoso J da C, de Almeida MAB, dos Santos E, da Fonseca DF, Sallum MAM, Noll CA, et al. Yellow fever virus in Haemagogus leucocelaenus and Aedes serratus mosquitoes, Southern Brazil, 2008. Emerg Infect Dis. 2010;16(12):1918–24.

de Castro Duarte AMR, Malafronte R dos S, Cerutti C, Curado I, de Paiva BR, Maeda AY, et al. Natural Plasmodium infections in Brazilian wild monkeys: Reservoirs for human infections? Acta Trop [Internet]. 2008 [cited 2019 Apr 24]; 107(2):179–85. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0001706X08001654#aep-article-footnote-id6

Causey OR, Hughes TP, Laemmert HW. The Invasion of Small Forests by Yellow Fever Virus as Indicated by Immunity in Cebus Monkeys 1. Am J Trop Med Hyg [Internet]. 1949 [cited 2018 Aug 26]; s1-29(4):555–65. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1949.s1-29.555

Causey OR, Laemmert HW, Kumm HW. Dispersion of forest mosquitoes in Brazil: Further studies 1. Am J Trop Med Hyg [Internet]. 1950 Mar [cited 2018 Sep 23]; s1-30(2):301–12. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1950.s1-30.301

Causey OR, Maroja OM. Mayaro Virus: A New Human Disease Agent. Am J Trop Med Hyg [Internet]. 1957 [cited 2019 Apr 23]; 6(6):1017–23. Available from:

http://www.ajtmh.org/content/journals/10.4269/ajtmh.1957.6.1017

CDC. International Task Force for Disease Eradication [Internet]. 1990 [cited 2019 Apr 23]. p.209–12. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/00001590.htm

Cerutti C, Boulos M, Coutinho AF, Hatab M do CL, Falqueto A, Rezende HR, et al. Epidemiologic aspects of the malaria transmission cycle in an area of very low incidence in Brazil. Malar J [Internet]. 2007 [cited 2018 Jul 27]; 6(1):33. Available from: http://malariajournal.biomedcentral.com/articles/10.1186/1475-2875-6-33

Chaudhury A, Venkataramana B. Plasmodium knowlesi : the fifth malaria parasite. Clin Sci Res [Internet]. 2017 [cited 2019 May 19]; 6:171-8. Available from: http://svimstpt.ap.nic.in/jcsr/jul-sep17_files/ra.17.04.003.pdf

Chevillon C, Briant L, Renaud F, Devaux C. The Chikungunya threat: an ecological and evolutionary perspective. Trends Microbiol [Internet]. 2008 [cited 2019 Apr 18]; 16(2):80–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18191569

Chiarello AG. Primates of the Brazilian Atlantic Forest: The Influence of Forest Fragmentation on Survival. In: Primates in Fragments [Internet]. Boston, MA: Springer US; 2003 [cited 2019 May 19]. p. 99–121. Available from: http://link.springer.com/10.1007/978-1-4757-3770-7_8

Clarke MR, Zucker EL, Scott NJ. Population Trends of the Mantled Howler Groups of La-Pacifica, Guanacaste, Costa-Rica. Am J Primatol. 1986;11(1):79–88.

Clements AN, Harbach RE. History of the discovery of the mode of transmission of yellow fever virus. J Vector Ecol [Internet]. 2017 [cited 2018 Sep 11]; 42(2):208–22. Available from: http://doi.wiley.com/10.1111/jvec.12261

Coatney GR. The Simian Malaria: Zoonoses, Anthroponoses, or Both? Am J Trop Med Hyg. 1971;20(6):795–803.

Consoli RAGB, Oliveira RL de. Principais mosquitos de importância sanitária no Brasil [Internet]. Editora FIOCRUZ; 1994 [cited 2018 Sep 12]. Available from: https://books.google.com.br/books/about/Principais_mosquitos_de_importância_san.html?id=c7YXBAAAQBAJ&redir_esc=y

Costa PL, Dantas-Torres F, da Silva FJ, Guimarães VCFV, Gaudêncio K, Brandão-Filho SP. Ecology of Lutzomyia longipalpis in an area of visceral leishmaniasis transmission in north-eastern Brazil. Acta Trop [Internet]. 2013 [cited 2019 Apr 23]; 126(2):99–102. Available from: https://www.sciencedirect.com/science/article/pii/S0001706X13000223

Costa ZGA, Romano APM, Elkhoury ANM, Flannery B. Evolução histórica da vigilância

epidemiológica e do controle da febre amarela no Brasil. Rev Pan-Amazônica Saúde [Internet]. 2011 [cited 2019 May 13]; 2(1):11–26. Available from: http://scielo.iec.pa.gov.br/scielo.php?script=sci_arttext&pid=S2176-62232011000100002&lng=en&nrm=iso&tlng=en

Coutinho JO, Rachou R. Data on the biology and the malaria vector capacity of anophelines of sub-group Kerteszia under natural conditions. Rev Bras Malariol Doencas Trop [Internet]. 1966 [cited 2019 May 13];18(3):557–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5998435

Couto-Lima D, Madec Y, Bersot MI, Campos SS, Motta M de A, Santos FB dos, et al. Potential risk of re-emergence of urban transmission of Yellow Fever virus in Brazil facilitated by competent Aedes populations. Sci Rep [Internet]. 2017 [cited 2018 Sep 24]; 7(1):4848. Available from: http://www.nature.com/articles/s41598-017-05186-3

Cox-Singh J, Singh B. Knowlesi malaria: newly emergent and of public health importance? Trends Parasitol [Internet]. 2008 [cited 2019 Apr 24]; 24(9):406–10. Available from: https://www.sciencedirect.com/science/article/pii/S1471492208001797

Cox FE. History of the discovery of the malaria parasites and their vectors. Parasit Vectors [Internet]. 2010 [cited 2019 May 26]; 3(1):5. Available from: http://parasitesandvectors.biomedcentral.com/articles/10.1186/1756-3305-3-5

Cunningham AA, Daszak P, Wood JLN. One Health, emerging infectious diseases and wildlife: two decades of progress? Philos Trans R Soc B Biol Sci [Internet]. 2017 [cited 2019 Apr 9]; 372(1725):20160167. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28584175

Curado I, Duarte AMR, Lal AA, Oliveira SG, Kloetzel JK. Antibodies anti Bloodstream and Circumsporozoite Antigens (Plasmodium vivax and Plasmodium malariae/P. brasilianum) in Areas of Very Low Malaria Endemicity in Brazil. Mem Inst Oswaldo Cruz [Internet]. 1997 [cited 2019 Apr 24]; 92(2):235–43. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02761997000200017&lng=en&tlng=en

Curado I, dos Santos Malafronte R, de Castro Duarte AMR, Kirchgatter K, Branquinho MS, Bianchi Galati EA. Malaria epidemiology in low-endemicity areas of the Atlantic Forest in the Vale do Ribeira, São Paulo, Brazil. Acta Trop [Internet]. 2006 [cited 2019 Mar 7]; 100(1–2):54–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17126279

Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: A One Health perspective. Trends Parasitol [Internet]. 2012 [cited 2019 May 13]; 28(10):437–46. Available from: http://dx.doi.org/10.1016/j.pt.2012.07.003

Davies TJ, Pedersen AB. Phylogeny and geography predict pathogen community similarity in wild primates and humans. Proc R Soc B Biol Sci [Internet]. 2008 [cited 2019 Apr 18]; 275(1643):1695–701. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18445561

Davis NC. Susceptibility of Capuchin (Cebus) monkeys to yellow fever virus. Am J Epidemiol [Internet]. 1930a [cited 2018 Nov 2]; 11(2):321–34. Available from: https://academic.oup.com/aje/article-lookup/doi/10.1093/oxfordjournals.aje.a113835

Davis NC. The transmission of Yellow fever: experiments with the Woolly monkey (Lagothrix lago-tricha humboldt), the spider monkey (Ateles ater F. Cuvier), and the Squirrel monkey (Saimiri scireus Linnaeus). J Exp Med [Internet]. 1930b [cited 2018 Nov 2]; 51(5):703–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19869721

Davis NC, Shannon RC. Studies on Yellow fever in South America : V. Transmission experiments with certain species of Culex and Aedes. J Exp Med [Internet]. 1929 [cited 2018 Aug 11]; 50(6):803–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19869666

Davis NC, Shannon RC. The location of Yellow Fever virus in infected mosquitoes and the possibility of hereditary transmission. Am J Epidemiol [Internet]. 1930 [cited 2018 Aug 11]; 11(2):335–44. Available from: https://academic.oup.com/aje/article-lookup/doi/10.1093/oxfordjournals.aje.a113836

Deane LM. Malaria vectors in Brazil. Mem Inst Oswaldo Cruz. 1986;81(suppl II):5–14.

Deane LM. Simian malaria in Brazil. Mem Inst Oswaldo Cruz [Internet]. 1992 [cited 2019 Jan 13]; 87(suppl 3):1–20. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02761992000700001&lng=en&tlng=en

Deane LM, Deane MP, Ferreira Neto J. Studies on transmission of simian malaria and on a natural infection of man with Plasmodium simium in Brazil. Bull World Health Organ [Internet]. 1966 [cited 2018 Jul 27]; 35(5):805–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5297817

Deane LM, Deane MP, Ferreira Neto JA, Barbosa de Almeida F. On the transmission of simian malaria in Brazil. Rev Inst Med Trop Sao Paulo [Internet]. 1971 [cited

2019 May 16]; 13(5):311–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5162258

Deane LM, Ferreira Neto JA, Lima MM. The vertical dispersion of Anopheles (Kerteszia) cruzi in a forest in southern Brazil suggests that human cases of malaria of simian origin might be expected. Mem Inst Oswaldo Cruz [Internet]. 1984 [cited 2018 Sep 26]; 79(4):461–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6533421

Dégallier N, Rosa APAT da, Hervé J-P, Rosa JEST da, Vasconcelos PFC, Silva CJM da, et al. A comparative study of yellow fever in Africa and South America. Cienc Cult [Internet]. 1992 [cited 2019 Mar 13]; 44(2–3):143–51. Available from: https://www.documentation.ird.fr/hor/fdi:38276

Dégallier N, Travassos da Rosa APA, Vasconcelos PFC, Travassos da Rosa ES, Rodrigues SG, Sa FGC, et al. New entomological and virological data on the vectors of sylvatic yellow fever in Brazil. Arthropod-Borne Virus Inf Exch [Internet]. 1993 [cited 2018 Sep 11]; 21–2. Available from: http://www.documentation.ird.fr/hor/fdi:010050683

Diallo D, Sall AA, Diagne CT, Faye O, Faye O, Ba Y, et al. Zika Virus Emergence in Mosquitoes in Southeastern Senegal, 2011. Attoui H, editor. PLoS One [Internet]. 2014 [cited 2019 Apr 24]; 9(10):e109442. Available from: http://dx.plos.org/10.1371/journal.pone.0109442

Diallo M, Ba Y, Sall AA, Diop OM, Ndione JA, Mondo M, et al. Amplification of the sylvatic cycle of dengue virus type 2, Senegal, 1999-2000: entomologic findings and epidemiologic considerations. Emerg Infect Dis [Internet]. 2003 [cited 2019 Apr 19]; 9(3):362–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12643833

Diallo M, Thonnon J, Traore-Lamizana M, Fontenille D. Vectors of Chikungunya virus in Senegal: current data and transmission cycles. Am J Trop Med Hyg [Internet]. 1999 [cited 2019 Apr 18]; 60(2):281–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10072152

Dick GW., Kitchen S., Haddow A. Zika Virus (I). Isolations and serological specificity. Trans R Soc Trop Med Hyg [Internet]. 1952 [cited 2019 Apr 24]; 46(5):509–20. Available from: https://academic.oup.com/trstmh/article-lookup/doi/10.1016/0035-9203(52)90042-4

Duarte AMRC, Pereira DM, De Paula MB, Fernandes A, Urbinatti PR, Ribeiro AF, et al. Natural infection in anopheline species and its implications for autochthonous

malaria in the Atlantic forest in Brazil. Parasites and Vectors. 2013;6(1):1–6.

Esposito DLA, Fonseca BAL da. Will Mayaro virus be responsible for the next outbreak of an arthropod-borne virus in Brazil? Brazilian J Infect Dis [Internet]. 2017 [cited 2019 May 13]; 21(5):540–4. Available from: http://dx.doi.org/10.1016/j.bjid.2017.06.002

Fall G, Di Paola N, Faye M, Dia M, Freire CC de M, Loucoubar C, et al. Biological and phylogenetic characteristics of West African lineages of West Nile virus. Beasley DWC, editor. PLoS Negl Trop Dis [Internet]. 2017 [cited 2019 Apr 21]; 11(11):e0006078. Available from: https://dx.plos.org/10.1371/journal.pntd.0006078

Faria NR, Kraemer MUG, Hill SC, Jesus JG de, Aguiar RS, Iani FCM, et al. Genomic and epidemiological monitoring of yellow fever virus transmission potential. Science [Internet]. 2018 [cited 2019 Feb 9]; 361(6405):894–9. Available from: http://science.sciencemag.org/content/361/6405/894.long

Favoretto SR, Araujo DB, Duarte NFH, Oliveira DBL, da Crus NG, Mesquita F, et al. Zika Virus in Peridomestic Neotropical Primates, Northeast Brazil. Ecohealth [Internet]. 2019 [cited 2019 Mar 8]; 1–9. Available from: http://link.springer.com/10.1007/s10393-019-01394-7

Fernandes NCC de A, Cunha MS, Guerra JM, Réssio RA, Cirqueira CDS, Iglezias SD, et al. Outbreak of Yellow Fever among Nonhuman Primates, Espirito Santo, Brazil, 2017. Emerg Infect Dis [Internet]. 2017 [cited 2019 May 7]; 23(12):2038–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29148378

Ferreira-de-Brito A, Ribeiro IP, Miranda RM de, Fernandes RS, Campos SS, Silva KAB da, et al. First detection of natural infection of Aedes aegypti with Zika virus in Brazil and throughout South America. Mem Inst Oswaldo Cruz [Internet]. 2016 [cited 2019 Apr 2]; 111(10):655–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27706382

Figueiredo LTM. Emergent arboviruses in Brazil. Rev Soc Bras Med Trop [Internet]. 2007 [cited 2019 Apr 23]; 40(2):224–9. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037-86822007000200016&lng=en&tlng=en

Figueiredo MAP, Di Santi SMF, Figueiredo TAP, Machado RZ. Natural Plasmodium infection in neotropical primates in the island of São Luís, state of Maranhão, Brazil. Rev Bras Parasitol Veterinária [Internet]. 2015; [cited 2019 Mar 13] 24(2):122–8. Available from:

http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1984-29612015000200122&lng=en&tlng=en

Finlay C. Yellow Fever: Its transmission by means of the Culex mosquito. Am J Med Sci [Internet]. 1886 [cited 2018 Sep 11]; 92:395–409. Available from: https://search.proquest.com/openview/a59a05fc10d5b9dd9205eca41220d61d/1?pq-origsite=gscholar&cbl=41361

Fioravanti C. O alerta dos macacos. Pesquisa FAPESP [Internet]. 2018 [cited 2019 Fev 13]; 19–23. Available from: http://revistapesquisa.fapesp.br/wp-content/uploads/2018/01/018-023_febre-amarela_263_novo.pdf

Fonseca F. Plasmódio de primata do Brasil. Mem Inst Oswaldo Cruz. 1951;49: 543–53.

Forrester NL, Coffey LL, Weaver SC. Arboviral bottlenecks and challenges to maintaining diversity and fitness during mosquito transmission. Viruses. 2014;6(10):3991–4004.

Franco O. História da Febre Amarela no Brasil. Rio de Janeiro: Ministério da Saúde. 1969, 210pp.

Fuentes-Ramírez A, Jiménez-Soto M, Castro R, Romero-Zuñiga JJ, Dolz G. Molecular Detection of Plasmodium malariae/Plasmodium brasilianum in Non-Human Primates in Captivity in Costa Rica. Culleton R, editor. PLoS One [Internet]. 2017 [cited 2018 Jul 27]; 12(1):e0170704. Available from: http://dx.plos.org/10.1371/journal.pone.0170704

Garnham PCC. Malaria Parasites and other Haemosporidia. 1st ed. Oxford: Blackwell Scientific Publications Ltd, 1966. 1132 pp.

Garrett-Jones C. The human blood index of malaria vectors in relation to epidemiological assessment. Bull World Health Organ [Internet]. 1964 [cited 2019 May 16]; 30(2):241–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14153413

Goenaga S, Fabbri C, Dueñas JCR, Gardenal CN, Rossi GC, Calderon G, et al. Isolation of Yellow Fever Virus from Mosquitoes in Misiones Province, Argentina. Vector-Borne Zoonotic Dis [Internet]. 2012 [cited 2019 Apr 18]; 12(11):986–93. Available from: http://online.liebertpub.com/doi/abs/10.1089/vbz.2011.0730

Gómez MM, de Abreu FVS, Dos Santos AAC, de Mello IS, Santos MP, Ribeiro IP, et al. Genomic and structural features of the yellow fever virus from the 2016-2017 Brazilian outbreak. J Gen Virol. 2018;99(4):536–48.

Goodman M, Porter CA, Czelusniak J, Page SL, Schneider H, Shoshani J, et al.

Toward a Phylogenetic Classification of Primates Based on DNA Evidence Complemented by Fossil Evidence. Mol Phylogenet Evol [Internet]. 1998 [cited 2019 Apr 18]; 9(3):585–98. Available from: https://www.sciencedirect.com/science/article/pii/S105579039890495X

Gould E, Pettersson J, Higgs S, Charrel R, de Lamballerie X. Emerging arboviruses: Why today? One Heal. 2017;4(July):1–13.

Grassi B, Bignami A, Bastianelli G. Ulteriori ricerche sul ciclo dei parassiti malarici umani nel corpo del zanzarone. Tip della R Accad dei Lincei [Internet]. 1898 [cited 2019 May 26]; Available from: https://scholar.google.com.br/scholar?hl=pt-BR&as_sdt=0%2C5&q=Ulteriore+ricerche+sul+ciclo+dei+parassiti+malarici+umani+sul+corpo+del+zanzarone&btnG=

Gratz N. Plague Manual: Rodent reservoirs & flea vectors of natural foci of plague. Plague Man Epidemiol Distrib Surveill Control. 1999;6:63–96.

Griffing SM, Tauil PL, Udhayakumar V, Silva-Flannery L, Griffing SM, Tauil PL, et al. A historical perspective on malaria control in Brazil. Mem Inst Oswaldo Cruz [Internet]. 2015 [cited 2019 May 26]; 110(6):701–18. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762015000600701&lng=en&tlng=en

Gubler D. Vector-borne diseases. Rev Sci Tech - Off Int des Épizooties [Internet]. 2009 [cited 2019 Apr 22]; 28(2):583–8. Available from: https://www.cabdirect.org/cabdirect/abstract/20103045683

Le Guenno B, Formenty P, Formentry P, Wyers M, Gounon P, Walker F, et al. Isolation and partial characterisation of a new strain of Ebola virus. Lancet [Internet]. 1995 [cited 2019 Apr 18]; 345(8960):1271–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7746057

Guimarães LO, Bajay MM, Wunderlich G, Bueno MG, Röhe F, Catão-Dias JL, et al. The genetic diversity of Plasmodium malariae and Plasmodium brasilianum from human, simian and mosquito hosts in Brazil. Acta Trop [Internet]. 2012 [cited 2019 Apr 18]; 124(1):27–32. Available from: http://dx.doi.org/10.1016/j.actatropica.2012.05.016

Haddow A, Williams M, Woodall J, Simpson D, Goma L. Twelve isolations of Zika Virus from Aedes (Stegomyia) Africanus (Theobald) taken in and above a Uganda forest. Bull World Health Organ [Internet]. 1964 [cited 2019 Apr 24]; 31(1):57–69. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14230895

Hamrick PN, Aldighieri S, Machado G, Leonel DG, Vilca LM, Uriona S, et al.

Geographic patterns and environmental factors associated with human yellow fever presence in the Americas. PLoS Negl Trop Dis. 2017;11(9):1–27.

Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis [Internet]. 2005 [cited 2019 Apr 21]; 11(8):1167–73. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16102302

Hervé JP, Travassos da Rosa APA, Vasconcelos PFC, Travassos da Rosa JFS, Dégallier N. Isolation of yellow fever virus from Haemagogus (Hag.) albomaculatus. Arthropod-Borne Virus Inf Exch [Internet]. 1984 [cited 2018 Sep 11];53. Available from: http://www.documentation.ird.fr/hor/fdi:010050675

Van Heuverswyn F, Peeters M. The origins of HIV and implications for the global epidemic. Curr Infect Dis Rep [Internet]. 2007 [cited 2019 Apr 18]; 9(4):338–46. Available from: http://link.springer.com/10.1007/s11908-007-0052-x

Hinnebusch BJ, Erickson DL. Yersinia pestis Biofilm in the Flea Vector and Its Role in the Transmission of Plague. In Springer, Berlin, Heidelberg;]Internet] 2008 [cited 2019 Apr 22]. p. 229–48. Available from: http://link.springer.com/10.1007/978-3-540-75418-3_11

Jacobson ER, Ginn PE, Troutman JM, Farina L, Stark L, Klenk K, et al. West Nile Virus infection in farmed american alligators (Alligator mississippiensis) in Florida. J Wildl Dis [Internet]. 2005 [cited 2019 Apr 21]; 41(1):96–106. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15827215

Jenkins EJ, Simon A, Bachand N, Stephen C. Wildlife parasites in a One Health world. Trends Parasitol [Internet]. 2015 [cited 2019 Apr 22]; 31(5):174–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25662272

Johnson BW, Chambers T V., Crabtree MB, Filippis AMB, Vilarinhos PTR, Resende MC, et al. Vector competence of Brazilian Aedes aegypti and Ae. albopictus for a Brazilian yellow fever virus isolate. Trans R Soc Trop Med Hyg [Internet]. 2002 [cited 2019 Apr 16]; 96(6):611–3. Available from: https://academic.oup.com/trstmh/article-lookup/doi/10.1016/S0035-9203(02)90326-3

Jongejan F, Uilenberg G. The global importance of ticks. Parasitology [Internet]. 2004 [cited 2019 Apr 22]; 129 Suppl:S3-14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15938502

Karna AK, Azar SR, Plante JA, Yun R, Vasilakis N, Weaver SC, et al. Colonized Sabethes cyaneus, a Sylvatic New World Mosquito Species, Shows a Low Vector

Competence for Zika Virus Relative to Aedes aegypti. Viruses [Internet]. 2018 [cited 2019 Mar 24]; 10(8):434. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30115888

Kaufmann B, Rossmann MG. Molecular mechanisms involved in the early steps of flavivirus cell entry. Microbes Infect [Internet]. 2011 [cited 2019 May 19]; 13(1):1–9. Available from: https://www.sciencedirect.com/science/article/pii/S1286457910002315?via%3Dihub

Kirchgatter K, Tubaki RM, Malafronte R dos S, Alves IC, Lima GFM de C, Guimarães L de O, et al. Anopheles (Kerteszia) cruzii (Diptera: Culicidae) in peridomiciliary area during asymptomatic malaria transmission in the Atlantic forest: molecular identification of blood-meal sources indicates humans as primary intermediate hosts. Rev Inst Med Trop Sao Paulo [Internet]. 2014 [cited 2019 Apr 18]; 56(5):403–9. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0036-46652014000500403&lng=en&tlng=en

Klenk K, Komar N. Poor replication of West Nile virus (New York 1999 strain) in three reptilian and one amphibian species. Am J Trop Med Hyg [Internet]. 2003 [cited 2019 Apr 21]; 69(3):260–2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14628941

Klenk K, Snow J, Morgan K, Bowen R, Stephens M, Foster F, et al. Alligators as West Nile virus amplifiers. Emerg Infect Dis [Internet]. 2004 [cited 2019 Apr 21]; 10(12):2150–5. Available from: http://wwwnc.cdc.gov/eid/article/10/12/04-0264_article.htm

Krause RM. The origin of plagues: old and new. Science [Internet]. 1992 [cited 2019 Apr 22]; 257(5073):1073–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1509258

Krawczak FS, Nieri-Bastos FA, Nunes FP, Soares JF, Moraes-Filho J, Labruna MB. Rickettsial infection in Amblyomma cajennense ticks and capybaras (Hydrochoerus hydrochaeris) in a Brazilian spotted fever-endemic area. Parasit Vectors [Internet]. 2014 [cited 2019 Apr 22]; 7(1):7. Available from: http://parasitesandvectors.biomedcentral.com/articles/10.1186/1756-3305-7-7

Kumm HW, Cerqueira NL. The Rôle of Aëdes leucocelaenus in the Epidemiology of Jungle Yellow Fever in Brazil. Bull Entomol Res [Internet]. 1951 [cited 2018 Sep 4]; 42(01):195. Available from:

http://www.journals.cambridge.org/abstract_S0007485300025281

Kumm HW, Laemmert HW. The geographical distribution of immunity to yellow fever among the primates of Brazil. Am J Trop Med Hyg [Internet]. 1950 [cited 2018 Aug 25]; 30(5):733–48. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14771397

Kuno G, Chang G-JJ. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. Arch Virol [Internet]. 2007 [cited 2019 Apr 24]; 152(4):687–96. Available from: http://link.springer.com/10.1007/s00705-006-0903-z

Laemmert HW, Ferreira LDC, Taylor RM. Part II—Investigations of vertebrate hosts and arthropod vectors. Am J Trop Med Hyg [Internet]. 1946 [cited 2018 Aug 26]; s1-26(6_Suppl_2):23–69. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1946.s1-26.23

Laemmert HW, Kumm HW. The Geographical Distribution of Immunity to Yellow Fever among the Primates of Brazil 1. Am J Trop Med Hyg [Internet]. 1950 [cited 2018 Aug 25]; s1-30(5):733–48. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1950.s1-30.733

Lainson R, Rangel EF. Lutzomyia longipalpis and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. Mem Inst Oswaldo Cruz [Internet]. 2005 [cited 2019 Apr 23]; 100(8):811–27. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762005000800001&lng=en&tlng=en

Lalremruata A, Magris M, Vivas-Martínez S, Koehler M, Esen M, Kempaiah P, et al. Natural infection of Plasmodium brasilianum in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon. EBioMedicine [Internet]. 2015 [cited 2019 Mar 6]; 2(9):1186–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26501116

Laveran A. Un nouveau parasite trouvé dans le sang des malades atteints de fièvre palustre: origine parasitaire des accidents de l'impaludisme. 1881. 164pp

Long KC, Ziegler SA, Thangamani S, Hausser NL, Kochel TJ, Higgs S, et al. Experimental Transmission of Mayaro Virus by Aedes aegypti. Am J Trop Med Hyg [Internet]. 2011 [cited 2019 Apr 23]; 85(4):750–7. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.2011.11-0359

Lourenço-de-Oliveira R, Deane LM. Simian malaria at two sites in the Brazilian Amazon. I--The infection rates of Plasmodium brasilianum in non-human

primates. Mem. Inst. Oswaldo Cruz. 1995;90:331–9.

Lourenço-de-Oliveira R, Failloux A-B. High risk for chikungunya virus to initiate an enzootic sylvatic cycle in the tropical Americas. Vasilakis N, editor. PLoS Negl Trop Dis [Internet]. 2017 [cited 2018 Sep 24];11(6):e0005698. Available from: http://dx.plos.org/10.1371/journal.pntd.0005698

Lourenço-de-Oliveira R, Luz SLB. Simian malaria at two sites in the Brazilian Amazon - II. Vertical distribution and frequency of anopheline species inside and outside the forest. Mem Inst Oswaldo Cruz. 1996;91(6):687–94.

Lourenço-de-Oliveira R, Vazeille M, de Filippis AMB, Failloux A-B. Large genetic differentiation and low variation in vector competence for dengue and Yellow Fever viruses of Aedes albopictus from Brazil, the United States, and the Cayman Islands. Am J Trop Med Hyg [Internet]. 2003 [cited 2018 Sep 11]; 69(1):105–14. Available from: http://www.ajtmh.org/content/69/1/105.abstract

Lutz A. Forest mosquitoes and forest malaria. J Trop Med. 1903;6:282–92.

Lutz A. Reminiscencias da febre amarella no Estado de São Paulo. Erinnerungen aus der Gelbfieberzeit im Staate São Paulo. Mem Inst Oswaldo Cruz [Internet]. 1930 [cited 2018 Sep 11]; 24(3):127–42. Available from: http://www.scielo.br/pdf/mioc/v24n3/tomo24(f3)_127-142.pdf

Machado RL, Ceddia MB, Carvalho DF de, Cruz ES da, Francelino MR. Spatial variability of maximum annual daily rain under different return periods at the Rio de Janeiro state, Brazil. Bragantia [Internet]. 2010 [cited 2019 May 13]; 69(suppl):77–84. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0006-87052010000500009&lng=en&tlng=en

MacNamara F. Zika virus : A report on three cases of human infection during an epidemic of jaundice in Nigeria. Trans R Soc Trop Med Hyg [Internet]. 1954 [cited 2019 Apr 24]; 4 8(2):139–45. Available from: https://www.sciencedirect.com/science/article/pii/0035920354900061

Manson P. On the Development of Filaria sanguinis hominis, and on the Mosquito considered as a Nurse*. J Linn Soc London, Zool [Internet]. 1878 [cited 2019 Apr 23]; 14(75):304–11. Available from: https://academic.oup.com/zoolinnean/article-lookup/doi/10.1111/j.1096-3642.1878.tb01837.x

Marcondes CB, Alencar J. Revisão de mosquitos Haemagogus Williston (Diptera : Culicidae) do Brasil. Rev Biomed. 2010; 21(3):221–38.

Martins LC, Silva EVP da, Casseb LMN, Silva SP da, Cruz ACR, Pantoja JA de S, et

al. First isolation of West Nile virus in Brazil. Mem Inst Oswaldo Cruz [Internet]. 2019 [cited 2019 Apr 20]; 114(0). Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762019000100305&lng=en&tlng=en

Martins TF, Barbieri ARM, Costa FB, Terassini FA, Camargo LMA, Peterka CRL, et al. Geographical distribution of Amblyomma cajennense (sensu lato) ticks (Parasitiformes: Ixodidae) in Brazil, with description of the nymph of A. cajennense (sensu stricto). Parasit Vectors [Internet]. 2016 [cited 2019 Fev 10]; 9(1):186. Available from: http://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-016-1460-2

Mascheretti M, Tengan CH, Sato HK, Suzuki A, de Souza RP, Maeda M, et al. Yellow fever: Reemerging in the state of Sao Paulo, Brazil, 2009. Rev Saude Publica. 2013;47(5):881–9.

McCulloch E, Ramage G, Jones B, Warn P, Kirkpatrick WR, Patterson TF, et al. Don't throw your blood clots away: use of blood clot may improve sensitivity of PCR diagnosis in invasive aspergillosis. J Clin Pathol [Internet]. 2009 [cited 2018 Jul 27]; 62(6):539–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19190008

Miguel RB, Albuquerque HG, Sanchez MCA, Coura JR, Santos S da S, Silva S da, et al. Asymptomatic Plasmodium infection in a residual malaria transmission area in the Atlantic Forest region: Implications for elimination. Rev Soc Bras Med Trop [Internet]. 2019 [cited 2019 Apr 24]; 52(0):1–9. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037-86822019000100316&lng=en&tlng=en

Milton K, Lozier JD, Lacey EA. Genetic structure of an isolated population of mantled howler monkeys (Alouatta palliata) on Barro Colorado Island, Panama. Conserv Genet. 2009;10(2):347–58.

Ministério de Saúde. Casos confirmados de febre maculosa. Brasil, Grandes Regiões e Unidades Federadas. 2000 a 2018*. 2018 [cited 2019 Apr 24]. Available from: https://portalarquivos2.saude.gov.br/images/pdf/2018/fevereiro/02/Casos-confirmados-febre-maculosa.pdf

Mir D, Delatorre E, Bonaldo M, Lourenço-de-Oliveira R, Vicente AC, Bello G. Phylodynamics of Yellow Fever Virus in the Americas: new insights into the origin of the 2017 Brazilian outbreak. Sci Rep [Internet]. 2017 [cited 2018 Sep 26];

7(1):7385. Available from: http://www.nature.com/articles/s41598-017-07873-7

Monath TP, Vasconcelos PFC. Yellow fever. J Clin Virol [Internet]. 2015 [cited 2019 Apr 24];64:160–73. Available from: http://dx.doi.org/10.1016/j.jcv.2014.08.030

Moreira-Soto A, Carneiro I de O, Fischer C, Feldmann M, Kümmerer BM, Silva NS, et al. Limited Evidence for Infection of Urban and Peri-urban Nonhuman Primates with Zika and Chikungunya Viruses in Brazil. Fernandez-Sesma A, editor. mSphere [Internet]. 2018 [cited 2019 Mar 8]; 3(1). Available from: http://www.ncbi.nlm.nih.gov/pubmed/29404420

Moreno ES, Agostini I, Holzmann I, Di Bitetti MS, Oklander LI, Kowalewski MM, et al. Yellow fever impact on brown howler monkeys (Alouatta guariba clamitans) in Argentina: A metamodelling approach based on population viability analysis and epidemiological dynamics. Mem Inst Oswaldo Cruz. 2015;110(7):865–76.

Moreno ES, Spinola R, Tengan CH, Brasil RA, Siciliano MM, Coimbra TLM, et al. Epizootias de febre amarela em primatas não humanos no estado de São Paulo, Brasil, 2008-2009. Rev Inst Med Trop Sao Paulo. 2013;55(1):45–50.

Mossoun A, Pauly M, Akoua-Koffi C, Couacy-Hymann E, Leendertz SAJ, Anoh AE, et al. Contact to Non-human Primates and Risk Factors for Zoonotic Disease Emergence in the Taï Region, Côte d'Ivoire. Ecohealth [Internet]. 2015 [cited 2019 Apr 18];12(4):580–91. Available from: http://link.springer.com/10.1007/s10393-015-1056-x

Mourão MPG, Bastos M de S, de Figueiredo RP, Gimaque JBL, dos Santos Galusso E, Kramer VM, et al. Mayaro Fever in the City of Manaus, Brazil, 2007–2008. Vector-Borne Zoonotic Dis. 2011;12(1):42–6.

Nasci RS, White DJ, Stirling H, Oliver J, Daniels TJ, Falco RC, et al. West Nile Virus Isolates from Mosquitoes in New York and New Jersey, 1999. Emerg Infect Dis [Internet]. 2001 [cited 2019 Apr 21]; 7(4):626–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11585523

Nehete PN, Abee CR, Wilkerson GK, Weaver SC, Brady AG, Vasilakis N, et al. Experimental Zika Virus Infection of Neotropical Primates. Am J Trop Med Hyg [Internet]. 2018 [cited 2019 May 5]; 98(1):173–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29182145

Neves A, Urbinatti PR, Malafronte R dos S, Fernandes A, Paganini W da S, Natal D. Malaria outside the Amazon region: Natural Plasmodium infection in anophelines collected near an indigenous village in the Vale do Rio Branco, Itanhaém, SP, Brazil. Acta Trop [Internet]. 2013 [cited 2019 Apr 24]; 125(1):102–6. Available

from: http://dx.doi.org/10.1016/j.actatropica.2012.08.014

Nott JC. The Cause of Yellow Fever. 1848 [cited 2019 Apr 15];IV:563–601. Available from: https://books.google.mw/books?id=ShZDAAAAYAAJ&printsec=frontcover&source=gbs_atb#v=onepage&q&f=false

Nunes MRT, Palacios G, Cardoso JF, Martins LC, Sousa EC, de Lima CPS, et al. Genomic and phylogenetic characterization of Brazilian yellow fever virus strains. J Virol [Internet]. 2012 [cited 2019 Feb 13]; 86(24):13263–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23015713

Oliveira-Ferreira J, Lacerda MV, Brasil P, Ladislau JL, Tauil PL, Daniel-Ribeiro CT. Malaria in Brazil: an overview. Malar J [Internet]. 2010 [cited 2019 May 26]; 9(1):115. Available from: http://malariajournal.biomedcentral.com/articles/10.1186/1475-2875-9-115

Paddock CD, Finley RW, Wright CS, Robinson HN, Schrodt BJ, Lane CC, et al. *Rickettsia parkeri* Rickettsiosis and Its Clinical Distinction from Rocky Mountain Spotted Fever. Clin Infect Dis [Internet]. 2008 [cited 2019 Jun 10]; 47(9):1188–96. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1086/592254

Paglia AP, Rylands AB, Herrmann G, Aguiar LMS, Chiarello AG, Leite YLR, et al. Lista anotada dos mamiferos do Brasil, segunda edicao. 2nd ed. Arlington: Conservation International; 2012.82pp.

PAHO/WHO. Data - Dengue cases [Internet]. 2019 [cited 2019 Apr 20]. Available from: http://www.paho.org/data/index.php/en/mnu-topics/indicadores-dengue-en/dengue-nacional-en/252-dengue-pais-ano-en.html

Pauvolid-Corrêa A, Campos Z, Juliano R, Velez J, Nogueira RMR, Komar N. Serological Evidence of Widespread Circulation of West Nile Virus and Other Flaviviruses in Equines of the Pantanal, Brazil. PLoS Negl Trop Dis. 2014;8(2):1–11.

Pauvolid-Corrêa A, Campos Z, Soares R, Nogueira RMR, Komar N. Neutralizing antibodies for orthobunyaviruses in Pantanal, Brazil. PLoS Negl Trop Dis. 2017;11(11):1–11.

Pereira Serra O, Fernandes Cardoso B, Maria Ribeiro AL, dos Santos FAL, Dezengrini Slhessarenko R. Mayaro virus and dengue virus 1 and 4 natural infection in culicids from Cuiabá, state of Mato Grosso, Brazil. Mem Inst Oswaldo Cruz. 2016;111(1):20–9.

Pina-Costa A de, Brasil P, Santi SM Di, Araujo MP de, Suárez-Mutis MC, Santelli ACF

e S, et al. Malaria in Brazil: what happens outside the Amazonian endemic region. Mem Inst Oswaldo Cruz [Internet]. 2014 [cited 2019 Apr 20]; 109(5):618–33. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762014000500618&lng=en&tlng=en

Plowright RK, Parrish CR, McCallum H, Hudson PJ, Ko AI, Graham AL, et al. Pathways to zoonotic spillover. Nat Rev Microbiol [Internet]. 2017 May 30 [cited 2019 Apr 21]; 15(8):502–10. Available from: http://www.nature.com/doifinder/10.1038/nrmicro.2017.45

Possas C, Lourenço-de-Oliveira R, Tauil PL, Pinheiro Fr de P, PIssinatti A, Cunha RV da, et al. Yellow fever outbreak in Brazil: the puzzle of rapid viral spread and challenges for immunization. Mem Inst Oswaldo Cruz [Internet]. 2018 [cited 2018 Sep 11];Fast Track:1–37. Available from: http://memorias.ioc.fiocruz.br/article/6475/0278-yellow-fever-outbreak-in-brazil-the-puzzle-of-rapid-viral-spread-and-challenges-for-immunization

Possas C, Martins RM, Oliveira RL de, Homma A, Possas C, Martins RM, et al. Urgent call for action: avoiding spread and re-urbanisation of yellow fever in Brazil. Mem Inst Oswaldo Cruz [Internet]. 2017 [cited 2018 Sep 24]; 113(1):1–2. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762018000100001&lng=en&tlng=en

Rachou R. Sobre o combate aos anofelinos do sub-género Kerteszia no sul do Brasil. Rev Bras Malar [Internet]. 1952 [cited 2019 May 16]; IV(3):245–54. Available from: https://scholar.google.com.br/scholar?hl=pt-BR&as_sdt=0%2C5&q=Sobre+o+combate+aos+anofelinos+do+sub-gênero+Kerteszia+no+Sul+do+Brasil&btnG=

Rachou RG. Da domesticidade dos anofelinos do subgênero Kerteszia no litoral do Estado de Santa Catarina. Folha méd, v. 1946;27(1):1–8.

Ramasamy R. Zoonotic Malaria - Global Overview and Research and Policy Needs. Front Public Heal [Internet]. 2014 [cited 2019 Apr 24]; 2:123. Available from: http://journal.frontiersin.org/article/10.3389/fpubh.2014.00123/abstract

Rawlins SC, Hull B, Chadee DD, Martinez R, LeMaitre A, James F, et al. Sylvatic yellow fever activity in Trinidad, 1988–1989. Trans R Soc Trop Med Hyg. 1990;84(1):142–3.

Reed W, Carrol J, Agramonte A. The etiology of Yellow Fever. J Am Med Assoc [Internet]. 1901 [cited 2018 Sep 11]; 36(7):415–40. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.1901.52470070017

001f

de Rezende Dias G, Fujii TTS, Fogel BF, Lourenço-de-Oliveira R, Silva-do-Nascimento TF, Pitaluga AN, et al. Cryptic diversity in an Atlantic Forest malaria vector from the mountains of South-East Brazil. Parasit Vectors [Internet]. 2018 [cited 2019 May 12]; 11(1):36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29335015

Rezende HR, Soares RM, Cerutti Jr C, Alves IC, Natal D, Urbinatti PR, et al. Entomological characterization and natural infection of anophelines in an area of the Atlantic Forest with autochthonous malaria cases in mountainous region of Espírito Santo State, Brazil. Neotrop Entomol [Internet]. 2009 [cited 2019 Feb 9]; 38(2):272–80. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1519-566X2009000200017&lng=en&nrm=iso&tlng=en

Rezende IM de, Sacchetto L, Mello ÉM, Alves PA, Iani FC de M, Adelino TÉR, et al. Persistence of Yellow fever virus outside the Amazon Basin, causing epidemics in Southeast Brazil , from 2016 to 2018. PLoS Negl Trop Dis. 2018;12(6):1–12.

Ribeiro M, Antunes CM de F. Febre amarela: estudo de um surto. Rev Soc Bras Med Trop [Internet]. 2009 [cited 2019 Feb 9]; 42(5):523–31. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037-86822009000500009&lng=pt&tlng=pt

Robinson MC. An Epidemic of Yiras Disease in Southern Province, Tanganyika Territory, In 1952-83. I. Clinical Features. Trans R Soc Trop Med Hyg [Internet]. 1955 [cited 2019 Apr 24]; 49(1):28–32. Available from: https://www.cabdirect.org/cabdirect/abstract/19552900753

Rodrigues MS, Lima L, Xavier SC das C, Herrera HM, Rocha FL, Roque ALR, et al. Uncovering Trypanosoma spp. diversity of wild mammals by the use of DNA from blood clots. Int J Parasitol Parasites Wildl [Internet]. 2019 [cited 2019 May 25]; 8:171–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30847276

Romano APM, Costa ZGA, Ramos DG, Andrade MA, de Jayme VS, de Almeida MAB, et al. Yellow Fever Outbreaks in Unvaccinated Populations, Brazil, 2008-2009. PLoS Negl Trop Dis. 2014;8(3):18–21.

Ross R. The rôle of the mosquito in the evolution of the malarial parasite. Lancet [Internet]. 1898 [cited 2019 May 26]; 152(3912):488–90. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673601814008

Rylands AB, Mittermeier RA. The Diversity of the New World Primates (Platyrrhini): An

Annotated Taxonomy. In: South American Primates [Internet]. New York: Springer, New York, NY; 2009 [cited 2019 May 21]. 23–54pp. Available from: https://link.springer.com/chapter/10.1007/978-0-387-78705-3_2

Scott NJ, Júnior AFS, Malmgren LA. capturing and marking howler monkeys for field behavioral studies. Primates. 1976;17:527–33.

Se Fum Wong S, Kuei JJ, Prasad N, Agonafer E, Mendoza GA, Pemberton TJ, et al. A simple method for DNA isolation from clotted blood extricated rapidly from serum separator tubes. Clin Chem [Internet]. 2007 [cited 2018 Jul 27]; 53(3):522–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9702967

Shannon RC. Methods for Collecting and Feeding Mosquitoes in Jungle Yellow Fever Studies 1. Am J Trop Med Hyg [Internet]. 1939 [cited 2018 Aug 29]; s1-19(2):131–40. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1939.s1-19.131

Shannon RC, Whitman L, Franca M. Yellow Fever virus in jungle mosquitoes. Science (80-) [Internet]. 1938 [cited 2018 Aug 27]; 88(2274):110–1. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.88.2274.110

Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. Lancet [Internet]. 2004 [cited 2019 Apr 24]; 363(9414):1017–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15051281

Smithburn KC, Hughes TP, Burke AW, Paul JH. A Neurotropic Virus Isolated from the Blood of a Native of Uganda 1. Am J Trop Med Hyg [Internet]. 1940 [cited 2019 Apr 21]; s1-20(4):471–92. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1940.s1-20.471

Soper FL. Jungle Yellow Fever. A New Epidemiological Entity in South America. Rev Hig e Saude Publica [Internet]. 1937 [cited 2018 Sep 4];10(4):107-44. Available from: https://www.cabdirect.org/cabdirect/abstract/19372900588

Soper FL. Yellow fever: The present situation (October 1938) with special reference to South America. Trans R Soc Trop Med Hyg [Internet]. 1938 Nov 26 [cited 2018 Sep 4];32(3):297–322. Available from: https://academic.oup.com/trstmh/article-lookup/doi/10.1016/S0035-9203(38)90045-8

Soper FL. The elimination of urban yellow fever in the Americas through the eradication of Aedes aegypti. Am J Public Health. 1963;53:7–16.

Soper FL, Penna H a, Cardoso E, Serafim Jr J, Frobisher Jr M, Pinheiro J. Yellow Fever without *Aedes aegypti*. Study of a rural epidemic in the Valle do Chanaan,

Espirito Santo, Brazil, 1932. Am J Hyg. 1933;18:555–87.

de Souza RP, Foster PG, Sallum MAM, Coimbra TLM, Maeda AY, Silveira VR, et al. Detection of a new yellow fever virus lineage within the South American genotype I in Brazil. J Med Virol [Internet]. 2010 [cited 2019 May 19]; 82(1):175–85. Available from: http://doi.wiley.com/10.1002/jmv.21606

de Souza RP, Petrella S, Coimbra TLM, Maeda AY, Rocco IM, Bisordi I, et al. Isolation of Yellow Fever virus (YFV) from naturally infected Haemagogus (Conopostegus) leucocelaenus (Diptera, Culicidae) in São Paulo state, Brazil, 2009. Rev Inst Med Trop Sao Paulo. 2011;53(3):133–9.

Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. J Clin Invest [Internet]. 2004 [cited 2019 Apr 22]; 113(8):1093–101. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15085185

Steverding D. The history of leishmaniasis. Parasit Vectors [Internet]. 2017 [cited 2019 Apr 11]; 10(1):82. Available from: http://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-017-2028-5

Stokes A, Bauer JH, Hudson NP. Experimental Transmission of Yellow Fever to Laboratory Animals 1. Am J Trop Med Hyg [Internet]. 1928 [cited 2019 May 16]; s1-8(2):103–64. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1928.s1-8.103

Strode GK. Yellow Fever. George K. Strode, Ed. New York-London: McGraw-Hill, 1951, 710pp.

Struchiner CJ, Luz PM, Dourado I, Sato HK, Aguiar SG, Ribeiro JGL, et al. Risk of fatal adverse events associated with 17DD yellow fever vaccine. Epidemiol Infect [Internet]. 2004 [cited 2019 May 19]; 132(5):939–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15473158

Tátila-Ferreira A, Maia D de A, Abreu FVS de, Rodrigues WC, Alencar J. Oviposition behavior of Haemagogus leucocelaenus (Diptera: culicidae), a vector of wild yellow fever in Brazil. Rev Inst Med Trop Sao Paulo [Internet]. 2017a [cited 2019 May 17]; 7;59(0). Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0036-46652017005000232&lng=en&tlng=en

Tátila-Ferreira A, Maia D de A, Alencar J. Development of Preimaginal Stages of *Haemagogus leucocelaenus* (Diptera: Culicidae) in Laboratory Conditions. Entomol News [Internet]. 2017b [cited 2019 May 17]; 127(2):142–50. Available

from: http://www.bioone.org/doi/10.3157/021.127.0209

Tavares C, Aragão AI, Leal NC, Leal-Balbino TC, de Oliveira MBM, de Oliveira Gonçalves Ferreira GM, et al. Plague in Brazil: From Now and Then. In Springer, New York, NY; 2012. 69–77pp. Available from: http://link.springer.com/10.1007/978-1-4614-3561-7_10

Taylor LH, Latham SM, woolhouse MEJ. Risk factors for human disease emergence. Woolhouse MEJ, Dye C, editors. Philos Trans R Soc London Ser B Biol Sci [Internet]. 2001 [cited 2019 Apr 21]; 356(1411):983–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11516376

Taylor RM, Da Cunha JF. Part I—Epidemiology of Human Infections. Am J Trop Med Hyg [Internet]. 1946 [cited 2018 Aug 26]; s1-26(6_Suppl_2):1–21. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1946.s1-26.1

Terzian ACB, Zini N, Sacchetto L, Rocha RF, Parra MCP, Del Sarto JL, et al. Evidence of natural Zika virus infection in neotropical non-human primates in Brazil. Sci Rep [Internet]. 2018 [cited 2019 Mar 8]; 8(1):16034. Available from: http://www.nature.com/articles/s41598-018-34423-6

de Thoisy B, Dussart P, Kazanji M. Wild terrestrial rainforest mammals as potential reservoirs for flaviviruses (yellow fever, dengue 2 and St Louis encephalitis viruses) in French Guiana. Trans R Soc Trop Med Hyg [Internet]. 2004 [cited 2019 Apr 20]; 98(7):409–12. Available from: https://academic.oup.com/trstmh/article-lookup/doi/10.1016/j.trstmh.2003.12.003

Thompson RCA. Parasite zoonoses and wildlife: One health, spillover and human activity. Int J Parasitol [Internet]. 2013 [cited 2019 Apr 9]; 43(12–13):1079–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23892130

Vasconcelos PF da C. Febre amarela Yellow fever. Rev. Soc. Bras. Med. Trop 2003;36(2):275–93.

Vasconcelos PF da C. Yellow fever in Brazil: thoughts and hypotheses on the emergence in previously free areas. Rev Saude Publica [Internet]. 2010 [cited 2019 Apr 23];44(6):1144–9. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0034-89102010000600021&lng=en&nrm=iso&tlng=en%0Ahttp://www.scielo.br/scielo.php?script=sci_arttext&pid=S0034-89102010000600021&lng=en&tlng=en

Vasconcelos PFC, Bryant JE, Travassos Da Rosa APA, Tesh RB, Rodrigues SG, Barrett ADT. Genetic divergence and dispersal of yellow fever virus, Brazil. Emerg Infect Dis. 2004;10(9):1578–84.

Vasconcelos PFC, Costa ZG, Travassos Da Rosa ES, Luna E, Rodrigues SG, Barros VLRS, et al. Epidemic of jungle yellow fever in Brazil, 2000: Implications of climatic alterations in disease spread. J Med Virol. 2001a;65(3):598–604.

Vasconcelos PFC, Luna EJ, Galler R, Silva LJ, Coimbra T, Barros V, et al. Serious adverse events associated with yellow fever 17DD vaccine in\nBrazil: a report of two cases. Lancet. 2001b;358(9276):91–7.

Vasconcelos PFC, Rodrigues SG, Degallier N, Moraes MAP, Travassos Da Rosa JFS, Travassos Da Rosa ES, et al. An epidemic of sylvatic yellow fever in the southeast region of Maranhao State, Brazil, 1993-1994: Epidemiologic and entomologic findings. Am J Trop Med Hyg. 1997;57(2):132–7.

Vasconcelos PFC, Rosa APAT, Rodrigues SG, Rosa EST, Monteiro HAO, Cruz ACR, et al. Yellow fever in Pará State, Amazon region of Brazil, 1998-1999: Entomologic and epidemiologic findings. Emerg Infect Dis. 2001c;7(3):565–9.

Vasconcelos PFC, Sperb AF, Monteiro HAO, Torres MAN, Sousa MRS, Vasconcelos HB, et al. Isolations of yellow fever virus from Haemagogus leucocelaenus in Rio Grande do Sul State, Brazil. Trans R Soc Trop Med Hyg. 2003;97(1):60–2.

Vasconcelos PFC, Travassos da Rosa APA, Rodrigues SG, Travassos da Rosa ES, Dégallier N, Travassos da Rosa JFS. Inadequate management of natural ecosystem in the Brazilian Amazon region results in the emergence and reemergence of arboviruses. Cad Saude Publica [Internet]. 2001d [cited 2019 Apr 23]; 17(suppl):S155–64. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-311X2001000700025&lng=en&tlng=en

Vasconcelos PFDC. Febre amarela: reflexões sobre a doença, as perspectivas para o século XXI e o risco da reurbanização. Rev Bras Epidemiol [Internet]. 2002 [cited 2019 Apr 23]; 5(3):244–58. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1415-790X2002000300004&lng=pt&nrm=iso&tlng=pt

Vasilakis N, Cardosa J, Hanley KA, Holmes EC, Weaver SC. Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. Nat Rev Microbiol [Internet]. 2011 [cited 2019 Apr 19]; 9(7):532–41. Available from: http://www.nature.com/articles/nrmicro2595

Vasilakis N, Weaver SC. Flavivirus transmission focusing on Zika. Curr Opin Virol [Internet]. 2017 [cited 2019 Apr 20]; 22:30–5. Available from: https://www.sciencedirect.com/science/article/pii/S1879625716301870?via%3Di

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Vega-Rua A, Zouache K, Girod R, Failloux A-B, Lourenco-de-Oliveira R. High Level of Vector Competence of Aedes aegypti and Aedes albopictus from Ten American Countries as a Crucial Factor in the Spread of Chikungunya Virus. J Virol [Internet]. 2014 [cited 2019 Apr 2]; 88(11):6294–306. Available from: http://jvi.asm.org/cgi/doi/10.1128/JVI.00370-14

Waltner-Toews D. Zoonoses, One Health and complexity: wicked problems and constructive conflict. Philos Trans R Soc B Biol Sci [Internet]. 2017 [cited 2019 Apr 9]; 372(1725):20160171. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28584179

Watsa M, Erkenswick G, Halloran D, Kane EK, Poirier A, Klonoski K, et al. A Field Protocol for the Capture and Release of Callitrichids. Neotrop Primates. 2015;22(2):59–68.

Weaver SC, Barrett ADT. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol [Internet]. 2004 [cited 2019 Mar 11]; 2(10):789–801. Available from: http://www.nature.com/articles/nrmicro1006

Weaver SC, Reisen WK. Present and future arboviral threats. Antiviral Res [Internet]. 2010 [cited 2019 Mar 11]; 85(2):328–45. Available from: https://www.sciencedirect.com/science/article/pii/S0166354209004951

Weinbren M., Haddow A., Williams M. The occurrence of chikungunya virus in Uganda I. Isolation from mosquitoes. Trans R Soc Trop Med Hyg [Internet]. 1958 [cited 2019 Apr 24]; 52(3):253–62. Available from: https://academic.oup.com/trstmh/article-lookup/doi/10.1016/0035-9203(58)90084-1

Whitman L, Antunes PCA. Studies on the capacity of various brazilian mosquitoes, representing the Genera Psorophora, Aedes, Mansonia, and Culex, to transmit Yellow Fever 1. Am J Trop Med Hyg [Internet]. 1937 [cited 2018 Sep 24]; s1-17(6):803–23. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1937.s1-17.803

WHO. West Nile virus [Internet]. [cited 2019 Apr 21]. Available from: https://www.who.int/en/news-room/fact-sheets/detail/west-nile-virus

WHO. Groupe Mixte OMS/FAO D’experrs des zoonoses. Genéve: World Health Organization; 1951. 51pp.

WHO. Joint WHO/FAO Expert Committee on Zonnoses. Geneva; World Health Organization 1959. 84pp.

WHO. Lyme borreliosis in Europe. Influences of climate and climate change, epidemiology, ecology and adaptation measures. Geneva: World Health Organization 2017. 34pp.

WHO. Update on the E-2020 initiative of 21 malaria-eliminating countries [Internet]. World Health Organization; 2018a. [cited 2019 apr 15]. Available from: https://www.who.int/malaria/publications/atoz/e-2020-progress-report/en/

WHO. World malaria report 2018. World Health Organization; 2018b 210pp.

Woolhouse MEJ, Gowtage-Sequeria S. Host range and emerging and reemerging pathogens. Emerg Infect Dis [Internet]. 2005 [cited 2019 Apr 18]; 11(12):1842–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16485468

Work TH, Hurlbult HS, Taylor RM. Indigenous wild birds of the Nile Delta as potential West Nile virus circulating reservoirs. Am J Trop Med Hyg [Internet]. 1955 [cited 2019 Apr 21]; 4(5):872–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13259011

Zanluca C, Melo VCA de, Mosimann ALP, Santos GIV dos, Santos CND dos, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz [Internet]. 2015 [cited 2019 Apr 2]; 110(4):569–72. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762015000400569&lng=en&tlng=en

Zietz BP, Dunkelberg H. The history of the plague and the research on the causative agent Yersinia pestis. Int J Hyg Environ Health [Internet]. 2004 [cited 2019 Apr 11];207(2):165–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15031959

Zinsstag J. Convergence of Ecohealth and One Health. Ecohealth [Internet]. 2012 [cited 2019 Apr 13]; 9(4):371–3. Available from: http://link.springer.com/10.1007/s10393-013-0812-z

Zuchi N, Heinen LB da S, Santos MAM dos, Pereira FC, Slhessarenko RD, Zuchi N, et al. Molecular detection of Mayaro virus during a dengue outbreak in the state of Mato Grosso, Central-West Brazil. Mem Inst Oswaldo Cruz [Internet]. 2014 [cited 2019 Apr 23]; 109(6):820–3. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762014000600820&lng=en&tlng=en

APÊNDICES E/OU ANEXOS

7.1 Anexo I – Artigo: Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation

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Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation

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Summary

Background Malaria was eliminated from southern and southeastern Brazil over 50 years ago. However, an increasing number of autochthonous episodes attributed to *Plasmodium vivax* have recently been reported from the Atlantic Forest region of Rio de Janeiro state. As the *P vivax*-like non-human primate malaria parasite species *Plasmodium simium* is locally enzootic, we performed a molecular epidemiological investigation to determine whether zoonotic malaria transmission is occurring.

Methods We examined blood samples from patients presenting with signs or symptoms suggestive of malaria as well as from local howler monkeys by microscopy and PCR. Samples were included from individuals if they had a history of travel to or resided in areas within the Rio de Janeiro Atlantic Forest, but not if they had malaria prophylaxis, blood transfusion or tissue or organ transplantation, or had travelled to known malaria endemic areas in the preceding year. Additionally, we developed a molecular assay based on sequencing of the parasite mitochondrial genome to distinguish between *P vivax* and *P simium*, and applied this assay to 33 cases from outbreaks that occurred in 2015, and 2016.

Findings A total of 49 autochthonous malaria cases were reported in 2015–16. Most patients were male, with a mean age of 44 years (SD 14·6), and 82% lived in urban areas of Rio de Janeiro state and had visited the Atlantic Forest for leisure or work-related activities. 33 cases were used for mitochondrial DNA sequencing. The assay was successfully performed for 28 samples, and all were shown to be *P simium*, indicative of zoonotic transmission of this species to human beings in this region. Sequencing of the whole mitochondrial genome of three of these cases showed that *P simium* is most closely related to *P vivax* parasites from South America. The malaria outbreaks in this region were caused by *P simium*, previously considered to be a monkey-specific malaria parasite, related to but distinct from *P vivax*, and which has never conclusively been shown to infect people before.

Interpretation This unequivocal demonstration of zoonotic transmission, 50 years after the only previous report of *P simium* in people, leads to the possibility that this parasite has always infected people in this region, but that it has been consistently misdiagnosed as *P vivax* because of an absence of molecular typing techniques. Thorough screening of local non-human primates and mosquitoes (*Anopheline*) is required to evaluate the extent of this newly recognised zoonotic threat to public health and malaria elimination in Brazil.

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Introduction

Zoonotic malaria occurs when people become infected with malaria parasite species that more commonly infect non-human primates. Species such as *Plasmodium knowlesi* and *Plasmodium cynomolgi*, both parasites of macaque monkeys (*Macaca*), can infect people via the bites of infected mosquitoes under natural and experimental conditions. *P knowlesi* is responsible for a high proportion of human malaria cases in Southeast Asia, mostly affecting individuals living or working in close contact with forests.[1] Zoonotic malaria poses a unique problem for malaria control efforts and complicates the drive towards eventual elimination of the disease; because of the nature of its reservoir and transmission dynamics, the interruption of its transmission might not be achievable with the available tools in areas of high forest coverage.

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For more on the **cases reported by the Ministry of Health** see www.saude.gov.br/malaria

Research in context

Evidence before this study

Autochthonous malaria infections in people leaving near the Atlantic Forest in Rio de Janeiro, Brazil, were diagnosed as *Plasmodium vivax*, a human malaria parasite. The diagnosis of *P vivax* was on the basis of the morphology of the parasites as observed through microscopy of thin blood smears stained with Giemsa's solution. As malaria was thought to have been eliminated from this area over 50 years ago, it was uncertain where and when this malaria parasite pool had emerged. Cases have been increasing in the past 5 years.

Added value of this study

This study shows that these parasites are, in fact, not *P vivax*, but rather *P simium*, a closely related parasite species whose natural hosts are non-human primates native to the Atlantic Forest. This diagnosis was made by molecular investigation of parasite DNA. Genotyping of malaria parasites from monkeys in this region revealed that the same parasites are infecting both monkeys and human beings in this area.

Implications of all the available evidence

Our study suggests that malaria transmission in the Atlantic Forest region of Rio de Janeiro has a zoonotic component, with parasites shared between human beings and monkeys. The implications of this finding for malaria control and elimination in this region are profound, as zoonotic reservoirs of disease are difficult to target with interventions.

Once prevalent throughout the country, malaria transmission in Brazil now occurs almost entirely within the northern Amazon region. However, a consistent number of autochthonous cases have been reported in southern and southeastern regions of Brazil from where human malaria was eliminated 50 years ago.[2] From 2006 to 2016, 1032 autochthonous cases (Ministry of Health Brazil 2017) were reported at sites scattered along the mountainous valleys covered by the Atlantic Forest in these regions. The Atlantic Forest is rich in bromeliads (Bromeliaceae), which provide a larval habitat for *Anopheles Kertezsia cruzii*, a vector of both human and non-human primate malaria parasites. Most of the malaria cases reported in the Atlantic Forest have been attributed to *Plasmodium vivax* and mainly occur among non-resident visitors, without any identifiable index case that could have introduced the parasite from a malaria endemic region.[3]

It has long been hypothesised that autochthonous human malaria in the Atlantic Forest could be the result of infection by non-human primate parasite species.[4] In 1966, Deane and colleagues[4] proposed that monkeys could serve as reservoirs of *Plasmodium* that could be transmitted to people by *A K cruzii*, because this species is known to bite both monkeys in the forest canopy and people at ground level.

Two malaria parasite species are known to infect new world monkeys (Ceboidea) in the Atlantic Forest of Brazil: *Plasmodium simium* and *Plasmodium brasilianum*.[5] These are similar at the morphological, genetic, and immunological levels to *P vivax* and *Plasmodium malariae*, respectively.[6] *P simium* has been observed to naturally infect howler monkeys of the genera *Alouatta* and *Brachyteles*, and capuchin monkeys of the genera *Cebus* and *Sapajus*.[7] Despite the distribution of the howler monkeys and capuchins across almost all biomes in South and Central America, the distribution of *P simium* is considered to be limited to the Atlantic Forest of south and southeastern Brazil.[5]

Here we describe the parasitological and molecular analyses of parasites causing autochthonous human malaria in the Atlantic Forest region of Rio de Janeiro in 2015 and 2016, with the aim of determining whether zoonotic malaria transmission occurs there.

Methods

Study area, population, and design

Rio de Janeiro state is located in southeast Brazil. It consists of urban areas with high population densities, mostly in the coastal lowlands, and mountainous areas covered by the Atlantic Forest containing small cities and settlements scattered in the valleys. Localities where malaria cases have been reported are situated in valleys between 280 m and 1300 m above sea level.[8]

We performed an epidemiological investigation to characterise the possible location of infection, by classifying each episode as autochthonous or imported. The cases considered here are from patients who attended the Instituto Nacional de Infectologia Evandro Chagas (INI), a reference centre for the diagnosis and treatment of infectious diseases at the Fundação Oswaldo Cruz (Fiocruz), in Rio de Janeiro, Brazil. Blood samples from patients with acute fever symptoms were collected from the Acute Febrile Illness Outpatient Clinic in INI. The INI-Fiocruz Ethical Board approved the study (number 0062.0.009.000-11). All participants provided informed written consent.

Procedures

Individuals were recruited upon presentation of signs or symptoms suggestive of malaria, a history of travel to or habitation in areas within the Rio de Janeiro Atlantic Forest, and a positive test by thick blood smear or PCR, or both. Individuals were excluded if they had malaria prophylaxis, blood transfusion or tissue or organ transplantation, used intravenous drugs, had a needlestick injury, resided or undertook recreation near ports or airports, or travelled to known malaria endemic areas in the preceding year. Following informed consent, venous blood was drawn for clinical laboratory analyses and molecular studies. Additional tests, such as blood

culture, viral serology and G6PD deficiency, were done for all patients at the attending physician's discretion.

Diagnosis by microscopy

Giemsa's solution-stained thin and thick blood smears were examined by bright-field microscopy, with a 100×/1·3 numerical aperture oil immersion objective lens for species identification and parasite density estimations. Blood films were examined for a minimum of 100 fields in the case of the presence of malaria parasites and 500 fields when no parasites could be detected. Parasite numbers were recorded per 200 white blood cells, or 500 white blood cells in the case of low parasitaemia. To estimate parasite density, a standard mean white blood cell count of 6000 white blood cells per µL of blood was assumed. All slides were subsequently examined by an independent Pan American Health Organization or WHO accredited malaria microscopist.

DNA extraction and *P vivax* species-specific PCR

DNA was extracted from whole blood with the QIAamp midi kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA samples were tested for *P vivax* by conventional and real-time PCR (rtPCR), both using the cysteine proteinase gene (GenBank number L26362) as a target.[9] For rtPCR, 2·5 µL of DNA were added to a 47·5 µL mixture containing the 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 300 nM of primer Pv1 (5′ATCAACGAGCAG ATGGAGAAATATA3′), 300 nM of primer Pv5 (5′GCT CTCGAAATCTTTCTTCGA3′), and 150 nM of PVIV probe (5′ FAM AACTTCAAAATGAATTATCTC MGB NFQ 3′) (Applied Biosystems). Amplification conditions involved two holds (50°C for 2 min and 95°C for 10 min) followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). The rtPCR was run at least in duplicate on the ABI PRISM Sequence Detection System 7500 (Applied Biosystems). The results were analysed using ABI Prism 7500, software version 1.1 RQ Study. TaqMan RNaseP Control 20x was used as an endogenous reaction control. To avoid DNA contamination, we used separated workstations for mix preparation and DNA extraction and we applied DNA Away for surface decontamination. Positive (DNA extracted from blood from patients with known *P vivax* infection) and negative (no DNA and DNA extracted from individuals who have never travelled to malaria-endemic areas) controls were used in each round of amplification. This PCR does not discriminate between *P vivax* and *P simium*.

Non-human primate samples

DNA was extracted from samples obtained from four howler monkeys from different sites and times in southeastern Brazil (MB CPRJ from Guapimirim in December, 2013; RJ 30 from Vale das Princesas, Miguel Pereira on March 21, 2016; RJ 59 from Macaé on Sept 22, 2016; and ATCC from São Paulo in 1966).

DNA extracted from the spleen and liver of one brown howler monkey (*Alouatta guariba clamitans*), found dead in Guapimirim (one of the municipalities where human malaria occurs in the Atlantic Forest of Rio de Janeiro), was used for *Plasmodium* species detection by nested-PCR.[10] Samples from both organs were positive for *P vivax* or *P simium* DNA, according to our PCR method.[7]

DNA was also extracted from the blood of two *A g clamitans*; one was captured at Vale das Princesas, Miguel Pereira (a site where human malaria cases have also been reported in Rio de Janeiro) in 2016, and tested positive by PCR analysis for both *P vivax* or *P simium* and *P brasilianum* or *P malariae*, and the other was from Macaé (another locality in Rio de Janeiro with human malaria cases), and was positive for *P vivax* or *P simium*. Additionally, a *P simium* reference sample (American Type Culture Collection [ATCC] 30130), derived from a howler monkey (*Alouatta fusca clamitans*) captured in São Paulo, southeast Brazil, in 1966, was also used. The DNA extracted from these four monkey samples also underwent mitochondrial genome analysis.

For more on the **reference sample** see https://www.atcc.org/~/ps/30130.ashx

Molecular phylogenetic analysis of *P simium* infections

Among samples derived from 39 individuals presenting at INI, 33 were subjected to parasite mitochondrial genome sequencing (20 from 2015 and 13 from 2016): 30 had partial analysis and three full-length mitochondrial genome sequencing. Samples from two monkeys collected from the Atlantic Forest of Rio de Janeiro and one ATCC *P simium* reference sample were also subjected to malaria parasite mitochondrial genome sequencing.

Because of the low amount of high-quality parasite DNA, full-length mitochondrial genome sequence was obtained for only four samples (three cases: AF 1, AF 2, and AF 3 and the ATCC reference sample), following the method reported by Culleton and colleagues,[11] and was compared with 794 *P vivax* mitochondrial genome sequences and three sequences of *P simium* (accession numbers AY800110, NC_007233 and AY722798, all of which have identical sequences) deposited in Genbank.[11–17] Using these sequences, a median-joining haplotype network was produced with NETWORK 4.5.0, as previously described.[11]

The mitochondrial genome of the remaining 30 samples was partially sequenced to distinguish *P simium* from *P vivax*. *P simium* differs from the most closely related *P vivax* isolate at two unique single-nucleotide polymorphisms (SNPs) in the mitochondrial genome, at positions 3535 (T→C) and 3869 (A→G), numbered according to the nucleotide sequences deposited by Culleton and colleagues.[11] These two SNPs are close together, and can be PCR amplified and sequenced with a single set of primers, or with a nested PCR if DNA concentrations are low. Primer pairs for the outer PCR were PsimOUTF 5′CAGGTGGTG TTTTAATGTTATTATCAG3′ and PsimOUTR 5′GCATAG GTAAGAATGTTAATACAACTCC3′, whereas inner

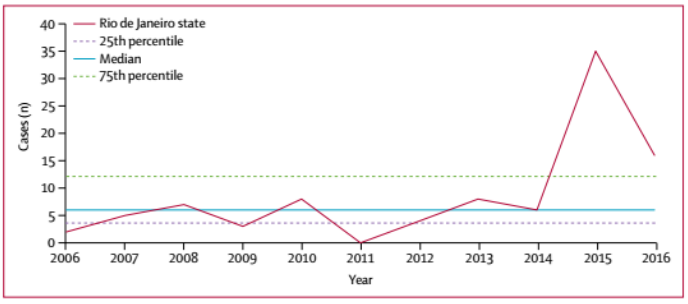

Figure 1: **Historical series of autochthonous malaria cases in the state of Rio de Janeiro, Brazil, from 2006 to 2016**
Historical series of autochthonous malaria cases from 2006 to 2016. In 2015–16, the number of cases exceeding the 75th percentile of maximum expected cases increased sharply, configuring an outbreak.

See Online for appendix

PCR primers were PsimINF 5′GCTGGAGATCCTATT TTATATCAAC3′ and PsimINR 5′ATGTAAACAATCCAA TAATTGCACC3′.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between 2006 and 2014, 43 autochthonous malaria cases were reported in the Atlantic Forest in the state of Rio de Janeiro, an average of 4·8 cases per year (SD 2·8), with an unexpected increase in the number of cases occurring during outbreaks in 2015 (n=33) and 2016 (n=16; figure 1). In 2015, 25 (76%) of the 33 cases were followed and processed at Fiocruz. In 2016 (until Oct 31), 14 of 16 (88%) cases were also investigated at Fiocruz, with a total of 39 (80%) of the 49 cases reported in the state.

Patients followed up at Fiocruz had a mean age of 44 years (SD 14·6) and median age of 50 years (range 7–82; table). Most patients were male (79%; table) and inhabitants of urban areas of Rio de Janeiro state (82%), who visited areas of the Atlantic Forest for leisure or work-related activities, spending a median of 5 days (range 1–30) in vegetation-dense areas and its close surroundings. Transmission occurred either in people who entered regions of dense vegetation coverage or in people who lived in rural areas with low-population density in mountain valleys (figure 2). The presence of monkeys was regularly reported in the neighbouring forest by the inhabitants of all areas.

Case clustering occurred only when individuals travelled together and developed symptoms in the same incubation period. Fever was the main symptom and was present in all malaria cases. Periodic tertian fever was observed in 35 cases (90%). No patient was admitted to hospital and all made full recoveries with complete cessation of symptoms following treatment. It was the first malaria episode for all patients and only one patient was G6PD deficient.

In 37 cases (95%) a diagnosis of *P vivax* was made by microscopy. The highest parasitaemia was 3000 parasites per µL of blood and, in more than 67% of the cases, it was lower than 500 parasites per µL. Two patients had negative tests for the presence of parasites by microscopy. A PCR for *P vivax*-species detection, which does not discriminate between *P vivax* and *P simium*, suggested the presence of *P vivax* in 38 patients (97%).

When compared with *P vivax* from the malaria endemic Amazonian regions, parasites from the Atlantic Forest diagnosed as *P vivax* were morphologically different (appendix). Trophozoites were pleomorphic but less amoeboid than those observed in *P vivax* (appendix). They had a large mass of chromatin and a more compact cytoplasm with malaria pigment (appendix). Usually stippling was mostly observed in infected cells with late developmental forms, but erythrocytes containing early trophozoites were also frequently stippled (figure 3A–F). Furthermore, developing schizonts contained fewer merozoites than in *P vivax* (figure 3G–L). The highest number of merozoites in mature schizonts was 14 (figure 3M). Gametocytes were round with compact cytoplasm and marked pigmentation (figure 3N–P).

Non-infected erythrocytes showed marked anisocytosis and poikilocytosis (figure 3). Poikilocytosis was represented mainly by acanthocytes, dacrocytes, and spherocytes, which occurred together on the same preparations (figure 3).

Analysis of the four usable mitochondrial genome samples from the 33 human cases used for DNA sequencing revealed that they shared identical sequences, and these were in turn identical to the mitochondrial genome sequence of *P simium* deposited at Genbank, which differs from the most closely related isolates of *P vivax* by two SNPs. Analysis of 794 full-length mitochondrial genome sequences from globally acquired *P vivax* samples showed that these SNPs were unique to *P simium*. A haplotype network tree (appendix) was constructed using these sequences, and shows that *P simium* is most closely related to the *P vivax* parasites of human beings isolated from South America.

On the basis of two informative SNPs that differentiate *P vivax* from *P simium*, we were able to diagnose an infection of *P simium* in 28 of 33 samples typed for their species (table). We were unable to achieve PCR amplification for the remaining five samples, because of technical constraints. The same informative SNPs were found in *P simium* infecting three local howler monkeys, MB CPRJ, RJ 30, and R J59 (table).

Discussion

The results of our study have important implications for public health and for the malaria elimination agenda. To our knowledge, this is the first demonstration of

P simium naturally infecting human beings in forest locations in a region considered to have eliminated transmission of malaria at least 50 years ago. The sudden increase in malaria cases in the past 2 years in that area is associated with the Atlantic Forest of Rio de Janeiro. No major environmental modifications appear to have occurred that might have modified the behaviour of *Anopheles* spp or monkeys during this

	Sample collection (year)	Age (years)	Sex	Main activity developed in the area	Visitor or resident	Entry into Atlantic Forest area	Time between onset of symptoms and diagnosis (days)	Triad of malaria*	Highest axillar temperature (°C)	Parasites density (mm³/µL)	*Plasmodium simium* SNPs†
Human samples											
AF 1	2015	34	Male	Photographer	Visitor	Yes	11	Yes	39·5	920	Yes‡
AF 2	2015	58	Male	Engineering	Visitor	No	12	Yes	38·6	560	Yes‡
AF 3	2015	50	Male	Geologist	Visitor	Yes	14	Yes	38·8	112	Yes‡
AF 4	2015	27	Male	Ecotourism	Visitor	Yes	15	Yes	38·1	1200	Yes
AF 5	2015	26	Male	Ecotourism	Visitor	Yes	14	Yes	39·0	64	Yes
AF 6	2015	51	Male	Inhabitant	Resident	Yes	14	No	38·5	480	Yes
AF 7	2015	40	Male	Ecotourism	Visitor	Yes	13	Yes	39·5	800	Yes
AF 8	2015	52	Male	Ecotourism	Visitor	Yes	10	Yes	39·8	416	Yes
AF 9	2015	29	Male	Ecotourism	Resident	Yes	6	Yes	38·0	64	Yes
AF 10	2015	35	Male	Architecture	Visitor	Yes	16	Yes	39·0	576	Yes
AF 11	2015	52	Female	Ecotourism	Visitor	Yes	12	Yes	38·8	320	Yes
AF 12	2015	48	Male	Inhabitant	Visitor	No	9	Yes	NA	208	Yes
AF 13	2015	52	Male	Forestal Garden	Resident	Yes	12	Yes	40·0	624	Yes
AF 14	2015	26	Male	Ecotourism	Visitor	Yes	13	Yes	40·0	128	Not determined§
AF 15	2015	44	Male	Ecotourism	Visitor	Yes	39	Yes	39·0	1296	Yes
AF 16	2015	59	Male	Ecotourism	Visitor	Yes	20	Yes	40·0	336	Yes
AF 17	2015	54	Male	Ecotourism	Visitor	Yes	6	Yes	39·8	96	Not determined§
AF 18	2015	39	Male	Ecotourism	Visitor	Yes	3	No	38·0	112	Yes
AF 19	2015	56	Male	Ecotourism	Visitor	Yes	16	Yes	39·0	480	Yes
AF 20	2015	22	Male	Engineering	Visitor	No	NA	No	39·5	80	Not determined§
AF 21	2016	82	Female	Tourism	Visitor	No	13	Yes	NA	3000	Yes
AF 22	2016	40	Male	Ecotourism	Resident	Yes	10	Yes	39·0	48	Yes
AF 23	2016	35	Female	Inhabitant	Resident	No	12	Yes	39·0	Negative	Yes
AF 24	2016	50	Male	Ecotourism	Visitor	Yes	14	Yes	NA	80	Yes
AF 25	2016	26	Male	Ecotourism	Visitor	Yes	9	Yes	38·5	672	Yes
AF 26	2016	55	Male	Ecotourism	Visitor	Yes	14	No	39·0	1160	Yes
AF 27	2016	54	Female	Ecotourism	Visitor	Yes	18	Yes	40·0	2600	Yes
AF 28	2016	54	Male	Ecotourism	Visitor	Yes	3	No	38·5	592	Yes
AF 29	2016	51	Female	Ecotourism	Resident	Yes	11	Yes	38·0	416	Not determined§
AF 30	2016	52	Male	Ecotourism	Visitor	Yes	16	Yes	41·9	384	Yes
AF 31	2016	54	Female	Ecotourism	Visitor	Yes	10	Yes	NA	80	Yes
AF 32	2016	72	Female	Ecotourism	Visitor	Yes	13	Yes	39·0	704	Yes
AF 33	2016	53	Male	Ecotourism	Visitor	Yes	NA	Yes	NA	144	Not determined§
AF 34	2015	39	Female	Ecotourism	Visitor	Yes	13	No	NA	48	Not done
AF 35	2015	7	Male	Tourism	Visitor	No	12	Yes	39·7	48	Not done
AF 36	2015	47	Male	Ecotourism	Visitor	Yes	18	No	38·5	122	Not done
AF 37	2015	53	Male	Ecotourism	Visitor	Yes	21	Yes	39·7	80	Not done
AF 38	2015	22	Male	Gardener	Resident	Yes	9	Yes	NA	Negative	Not done
AF 39	2016	42	Male	Ecotourism	Visitor	Yes	20	Yes	39·0	256	Not done

(Table continues on next page)

	Sample collection (year)	Age (years)	Sex	Main activity developed in the area	Visitor or resident	Entry into Atlantic Forest area	Time between onset of symptoms and diagnosis (days)	Triad of malaria*	Highest axillar temperature (°C)	Parasites density (mm³/µL)	*Plasmodium simium* SNPs†
(Table continued from previous page)											
Non-human primates											
ATCC 30130	1966	..	..	..	..	..	..	..	..	..	Yes‡
MB CPRJ	2013	..	..	..	..	..	..	..	..	..	Yes
RJ 30	2016	..	..	..	..	..	..	..	..	..	Yes
RJ 59	2016	..	..	..	..	..	..	..	..	..	Yes

SNP=single-nucleotide polymorphisms. NA=not available. ATCC=American Type Culture Collection. *Fever, chills or rigors, and sweating. †SNPs identified by partial mitochondrial genome sequencing. ‡SNPs identified by whole mitochondrial genome sequencing. §Unable to achieve PCR amplification because of technical constraints.

Table: **Clinical, epidemiological, and parasitological characteristics of studied samples and identification of *Plasmodium simium* SNPs through whole or partial mitochondrial genome sequencing**

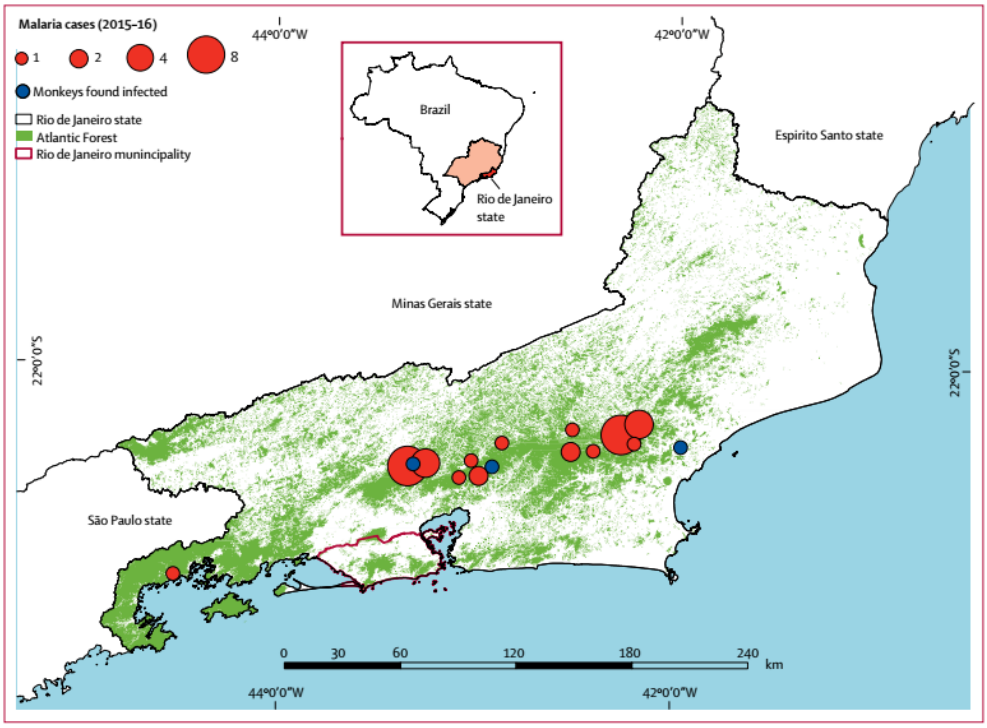

Figure 2: **Map of the Rio de Janeiro state, Brazil, showing the Atlantic Forest and indicating where human malaria cases of simian origin and monkeys infected with *Plasmodium simium* have been detected**

Human cases are represented by red spots of different sizes (symbolising one to eight cases), and the three captured, infected, wild howler monkeys are shown as blue spots. The extension of the area covered by the Atlantic Forest vegetation is indicated in green. All cases were reported in forest fragments located in Serra do Mar, and monkeys carrying *P simium* were found in the vicinity of each area. The municipality of Rio de Janeiro, delimitated with the red bold line, is free of malaria transmission.

time. However, the recent rise of ecotourism and the so-called back to nature movement might increase the opportunities for vector sharing between monkeys and human beings in this region. Despite increasing urbanisation, most of Brazil remains forested, with many human populations living in close contact with forests. The 2017 outbreak of sylvatic yellow fever in southeastern Brazil, a well established zoonosis that

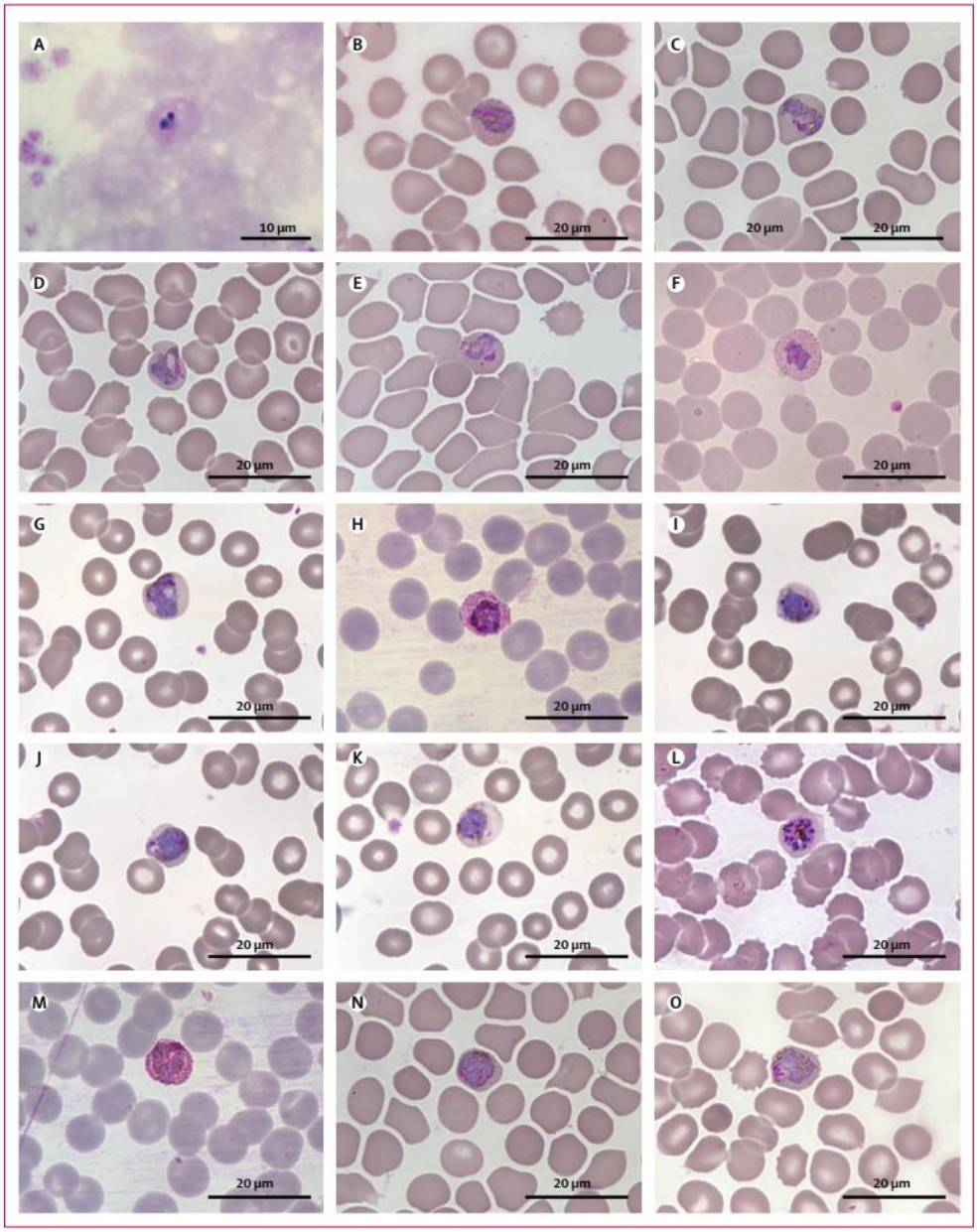

Figure 3: **Giemsa's solution-stained preparations of blood samples from human beings naturally infected with *Plasmodium simium* in Rio de Janeiro state, Brazil**
All preparations are thin blood films, except A (thick blood smear). (A) Early trophozoite. (B–F) Pleomorphic developing trophozoites. (G–L) Immature schizonts. (M) Mature schizont. (N–P) Gametocytes.

affected at least five Brazilian states, should raise concern for the possibility of the extension of occurrence of zoonotic malaria, because of the resemblance of the environmental and demographic characteristics in which both infections occur.[18] Further research is needed to elucidate these aspects.

P simium, a tertian malaria parasite found in New World non-human primates was first identified in 1951 in a monkey from the state of São Paulo and appears to be restricted to the Atlantic Forest regions of southern and southeastern Brazil.[5,19,20] Fonseca (1951),[21] Garnham (1966),[22] and Deane and colleagues (1966)[4] highlighted the morphological differences between *P vivax* and *P simium*; the trophozoites of *P simium* being less amoeboid and with coarser and more precocious and very prominent Schüffner's dots than *P vivax*. Garnham (1966)[22] reported that the detection of stippling in *P simium* early parasitised cells depends on the staining procedures. These morphological characteristics of *P simium* are consistent with those described here for the infections of human beings from the Atlantic Forest.

Although the initial diagnoses for these infections was *P vivax*, molecular evidence has revealed that these parasites are *P simium*. This misdiagnosis of a zoonotic non-human primate malaria parasite as a human parasite species has precedent and parallels the discovery of the large focus of *P knowlesi* in Borneo, which was initially attributed to *P malariae* on the basis of morphological characteristics.[23]

Despite the apparent genetic similarity of *P simium* to *P vivax*, attempts at inducing infections of *P simium* in human beings under laboratory conditions have been unsuccessful.[24] In 1966, however, Deane and colleagues[4] described the infection of a man with a *P vivax*-like parasite that they considered to be *P simium* on the basis of morphological characteristics of the parasite and because infection had occurred in a forest reserve outside São Paulo, where *P simium* was known to be transmitted. This infection remains the only previous case report of a possible human infection with *P simium*.

The clinical and parasitological features of our cases reveal that the pyrogenic threshold of *P simium* infection is considerably low. Whether this low fever threshold is related to the naive status of the individuals or specific parasitic-associated characteristics (eg, GC-content and other inflammatory factors) are yet to be better investigated.[25,26]

Patients who were naturally infected with *P simium* reported clinical symptoms congruent to symptoms of *P vivax* malaria, and responded successfully to chloroquine and primaquine, with no hospital admission, relapses, or deaths. It is not known whether *P simium* is capable of producing hypnozoites in human beings and, thus, relapses, as does *P vivax*. However, one patient (AF 3) who was treated solely with chloroquine because of G6PD deficiency and one other patient (AF 21) who discontinued primaquine treatment due to adverse events did not present any symptomatic relapse and were always negative for *Plasmodium* in all parasitological and molecular tests done during 18 months' follow-up. Further studies will be required to establish if *P simium* is capable of producing hypnozoites.

Whether this parasite can be transmitted from person to person is not known. All patients who presented with disease had entered the forest or visited the forest surroundings inhabited by howler monkeys, the main host of *P simium*. Case clustering occurred only when patients had entered such regions together, and in these cases the same time to onset of disease symptoms was observed. Although gametocytes were detected in blood smears of *P simium*-infected individuals in the present study, the infectivity of human infections of *P simium* mosquitoes is yet to be determined. Vector competence of primatophilic mosquitoes other than *A K cruzii* for *P simium* has not been studied and is a subject that needs to be urgently addressed.

Thorough screening of a large number of the local non-human primate and mosquito (*Anopheline*) populations in this area is required to evaluate the extent of this newly recognised zoonotic threat to public health. Moreover, one limitation of this study is the inclusion of samples from only one state covered by the Atlantic Forest. The analysis of both human and non-human primate samples from other areas that have been collected at different times will clarify whether the SNPs used to distinguish *P vivax* from *P simium* are specific to this region and this specific timeframe. However, the ATCC monkey sample was collected in a different region and time (50 years before) and it contains the same *P simium*-specific SNPs observed in the Rio de Janeiro Atlantic Forest. The small number of sequences from *P simium* hampers further analysis, and precludes drawing any conclusions regarding the evolution, natural history, and species status of this parasite.

This unequivocal demonstration of zoonotic *P simium* transmission leads to the possibility that this parasite, consistently misdiagnosed as *P vivax* because of an absence of molecular typing techniques, has always infected human beings in this region. Alternatively, it might be the case that *P simium* has only recently acquired the ability to frequently infect human beings, and this scenario has extremely important implications in terms of parasite–host relationships and evolution.

In summary, we report that the malaria outbreaks in 2015 and 2016 in the Atlantic Forest of southeastern Brazil were caused by *P simium*, previously considered to be a monkey-specific species of malaria parasite that is related to but distinct from *P vivax*, and which has never conclusively been shown to infect human beings before. Such zoonotic transmission of a malaria parasite from a monkey reservoir to human beings has immediate consequences for public health in this region, and for future attempts to control and eventually eliminate malaria in Brazil. Thorough screening of the local non-human primate and mosquito (*Anopheline*) populations in this area is required to evaluate the extent of this newly recognised zoonotic threat to public health.

Contributors

PB and CTD-R conceived the study. PB and AMS clinically followed-up the patients and AdP-C and CBJ obtained patients' data. FVSdA, RL-d-O, DAMdA, CBJ, AdP-C, and AP worked with the non-human samples. ACFdSS and CLP provided data from the National Program of Malaria Control from the Brazilian Ministry of Health. SS and GMZ examined (and RL-d-O reviewed) the microscopic slides and analysed the parasitological data. RL-d-O and SS contributed to the description of parasite morphological characteristics and SS did the slide photographs. MP-M described the red blood cell morphological characteristics. DAMdA and CFAdB undertook the molecular diagnosis of non-human primate samples. MdFF-d-C undertook the molecular diagnosis of human samples. ALLA carried out the mitochondrial genome sequence. MGZ, DAMdA, CFAdB, PC, MdFF-d-C, and RC did the analysis and interpretation of molecular data, and MGZ and RC did the DNA sequence analysis and the haplotype network in human and non-human primate samples. FVSdA and RL-d-O captured, made parasitological analysis, and interpreted non-human primate data. HGA and MCSM did the geographical description of the Atlantic Forest sites and the maps. PB, AdP-C, AMS, CFAdB, RL-d-O, RC, and CTD-R drafted and finalised the manuscript. All authors read, made suggestions, and approved the final manuscript.

Declaration of interests

We declare no competing interests.

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References

1 Ahmed MA, Cox-Singh J. *Plasmodium knowlesi*—an emerging pathogen. *Isbt Science Series* 2015; **10** (suppl 1): 134–40.
2 Siqueira AM, Mesones-Lapouble O, Marchesini P, et al. *Plasmodium vivax* Landscape in Brazil: scenario and challenges. *Am J Trop Med Hyg* 2016; **95** (6 suppl): 87–96.
3 de Pina-Costa A, Brasil P, Di Santi SM, et al. Malaria in Brazil: what happens outside the Amazonian endemic region. *Mem Inst Oswaldo Cruz* 2014; **109:** 618–33.
4 Deane LM, Deane MP, Ferreira Neto J. Studies on transmission of simian malaria and on the natural infection of man with *Plasmodium simium* in Brazil. *Bull World Health Organ* 1966; **35:** 805–08.
5 Deane LM. Simian malaria in Brazil. *Mem Inst Oswaldo Cruz* 1992; **87:** 1–20.
6 Coatney GR, Collins WE, Warren M, Contacos PG. The primate malarias. Bethesda, MD: US Department of Health, Education, and Welfare, 1971.
7 Alvarenga DA, de Pina-Costa A, de Sousa TN, et al. Simian malaria in the Brazilian Atlantic forest: first description of natural infection of capuchin monkeys (Cebinae subfamily) by *Plasmodium simium*. *Malar J* 2015; **14:** 81.
8 Brasil P, Costa AP, Longo CL, Silva S, Ferreira-da-Cruz MF, Daniel-Ribeiro CT. Malaria, a difficult diagnosis in a febrile patient with sub-microscopic parasitaemia and polyclonal lymphocyte activation outside the endemic region, in Brazil. *Malar J* 2013; **12:** 402.
9 Torres KL, Figueiredo DV, Zalis MG, Daniel-Ribeiro CT, Alecrim W, Ferreira-da-Cruz MF. Standardization of a very specific and sensitive single PCR for detection of *Plasmodium vivax* in low parasitized individuals and its usefulness for screening blood donors. *Parasitol Res* 2006; **98:** 519–24.
10 Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* 1993; **58:** 283–92.
11 Culleton R, Coban C, Zeyrek FY, et al. The origins of African *Plasmodium vivax*; insights from mitochondrial genome sequencing. *PLoS One* 2011; **6:** e29137.
12 Jongwutiwes S, Putaporntip C, Iwasaki T, Ferreira MU, Kanbara H, Hughes AL. Mitochondrial genome sequences support ancient population expansion in *Plasmodium vivax*. *Mol Biol Evol* 2005; **22:** 1733–39.
13 Mu J, Joy DA, Duan J, et al. Host switch leads to emergence of *Plasmodium vivax* malaria in humans. *Mol Biol Evol* 2005; **22:** 1686–93.
14 Iwagami M, Hwang SY, Fukumoto M, et al. Geographical origin of *Plasmodium vivax* in the Republic of Korea: haplotype network analysis based on the parasite's mitochondrial genome. *Malar J* 2010; **9:** 184.
15 Miao M, Yang Z, Patch H, Huang Y, Escalante AA, Cui L. *Plasmodium vivax* populations revisited: mitochondrial genomes of temperate strains in Asia suggest ancient population expansion. *BMC Evo Biol* 2012; **12:** 22.
16 Taylor JE, Pacheco MA, Bacon DJ, et al. The evolutionary history of *Plasmodium vivax* as inferred from mitochondrial genomes: parasite genetic diversity in the Americas. *Mol Biol Evol* 2013; **30:** 2050–64.
17 Rodrigues PT, Alves JM, Santamaria AM, et al. Using mitochondrial genome sequences to track the origin of imported *Plasmodium vivax* infections diagnosed in the United States. *Am J Trop Med Hyg* 2014; **90:** 1102–08.
18 Bonaldo MC, Gómez MM, Dos Santos AA, et al. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. *Mem Inst Oswaldo Cruz* 2017; **112:** 447–51.
19 Wanderley DM, da Silva RA, de Andrade JC. Epidemiological aspects of malaria in the State of São Paulo, Brazil, 1983 to 1992. *Rev Saude Publica* 1994; **28:** 192–97.
20 Cerutti-Junior C, Boulos M, Coutinho AF, et al. Epidemiologic aspects of the malaria transmission cycle in an area of very low incidence in Brazil. *Malar J* 2007; **6:** 33.
21 Fonseca F. Plasmódio de primata do Brasil. *Mem Inst Oswaldo Cruz* 1951; **49:** 543–53 (in Portuguese).
22 Garnham PCC. Malaria parasites and other haemosporidia. Blackwell Scientific Publication, Oxford, 1966: 1114.
23 Singh B, Kim Sung L, Matusop A, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 2004; **363:** 1017–24.
24 Coatney GR. The simian malarias: zoonoses, anthroponoses, or both? *Am J Trop Med Hyg* 1971; **20:** 795–803.
25 Anstey NM, Russell B, Yeo TW, Price RN. The pathophysiology of vivax malaria. *Trends Parasitol* 2009; **25:** 220–27.
26 Karunaweera ND, Ferreira MU, Hartl DL, Wirth DF. Fourteen polymorphic microsatellite DNA markers for the human malaria parasite *Plasmodium vivax*. *Mol Ecol Notes* 2007; **7:** 172–75.

7.2 Anexo II – Artigo: An assay for the identification of *Plasmodium simium* infection for diagnosis of zoonotic malaria in the Brazilian Atlantic Forest

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An assay for the identification of *Plasmodium simium* infection for diagnosis of zoonotic malaria in the Brazilian Atlantic Forest

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Zoonotic malaria poses a unique problem for malaria control. Autochthonous cases of human malaria in the Atlantic Forest have recently been attributed to *Plasmodium simium*, a parasite that commonly infects non-human primates in this Brazilian biome. However, due to its close similarity at both the morphological and molecular level to *Plasmodium vivax*, the diagnosis of *P. simium* in this region remains problematic. Therefore, a diagnostic assay able to accurately identify *P. simium* is important for malaria surveillance. Based on mitochondrial genome sequences, primers were designed to amplify a region containing a SNP specific to *P. simium*. This region can then be digested with the restriction enzyme *Hpy*CH4III, which results in digestion of *P. simium* sequences, but not of any other malaria parasite. Fifty-two human and monkey blood samples from different regions and infected with different *Plasmodium* species were used to validate this protocol. This easy and inexpensive tool can be used for the diagnosis of *P. simium* in non-human primates and human infections from the Atlantic Forest region to monitor zoonotic malaria transmission in Brazil.

Zoonotic malaria constitutes a major challenge to malaria elimination. Until recently, it was thought that there were four species of malaria parasite responsible for disease in humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. However, recent studies in Southeast Asia have demonstrated the widespread zoonotic transmission of *Plasmodium knowlesi*, a species previously thought to be confined to macaques[1]. These findings led to the recognition of *P. knowlesi* as a fifth human malaria parasite and demonstrated the zoonotic potential of non-human primate (NHP) malaria in sylvatic settings[2–4]. In addition to

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P. knowlesi, there are other *Plasmodium* species that infect NHPs and can also be transmitted to humans, such as *Plasmodium cynomolgi* in Asia and *Plasmodium brasilianum* and *Plasmodium simium* in the Americas[5–8].

Malaria transmission in Brazil occurs almost entirely within the Amazon region[9]. However, there are a consistent number of autochthonous cases reported in Southern and Southeastern Brazil, predominantly in areas under influence of the Atlantic Forest. From 2006 to 2016, 1047 autochthonous cases were reported in these areas[9–11]. Most of these episodes are attributed to *P. vivax* and essentially affect non-resident visitors[11]. These areas are particularly rich in bromeliads, which provide a highly suitable habitat for *Kerteszia* mosquito species, especially *Anopheles Kerteszia cruzii* and *Anopheles Kerteszia bellator*[12–14]. These mosquitoes are the primary vectors of the called "bromeliad malaria"[12,15–17]. These species may bite humans and non-human primates (NHPs) with similar frequencies, depending on the area and environmental/climatic situation[16,18–20]. Based on this, zoonotic transmission has been hypothesized in these areas, with NHPs acting as a reservoir of *Plasmodium* species capable of infecting humans[5]. There are two species of malaria parasites infecting non-human primates in this region; *Plasmodium simium*, a tertian malaria and *Plasmodium brasilianum*, a quartan malaria. *Plasmodium simium* naturally infects primates of the Atelidae and Cebidae families, genera *Alouatta* (howler monkeys), *Brachyteles* (wooly spider monkeys), *Cebus* and *Sapajus* (capuchin monkeys) in South and Southeastern Brazil[21,22]. *Plasmodium brasilianum* has been detected in nearly 50 NHPs species belonging to 13 genera distributed from Panama to the South of Brazil[21,23,24].

Plasmodium simium is highly similar at the morphological, genetic and immunological levels to *P. vivax*, the most prevalent human malaria parasite in Brazil[21,25,26]. Due to this, human malaria in the Atlantic Forest has previously been misdiagnosed as *P. vivax*, either by microscopy or through molecular diagnosis based on PCR of the 18S rRNA gene. However, recently, a study in the Rio de Janeiro Atlantic Forest described morphological and genetic differences (based on mitochondrial genome sequencing) between these two species, and incriminated *P. simium* as the causative agent of the great majority of human malaria cases in the region[27]. This observation was confirmed by other authors analyzing different Brazilian states under the influence of the same biome[28,29].

As morphological discrimination is highly dependent on the subjective consideration of very well-trained microscopists, we have developed a new methodology able to identify *P. simium* samples using nested PCR followed by enzyme digestion without the need for nucleotide sequencing. This methodology relies on the fact that all known *P. simium* mitochondrial genome sequences (derived from infection of NHPs from Atlantic forest) differ from the most closely related *P. vivax* sequences (derived from infection of humans) by two unique single nucleotide polymorphisms (SNPs). This protocol can be used for the screening of the local NHPs and humans malaria cases during surveillance of zoonotic malaria transmission in the Brazilian Atlantic Forest.

Methods

Non-human primate samples from the Atlantic Forest. Sixteen *P. simium* and two *P. brasilianum* infected whole blood samples were obtained from captive and free-living NHPs from the Brazilian Atlantic Forest of Rio de Janeiro and Santa Catarina States (Additional file 1)[22,24]. Animals used in this study were captive primates from the Primate Centre of Rio de Janeiro (CPRJ) (n = 2) and from Biological Research Centre of Indaial (CEPESBI) (n = 7) and free living NHPs from Santa Catarina State (n = 6) and Rio de Janeiro State (n = 1). The CPRJ (Brazilian Institute of Environment and Renewable Natural Resources-IBAMA, register number 458460) is a unit for wild monkey protection. It is geographically located between latitude 22°7′ and 22°32′ S and longitude 42°50′ and 42°56′ W in the municipality of Guapimirim (one of the municipalities where human malaria occurs in the Rio de Janeiro Atlantic Forest), on the Serra dos Órgãos slopes, in an area of the Atlantic Forest, about 100 km from the city of Rio de Janeiro. Serra dos Órgãos is part of the Serra do Mar, a large coastal mountain chain in southeast Brazil. As the handling of NHPs was exclusively performed by CPRJ technicians, the Fiocruz Animal Ethics Committee agreed to the protocol for sample collection. Captive Southern brown howler monkeys (*Alouatta guariba clamitans)* from CEPESBI (Indaial, SC) (IBAMA register number 197351) were also included[30]. Free-living NHPs from the Atlantic Forest in Joinville municipality, in Santa Catarina State in southern Brazil, were obtained from a behavioral study of *Alouatta g. clamitans* in the Brown Howler monkeys conservation program (Perini Business Park). The Brazilian government authorized this study and the access to and transport of biological samples through Biodiversity Information and Authorization System (SISBIO) no. 43375-4/2015. *P. simium* DNA extracted from the spleen and liver of a single *Alouatta g. clamitans* found dead in Guapimirim was used as positive control in the PCR because it was previously sequenced by us[22]. Capture, handling and blood sampling of free living primate in Rio de Janeiro State were approved by the SISBIO licenses 54707-137362-2 and 52472-1, and INEA license 012/2016012/2016) and the Institutional Ethics Committee of animal use (CEUA license L037/2016). All experiments were performed according to relevant guidelines and regulations.

Human DNA samples from the Atlantic Forest. Whole blood samples from patients with symptoms suggestive of malaria were used for DNA extraction. Patients were selected based on history of travel to or habitation in areas within the Atlantic Forest and a positive test by thick blood smear or PCR. The following exclusion criteria were applied: malaria prophylaxis, previous history of malaria, blood transfusion or tissue/organ transplantation, use of intravenous drugs, needle stick injury, residence or recreation near ports or airports or travel to known malaria endemic areas in the preceding year. Cases considered here are from patients who attended the Acute Febrile Illnesses Unit of the *Instituto Nacional de Infectologia Evandro Chagas*, which integrates the Center for Malaria Research, Diagnosis and Training of Fiocruz, a reference center for malaria in the Extra-Amazonian Region for the Ministry of Health. Patients' blood samples from Atlantic Forest area diagnosed as positive for *P. vivax* by optical microscopy or PCR[31] were included here (Additional file 2).

DNA extraction. DNA was extracted from whole blood using the QIAGEN Kit (PUREGENE®, Gentra Systems, Minneapolis, USA) according to the manufacturer's protocol. The DNA was diluted in 30 µl of TE Buffer and stored at −20 °C until used in the experiments.

Human DNA samples from Amazon region. Samples of three *Plasmodium* species from patients previously diagnosed by microscopy and nested PCR[32] were utilized to test the specificity of the novel *P. simium* primers: 2 *P. falciparum*, 15 *P. vivax* and 2 *P. malariae*, besides 2 malaria negative samples (Additional file 2). The *P. vivax* DNA samples selected for use in this study were from different parts of Amazon (Amazonas, Rondônia, Pará and Acre states of Brazil, Venezuela, French Guiana and Guyana) and were stored at the biorepository of Laboratory of Malaria at IRR.

Ethical clearance for human samples. The Ethics and Research Committee of INI-IPEC and Fiocruz Ethical Committee in Research approved the study (No. 0062.0.009.000-11). The methods used for human DNA collection were performed according to relevant guidelines and regulations. All participants and/or their legal guardians provided written informed consent.

Nested PCR and RFLP. Primer pairs for the nested PCR are: first reaction - *PsimOUTF* 5′CA GGTGGTGTTTTAATGTTATTATCAG3′ (forward) and *PsimINR* 5′ATGTAAACAATCCAATAATTGCACC3′ (reverse); and second reaction - *PsimEDF* 5′ATCCTACATTTGCTGGAGATCCTA3′ (forward) and *PsimEDR* 5′GCTCTTGTATCTACTTCTAAACCTGTAG3′ (reverse). The first reaction was performed in 20 µL volumes containing 0.5 µM of each oligonucleotide primer, 2 µL DNA, 0.2 µL Taq DNA Polymerase (Invitrogen, 5U/µL), 0.2 mM each deoxyribonucleotide triphosphates and 1.5 mM $MgCl_2$. The PCR assays were performed in a thermocycler (Veriti 96 wells, Applied Biosystems) with the following cycling parameters: an initial denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 20 sec and extension at 72 °C for 30 sec followed by a final extension incubation at 72 °C for 2 min. The temperature was then reduced to 4 °C until the samples were taken. For the second PCR 1–3 µL of the primary product was used as template. The cycling parameters for the second round of PCR were the same as the first round. The amplified fragments were visualized by electrophoresis on 2% agarose gels in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) with 5 µg/mL ethidium bromide (Invitrogen) in a horizontal system (Bio-Rad) at 100 V for 30 min. Gels were examined with a UV transilluminator (UVP - Bio-Doc System it).

To prevent cross-contamination, the DNA extraction and mix preparation were performed in "parasite DNA-free rooms" distinct from each other. Furthermore, each of these separate areas has different sets of pipettes and all procedures were performed using plugged pipette tips. DNA extraction was performed twice on different days. Positive (DNA extracted from blood from patients with known *P. vivax* infection) and negative (no DNA and DNA extracted from individuals who have never traveled to malaria-endemic areas) controls were used in each round of amplification. The sources of genomic DNA samples that served as positive controls in the nested PCR assays are: i) *P. falciparum* DNA, strain 3D7 maintained in Malaria Laboratory (IRR-Fiocruz MINAS); (ii) DNA of extracted from the blood of a patient with high parasitemia for *P. vivax* and DNA of *P. simium* of a non-human primate with an acute infection and parasitemia confirmed by optical microscopy; (iii) DNA of *P. brasilianum* from the MR4 (ATCC, USA).

Following amplification (verified by agarose gel electrophoresis), PCR products were digested with the restriction enzyme *Hpy*CH4III (New England biolabs, Ipswich, MA, USA). The restriction enzyme profile was identified using NEBcutter[33]. The digestion was performed in 10 µL containing 0.5 µL of the enzyme (5U/µL), 1 µL of the enzyme's buffer and 5 µL of PCR product (2–7 µL according to the intensity of PCR products on agarose gels). The digestion was incubated at 37 °C for 3 hours. All digestion reaction and the equivalent amount of non-digested DNA were visualized in electrophoresis on 3% agarose gel and examined under a UV transilluminator. Electrophoresis was performed in a room specific for amplified DNA, with appropriated sets of pipettes and plugged pipettes tips. For some samples, it was necessary to modify the amount of DNA in the PCR, PCR product in the second reaction or PCR product used in the digestion.

Limits of detection of PCR amplification. The analytical sensitivity of the assay was determined using a well-quantified parasitemia of *P. simium* obtained from a patient infected in the Atlantic Forest. The percent parasitemia was determined by Giemsa's solution-stained blood smears. To estimate parasite density per µL of blood, a standard mean WBC count of 6,000/µL blood was assumed. All slides were subsequently examined by an independent malaria microscopist qualified by the PAHO/WHO malaria accreditation course.

The resulting parasitemia was determined to be nearly 100 parasites/µL in this patient. DNA was extracted using 300 µL of blood sample. This standard DNA sample was then serial diluted two-fold to 1.65 parasites/µL. These diluted DNA samples were used to test the limits of detection of the novel *P. simium* primers set described here.

DNA Sequencing. 1–2 µL of PCR products were amplified using 5 µM of forward or reverse species-specific primer (*PsimEDF* or *PsimEDR*), 0.5 µL of BigDye terminator and 1 µL of BigDye Buffer (Applied Biosystems) for DNA sequencing. In addition, PCR products were sequenced using the primers *PsimINF* 5′ GCTGGAGATCCTATTTTATATCAAC 3′ (forward) or *PsimINR* 5′ATGTAAACAATCCAATAATTGCACC 3′ (reverse), with the same PCR conditions, resulting in sequences that covered both SNPs we consider diagnostic of *P. simium*. The following cycling parameters were used in both situations: 96 °C for 1 min, 35 cycles of 96 °C for 15 sec, followed by the temperature of primer annealing (54 °C) for 15 sec and 60 °C for 4 min. The fragments were precipitated using ammonium acetate, suspended in formamide HI-DI (Applied Biosystems) and electrophoretically separated in ABI 3730 DNA automatic sequencer. Sequences were aligned using ClustalW software in Bioedit package[34] and Chromas software[35].

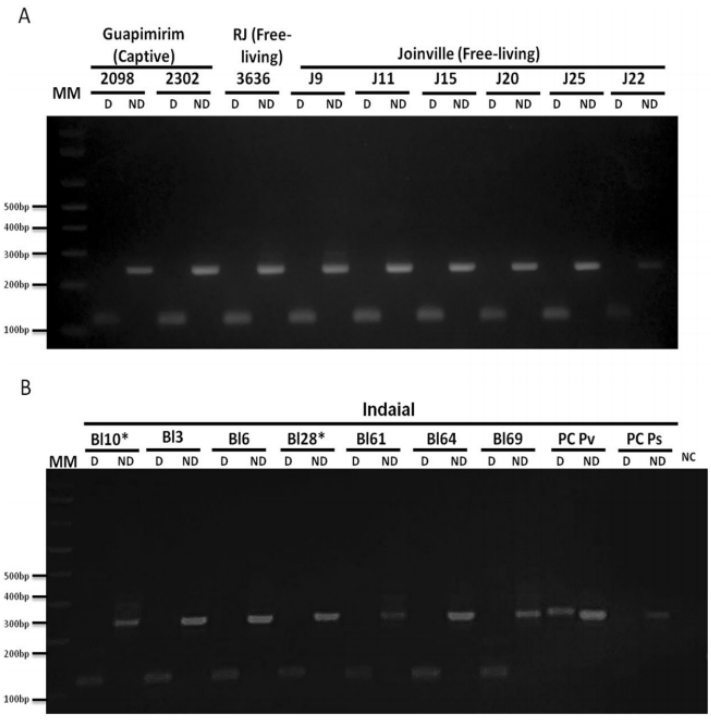

Figure 1. Differential Diagnosis of *Plasmodium simium* infection by nested/PCR followed by a digestion with *Hpy*CH4III restriction enzyme of 16 non-human primate samples: (**A**) 2 captive NHP from Rio de Janeiro/RJ (*Sapajus xanthosternos* 2098; *Cacajao melanocephalus* 2302), one free-living *Alouatta g. clamitans* from Rio de Janeiro State (3636) and 6 free-living *Alouatta g. clamitans* from Joinville/SC, Brazil (J9, J11, J15, J20, J22, J25); (**B**) 7 *Alouatta g. clamitans* from CEPESBI, Indaial, SC, Brazil (Bl3, Bl6, Bl10, Bl28, Bl61, Bl64, Bl69), *Captive NHPs, all the other were free-living. More details about each sample see Additional file 1. 3% agarose gel stained with ethidium bromide. MM:1 kb Plus Ladder (ThermoFischer). Reactions were performed simultaneously in the same thermocycler and splited in different gels. PC Pv: Positive Control for *P. vivax*, PC Ps: positive control for *P. simium*. NC: Negative Control (without DNA).

Results

Plasmodium simium primer design. *Plasmodium simium* whole mitochondrial genome sequence (accession numbers AY800110, NC_007233 and AY722798, all of which have identical sequences) was compared with 794 *P. vivax* mitochondrial genome sequences deposited in Genbank[27]. *Plasmodium simium* differs from the most closely related *P. vivax* at two unique single nucleotide polymorphisms (SNPs) in the mitochondrial genome, at positions 3535 (T>C) and 3869 (A>G) according to the nucleotide numbering system employed in Brasil *et al.*[27]. Restriction enzyme profiling of the fragment containing these two polymorphisms was performed using NEBcutter. Restriction enzyme *Hpy*CH4III recognizes the site ACNGT which includes one of the two *P. simium* specific SNPs. The "**T**" in *P. vivax*, as well in all other *Plasmodium* species tested, is substituted by a "**C**" in *P. simium* (position 3535). This polymorphism adds a new restriction site for *Hpy*CH4III. Based in the alignment of this region, primers were manually designed for a nested PCR to increase the sensitivity according to the expected low parasitemia. Primer candidates were screened for GC-content, melting temperature, secondary structure, and primer-dimer forming potential. The best pairs were selected with an amplified region of 244 bp, the *P. simium* type of which being digested by *Hpy*CH4III to two bands, one 118 bp and the other 126 bp.

Standardization of Nested/PCR-RFLP. In order to optimize the Nested/PCR, different annealing temperatures from 45 °C to 60 °C were tested. Primer concentrations from 0.5 mM to 1.0 mM were evaluated at 1.5 mM $MgCl_2$ concentration (data not shown). Optimal amplification was obtained with the use of 54 °C annealing temperature, with 0.5 mM of primer. Amplified products (verified in agarose gel) were digested using the restriction enzyme *Hpy*CH4III.

Differential diagnosis of _P. simium_ using Nested/PCR – RFLP. Samples from NHPs infected by *P. simium* were analyzed using the standardized protocol. Captive and free living NHPs were from 3 different areas and samples were collected at different times (additional file 1). All 16 samples showed the digestion profile characteristic of *P. simium* (Fig. 1). Also, 8 from 9 humans infected in the Atlantic Forest showed this profile (Fig. 2).

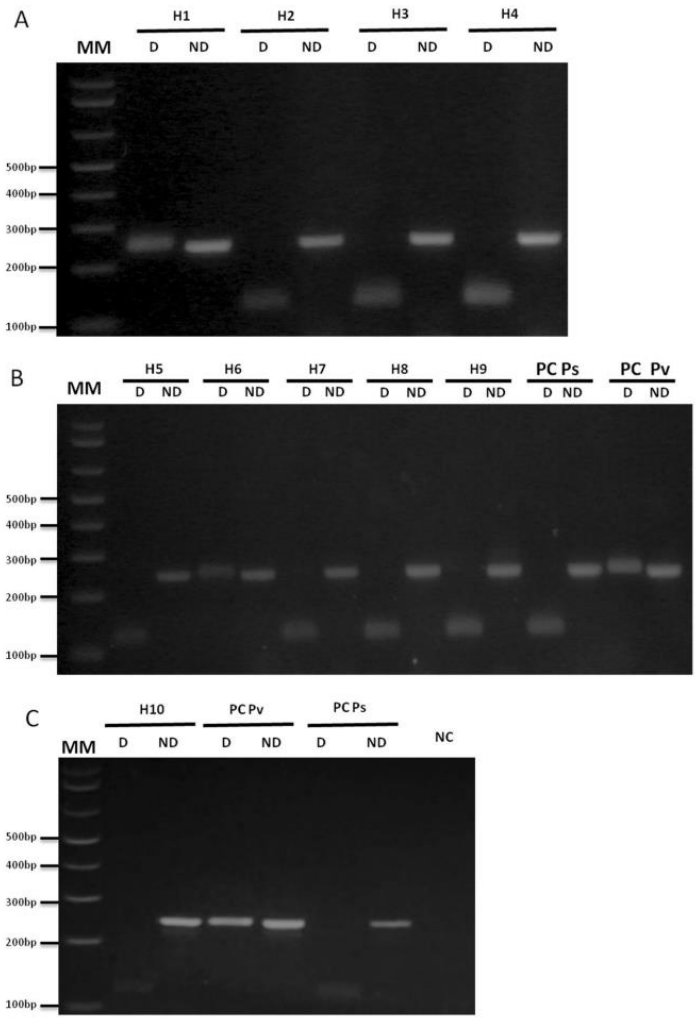

Figure 2. Differential diagnosis of *Plasmodium simium* infection by nested/PCR followed by a digestion with *Hpy*CH4III restriction enzyme of 9 infected human samples from Atlantic Forest in Rio de Janeiro/RJ (H2 – H9) and one from Amazon endemic region (H1) according to Additional file 1. 3% agarose gel stained with ethidium bromide. MM:1 kb Plus Ladder (ThermoFischer, Calrsbad, CA, USA). Reactions were performed simultaneously in the same thermocycler and splited in different gels.D: Digested; ND: Non Digested. PC Pv: Positive Control for *P. vivax*, PC Ps: positive control for *P. simium*. NC: Negative Control (without DNA).

Plasmodium vivax from different areas of Amazon showed the profile of absence of digestion (Fig. 3). To confirm that the absence of digestion was not due to low levels of DNA, increased amounts of PCR product from three *P.vivax*-infected patients were used in the digestion with no digestion observed (supplementary Fig. S1). Similarly, DNA from other *Plasmodium* species (*P. falciparum*, *P. brasilianum*/*P.malariae*) were amplified by PCR but were not digested by *Hpy*CH4III (Fig. 4A and B). DNA extracted from the blood of uninfected humans, uninfected non-human primate and non-DNA solutions were included as negative controls in Nested/PCR (Fig. 4C).

Sequencing of PCR products. Products of Nested/PCR were sequenced. Sequences obtained were aligned with sequences available at GenBank (Fig. 5). The *P. simium* specific SNP was confirmed in the majority of Atlantic Forest samples, including all NHPs and 8 out 9 of human-derived samples. In addition, all samples were subjected to PCR and sequencing of a larger product that included both potentially diagnostic SNPs. All samples that contained the 3535 (T>C) SNP also carried the 3869 (A>G) SNP, and no hybrids carrying one or the other SNP in isolation were observed (Fig. S2).

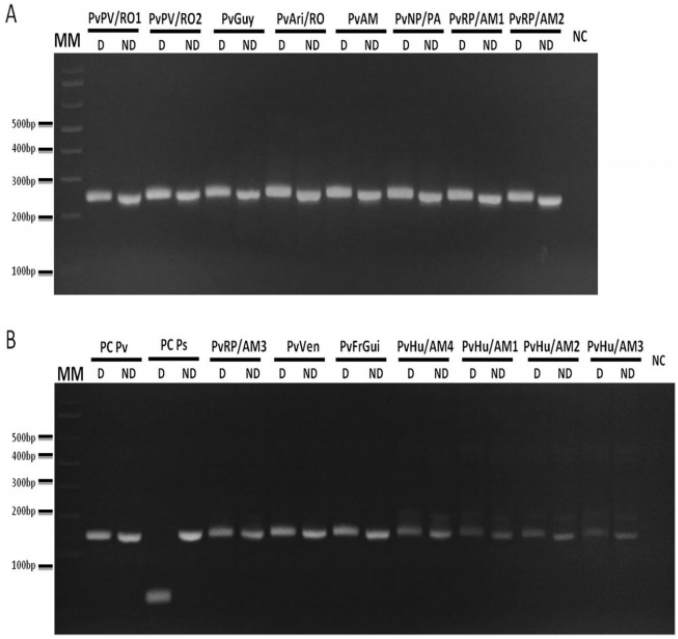

Figure 3. Nested/PCR-RFLP of *P. vivax* DNA samples. 15 DNA samples from *P. vivax* infected individuals from different parts of Amazon: Porto Velho/Rondônia State, Brazil (PvPV/RO1 and 2), Guyana (PvGuy), Ariquemes/Rondônia State, Brazil (PvAri/RO), Venezuela (PvVen), French Guiana (PvFrGui), Novo progresso/Pará State, Brazil (PvNP/PA), Rio Pardo/Amazonas State, Brazil (PvRP/AM1, 2 and 3), Humaita/Amazonas State, Brazil (PvHu/Am1, 2, 3 and 4) and unknown city in Amazonas State, Brazil (PvAM) were used for Nested PCR amplification followed by digestion with *Hpy*CH4III restriction enzyme. 3% Agarose gel stained with ethidium bromide. Name tags above gels indicated the patients according to Additional file 2. Reactions were performed simultaneously in the same thermocycler and splited in different gels. MM: 1 kb Plus Ladder. D: Digested (8 μL of digestion); ND: Non Digested (the equivalent amount of PCR product used in the digestion, 6.5 μL of samples and 5 μL of controls); PC Pv: positive control of *P. vivax* (pool of samples from infected patient from Amazonia); PC Ps: positive control of *P. simium* (*Alouatta g. clamitans* infected with *P. simium* previously sequenced[30]); NC: negative Control.

Limits of detection of the Nested PCR. Limits of detection of the Nested-PCR as were tested with *P. simium* DNA extracted from an infected human serially diluted two-fold with a starting parasitemia of 100 parasites/μL to 1.65 parasites/μL. The novel Nested PCR assay was able to detect down to 3.12 parasites/μL (Fig. 6).

Discussion

Zoonotic transmission of *Plasmodium* spp. complicates the control of malaria as NHPs can act as reservoirs. The process of urban expansion potentially increases contact between free-living NHPs and humans, as towns and cities encroach on previously untouched forested areas. There is a large human population worldwide currently living in the close vicinity of forests where many different species of NHPs potentially carry *Plasmodium* spp.

Neotropical Platyrrhine primates are distributed from Mexico to Argentina. To date, *P. simium* has been described only in the Atlantic Forest of South and Southeastern Brazil. However, it is possible that this species might also be prevalent in other biomes in South America, and that its zoonotic transmission could be a much larger problem than currently recognized. Herein we described an inexpensive tool for the discrimination of *P. vivax* from the agent we currently consider to be *P. simium*.

Only two unique single nucleotide polymorphisms (3535 T>C and 3869 A>G) in the whole mitochondrial genome sequence differentiate *P. simium* from *P. vivax*[27,28]. All 16-monkey samples from 3 different areas of Brazilian Atlantic Forest show both these polymorphisms. Up to now, there has not been a single case of a parasite detected in a NHP in Brazil which did not harbor the *P. simium* version of these SNPs. Every single *P. vivax*-like parasite found in New World NHPs carries it, including the 'type' *P. simium* strain deposited at the US Centers for Disease Control and Prevention (CDC) repositories[36].

Buery and colleagues have recently reported the presence of the two *P. simium* SNPs in one monkey sample and in 17 of 22 human infections from the Atlantic Forest area[29].Two out of the 22 infections carried *P. vivax* versions of the SNPs at both loci. It is possible that these latter cases are due to imported *P. vivax* from Amazonia. However, they also found 3 of the 22 Atlantic Forest-human samples harbouring only one of the *P. simium*-specific SNPs that have not previously been observed in isolation. It is possible that these latter samples

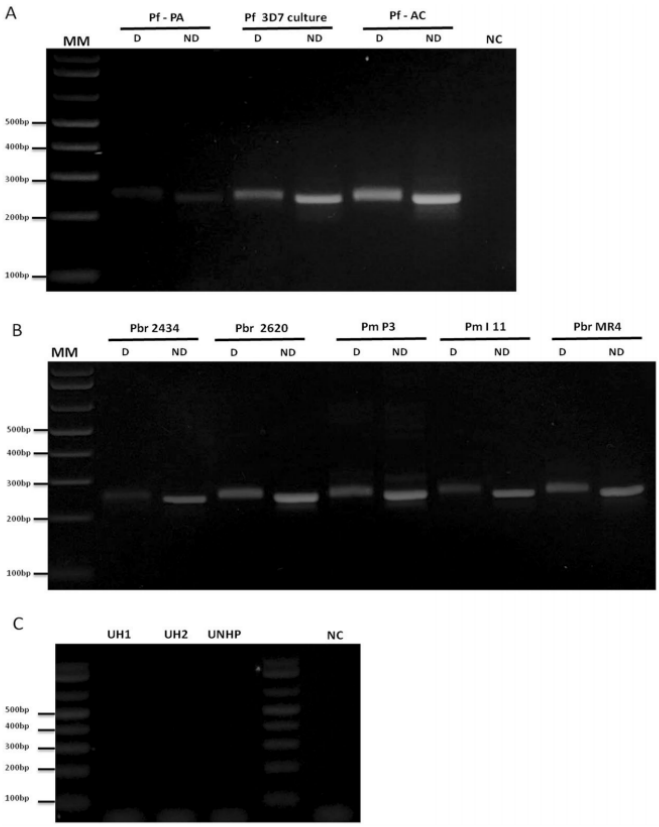

Figure 4. Nested/PCR-RFLP of (**A**) *Plasmodium falciparum* samples, two patients from Pará State (Pf – PA) and Acre State (Pf – AC) and Pf 3D7 culture (diluted 1:100); (**B**) *Plasmodium brasilianum/Plasmodium malariae*, two NHPs (Pbr 2434 and Pbr 2620), two patients (Pm P3 and Pm l11) and *Plasmodium brasilianum* from MR4 (diluted 1:100 in water); (**C**) Negative controls of PCR: two uninfected humans (UH - 1 and 2), one uninfected NHP (UNHP), and negative control of PCR (NC - without DNA). Reactions were performed simultaneously in the same thermocycler and splited in different agarose gels. 3% Agarose gelstained with ethidium bromide. MM: 1 kb Plus Ladder. D: Digested; ND: Non Digested.

are the result of mixed infections (*P. vivax* and *P. simium*) or are *P. vivax* variants that do not infect NHPs (as these haplotypes have never been observed in NHPs). It is unlikely that these infections were caused by *P. simium* variants because of the low genetic variability of this parasite[27,28,30]. Espirito Santo State has a history of intense migration to Amazon area, where *P. vivax* is endemic[37], and it may be expected that both parasites circulate in this area due to the presence of different anopheline species[38]. However any firm conclusions concerning the origins and relatedness of parasites in this study are hampered by the small number of samples collected from NHPs (n = 1), and differing times and locations of collection of samples.

It is possible that "*P. simium*" infecting NHPs in Brazil is, in fact, a variant strain of the human parasite *P. vivax*, and does not constitute a separate species based on a strict biological definition. It is possible that what we now consider to be *P. simium* and *P. vivax* are actually one and the same species, variants of which circulate freely between humans and NHPs in the Atlantic Forest when vectors and ecology allow. It will only be possible to conclusively address this issue with thorough surveillance of parasites from NHPs, and whole genome sequencing data. However, whether we consider *P. simium* a separate species, a sub-species or a strain *of P. vivax* does not alter the epidemiology and public health consequences of a monkey harbored parasite that is able to infect humans.

The *P. simium* specific SNP that forms the basis of our diagnostic method is carried by all parasites isolated from NHPs so far. It was also observed in the majority (8 out 9) of Atlantic Forest human samples. The other human case was a *P. vivax* infection that might be due to an undetected index case from Amazon area. No other *Plasmodium* strain or species has this polymorphism, as show in Fig. 5. We can be reasonably certain, in the absence of evidence to the contrary, that this SNP is exclusive to *P. simium*, and can be used to distinguish this

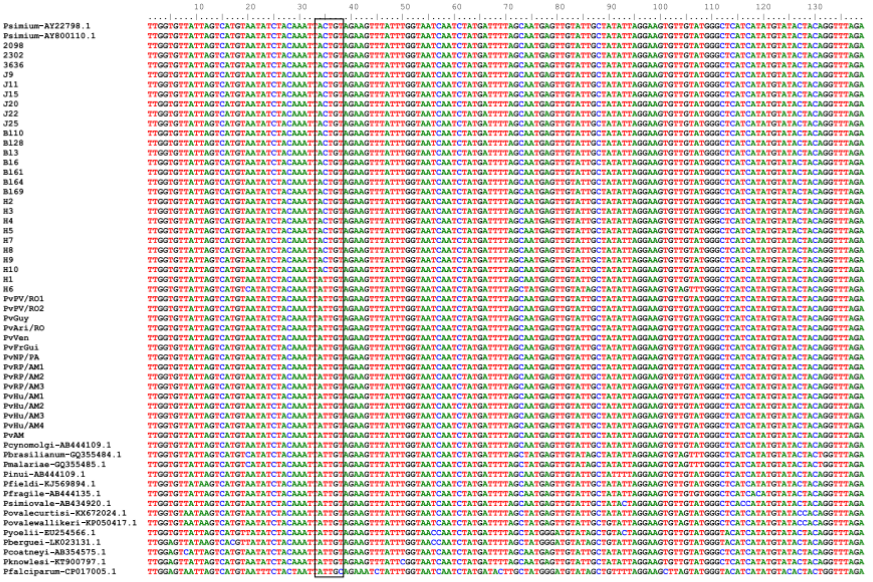

Figure 5. Alignment of partial mitochondrial sequences of *Plasmodium simium* isolated from captive (2098, 2302, 3636, BL10 and BL28) and free living NHPs (BL3, BL6, BL61, BL64, BL69, J9, J11, J15, J20, J22 and J25) from Atlantic forest; humans (H2, H3, H4, H5, H7, H8, H9 and H10) infected with *P. simium* at Atlantic Forest; human samples obtained in Atlantic Forest infected with *P. vivax* (H1 and H6); *P. vivax* isolated from human from Brazilian Amazonia: (PvPV/RO1 and PvPV/RO2 (Porto Velho, Rondonia), PvGuy (Guyana), PvAri/RO (Ariquimedes, Rondônia), PvVen (Venezuela), PvFrGui (French Guiana), PvNP/PA (Novo Progresso, Pará), PvRP/AM1- PvRPAM3 (Rio Pardo, Amazonia), PvHu/AM1 - PvHu/AM4 (Humaita, Amazonia) and PvAM (Amazonia State). These sequences were identified herein. Genbank sequences from *P. simium* (two sequences), *P. cynomolgi*, *P. inui*, *P.fieldi*, *P. fragile*, *P. coatneyi*, *P. simiovale*, *P. berguei*, *P. falciparum*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. yoelii* and *P. knowlesi* (accession number at genbank included in the name of sequence). Box delimited the site of *Hpy*CH4III restriction enzyme (ACNGT), including SNP T>C at position 3535[27,28].

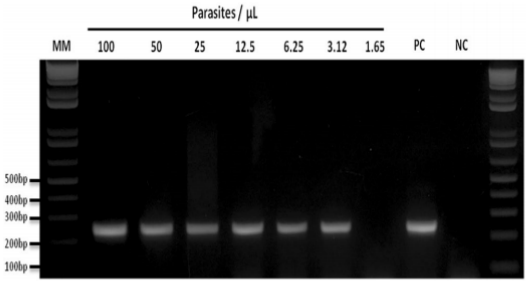

Figure 6. Detection limit of the Nested-PCR for a differential diagnosis of *Plasmodium simium. P. simium* human DNA sample were serially diluted 2-fold with a starting parasitemia of 100 parasites/µL to 1.65 parasites/µL. 2% Agarose gel stained with ethidium bromide. MM:1 kb Plus Ladder, PC: positive control, NC: negative control (without DNA).

zoonotic species from other species of malaria parasite. The novel Nested PCR assay was able to detect ≥3.12 parasites/µL, which is similar to the detection limit of the most used 18S-based PCR protocol in our laboratory[30,32].

In conclusion, we show that humans and NHPs in the Atlantic Forest region harbor the same parasite. We, therefore, confirm our previous findings showing that NHPs constitute a potential zoonotic reservoir, now with a greater number of samples, and conclude that zoonotic transmission is probably occurring in this biome[27]. The assay described here will, therefore, aid the surveillance of zoonotic malaria transmission in Brazil. Further

sampling from NHPs, and further genetic characterization of their parasites and those from humans in the same region will further elucidate the relationships between the parasites circulating between different host species in the Atlantic Forest as well in regions of Brazil and South America.

Data availability. All data provided in this manuscript is available for open access.

References

1. Cox-Singh, J. Zoonotic malaria: *Plasmodium knowlesi*, an emerging pathogen. *Curr Opin Infect Dis* **25**, 530–536, https://doi.org/10.1097/QCO.0b013e3283558780 (2012).
2. Singh, B. *et al.* A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* **363**, 1017–1024, https://doi.org/10.1016/S0140-6736(04)15836-4 (2004).
3. Antinori, S., Galimberti, L., Milazzo, L. & Corbellino, M. *Plasmodium knowlesi*: the emerging zoonotic malaria parasite. *Acta Trop* **125**, 191–201, https://doi.org/10.1016/j.actatropica.2012.10.008 (2013).
4. Yusof, R. *et al.* High proportion of knowlesi malaria in recent malaria cases in Malaysia. *Malar J* **13**, 168, https://doi.org/10.1186/1475-2875-13-168 (2014).
5. Deane, L. M., Deane, M. P., Ferreira & Neto, J. Studies on transmission of simian malaria and on the natural infection of man with *Plasmodium simium* in Brazil. *Bull World Health Organ* **35**, 805–808 (1966).
6. de Arruda, M., Nardin, E. H., Nussenzweig, R. S. & Cochrane, A. H. Sero-epidemiological studies of malaria in Indian tribes and monkeys of the Amazon Basin of Brazil. *Am J Trop Med Hyg* **41**, 379–385 (1989).
7. Lalremruata, A. *et al.* Natural infection of *Plasmodium brasilianum* in humans: man and monkey share quartan malaria parasites in the Venezuelan Amazon. *EBioMedicine* **2**, 1186–1192, https://doi.org/10.1016/j.ebiom.2015.07.033 (2015).
8. Ta, T. H. *et al.* First case of a naturally acquired human infection with *Plasmodium cynomolgi*. *Malar J* **13**, 68, https://doi.org/10.1186/1475-2875-13-68 (2014).
9. Ministério da Saúde. Situação Epidemiológica da Malária no Brasil, 2000 a 2011. Boletim Epidemiológico. vol 44 n1 Brasília, DF, Ministério da Saúde, Secretaria de Vigilância em Saúde (2013).
10. Oliveira-Ferreira, J. *et al.* Malaria in Brazil: an overview. *Malar J* **9**, 115, https://doi.org/10.1186/1475-2875-9-115. (2010).
11. de Pina-Costa, A. *et al.* Malaria in Brazil: what happens outside the Amazonian endemic region. *Mem Inst Oswaldo Cruz* **109**, 618–633 (2014).
12. Aragão, M. B. Sobre a biomassa dos Anopheles do subgênero Kerteszia em seus criadouros. *Mem Inst Oswaldo Cruz* **66**, 227–237 (1968).
13. Carvalho-Pinto, C. J. & Lourenço-de-Oliveira, R. Isoenzimatic analysis of four *Anopheles (Kerteszia) cruzii* (Diptera: Culicidae) populations of Brazil. *Mem Inst Oswaldo Cruz* **99**, 471–475 (2004).
14. Laporta, G. Z., Ramos, D. G., Ribeiro, M. C. & Sallum, M. A. M. Habitat suitability of *Anopheles vector species and association with human* malaria in the Atlantic Forest in south-eastern Brazil. *Mem Inst Oswaldo Cruz* **106**(Suppl. I), 239–245 (2011).
15. Forattini, O. P. Entomologia Médica. Vol. 1, S. Paulo, 662 pp (Faculdade de Higiene e Saúde Pública, 1962).
16. Deane, L. M. Malaria vectors in Brazil. *Mem Inst Oswaldo Cruz* **81**(Suppl II), 5–14 (1986).
17. Duarte, A. M. *et al.* Natural infection in anopheline species and its implications for autochthonous malaria in the Atlantic Forest in Brazil. *Parasit Vectors* **6**, 58, https://doi.org/10.1186/1756-3305-6-58 (2013).
18. Azevedo, A. L. Aspectos da Epidemiologia da Malária e da Biologia de Anopheles (Kerteszia) cruzii Dyar & Knab em Vales Montanhosos do Sistema de Mata Atlântica. Rio de Janeiro [Dissertação de mestrado]. Instituto Oswaldo Cruz, Fiocruz, 94p (1997).
19. Cerutti-Junior, C. *et al.* Epidemiologic aspects of the malaria transmission cycle in an area of very low incidence in Brazil. *Malar J* **6**, 33 (2007).
20. Ueno, H. M., Foratinni, O. P. & Kakitani, I. Distribuição vertical e sazonal de *Anopheles (Kerteszia)* em Ilha Comprida, SP. *Rev Saúde Pública* **41**(2), 269–275, https://doi.org/10.1186/1475-2875-6-33 (2007).
21. Deane, L. M. Simian malaria in Brazil. *Mem Inst Oswaldo Cruz* **87**(Suppl 3), 1–20 (1992).
22. Alvarenga, D. A. *et al.* Simian malaria in the Brazilian Atlantic forest: first description of natural infection of capuchin monkeys (Cebinae subfamily) by *Plasmodium simium*. *Malar J* **14**, 81, https://doi.org/10.1186/s12936-015-0606-6 (2015).
23. Lourenço-de-Oliveira, R. & Deane, L. M. Simian malaria at two sites in the Brazilian Amazon. I—the infection rates of *Plasmodium brasilianum* in non-human primates. *Mem Inst Oswaldo Cruz* **90**, 331–339 (1995).
24. Alvarenga, D. A. *et al.* New potential Plasmodium brasilianum hosts: tamarin and marmoset monkeys (family Callitrichidae). *Malar J* **16**(1), 71, https://doi.org/10.1186/s12936-017-1724-0 (2017).
25. Leclerc, M., Hugot, J., Durand, P. & Renaud, F. Evolutionary relationships between 15 *Plasmodium* species from new and old world primates (including humans): an 18S rDNA cladistic analysis. *Parasitology* **129**, 677–684 (2004).
26. Duarte, A. M. *et al.* Widespread occurrence of antibodies against circumsporozoite protein and against blood forms of *Plasmodium vivax*, *P. falciparum* and *P. malariae* in Brazilian wild monkeys. *J Med Primatol* **35**, 87–96, https://doi.org/10.1111/j.1600-0684.2006.00148.x (2006).
27. Brasil, P. *et al. Plasmodium simium* causing human malaria: a zoonoses with outbreak potential in the Rio de Janeiro Brazilian Atlantic forest. *Lancet. Global Health* **5**, e1038–e1046, https://doi.org/10.1016/S2214-109X(17)30333-9 (2017).
28. Rodrigues, P. T. *et al.* Human migration and the spread of malaria parasites to the New World. bioRxiv doi:10.1101/141853 (2017).
29. Buery, J. C. *et al.* Mitochondrial genome of *Plasmodium vivax/simium* detected in an endemic region for malaria in the Atlantic Forest of Espirito Santo state, Brazil: do mosquitoes, simians and humans harbour the same parasite? *Malar J* **16**, 437, https://doi.org/10.1186/s12936-017-2080-9 (2017).
30. Costa, D. C. *et al. Plasmodium simium/Plasmodium vivax* infections in southern brown howler monkeys from the Atlantic Forest. *Mem Inst Oswaldo Cruz* **109**, 641–653 (2014).
31. Torres, K. L. *et al.* Standardization of a very specific and sensitive single PCR for detection of *Plasmodium vivax* in low parasitized individuals and its usefulness for screening blood donors. *Parasitol Res* **98**(6), 519–524, https://doi.org/10.1007/s00436-005-0085-8 (2006).
32. Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S. & Brown, K. N. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* **58**, 283–292 (1993).
33. Vincze, T., Posfai, J. and Roberts, R. J. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Res*. **31**, 3688–3691, http://nc2.neb.com/NEBcutter2/ (2003).
34. Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98, http://www.mbio.ncsu.edu/BioEdit/page2.html/ (1999).
35. Chromas Lite software 2.1.1 by Australia Technelysium Pty Ltd. http://technelysium.com.au/wp/chromas/.
36. Collins, W. E., Contacos, P. G. & Guinn, E. G. Observations on the sporogonic cycle and transmission of *Plasmodium simium* do Fonseca. *J Parasitol* **55**, 685–686 (1969).
37. Secretary of Health of the Espirito Santo State (http://saude.es.gov.br/malaria).
38. Sallum, M. A., Marrelli, M. T., Nagaki, S. S., Laporta, G. Z. & Dos Santos, C. L. Insight into Anopheles (Nyssorhynchus) (Diptera: Culicidae) species from Brazil. *J Med Entomol* **45**, 970–981 (2008).

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Author Contributions

R.C., D.A.M.A., C.F.A.B. and C.T.D.R. conceived and designed the study. A.P.C., C.B.J. and P.B. atendded the patients, collected human samples and diagnosed malaria in humans. A.J.N., J.C.S. jr., Z.H., S.B.M., A.P., F.V.S.A. and R.L.O. collected the non-human primates samples. A.L.A., M.Z. and M.F.F.C. performed the molecular diagnosis and molecular experiments of mitochondrial genome. D.A.M.A. and D.F.R. performed nested-PCR/RFLP experiments. D.A.M.A. and C.F.A.B. performed and analysed DNA sequencing. D.A.M.A., R.C. and C.F.A.B. wrote the manuscript. All authors read, made suggestions and approved the final version of the manuscript.

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7.3 **Anexo III – Artigo:** Potential of *Aedes albopictus* as a bridge vector for enzootic pathogens at the urban-forest interface in Brazil

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Potential of *Aedes albopictus* as a bridge vector for enzootic pathogens at the urban-forest interface in Brazil

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Abstract

The invasive species *Aedes albopictus* is present in 60% of Brazilian municipalities, including at the interfaces between urban settings and forests that are zoonotic arbovirus hotspots. We investigated *Ae. albopictus* colonization, adult dispersal and host feeding patterns in the anthropic-natural interface of three forested sites covering three biomes in Brazil in 2016. To evaluate whether an ecological overlap exists between *Ae. albopictus* and sylvatic yellow fever virus (YFV) in forests, we performed similar investigations in seven additional urban-forest interfaces where YFV circulated in 2017. We found *Ae. albopictus* in all forested sites. We detected eggs and adults up to 300 and 500 m into the forest, respectively, demonstrating that *Ae. albopictus* forest colonization and dispersal decrease with distance from the forest edge. Analysis of the host identity in blood-engorged females indicated that they fed mainly on humans and domestic mammals, suggesting rare contact with wildlife at the forest edge. Our results show that *Ae. albopictus* frequency declines as it penetrates into the forest and highlight its potential role as a bridge vector of zoonotic diseases at the edge of the Brazilian forests studied.

Introduction

The risk of the emergence of zoonotic infectious diseases is particularly high in regions under the influence of tropical forests because these important biodiversity hotspots are undergoing anthropogenic land use changes[1]. Landscape anthropization, especially urbanization, shifts the risk of mosquito-borne pathogen emergence by affecting the mosquito and host communities both quantitatively and qualitatively[2,3]. Landscape disturbances at the urban-forest interface may facilitate the dispersion of anthropophilic mosquito species into previously unfavorable habitats[4], thus modifying vector-host interactions and potentially leading to more contact with sylvatic (i.e., enzootic) reservoirs of zoonotic pathogens. In this scenario, opportunistic mosquito species can act as bridge vectors between sylvatic and urban pathogen transmission cycles.

During the last 35 years, *Aedes* (*Stegomyia*) *albopictus* (Skuse) (Diptera: Culicidae) has expanded geographically from Southeast Asia to other continents, withimportant human health consequences and risks related particularly to arbovirus transmission[5–9]. This invasive mosquito species, which was considered sylvatic in its native area, has adapted to human settings (domestication) by exploiting man-made water containers as larval habitats and humans and/or domestic animals as its main blood source[10]. Domestication is one of the key features (together with other biological traits, such as desiccation-resistant eggs and overwintering ability) that facilitated the successful expansion of *Ae. albopictus* throughout

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anthropogenic environments worldwide[7]. Nevertheless, it remains unclear to what extent invasive (and "domesticated") *Ae. albopictus* populations have preserved their capacity to colonize forest environments. Indeed, *Ae. albopictus* was previously detected at the forest border of rural or urban habitats in colonized areas[5,11,12], and its opportunistic blood feeding behavior has been extensively described (i.e., a wide range of vertebrate hosts with a marked preference for mammals, especially humans, compared to birds)[13–15]. In opportunistic species, the degree of blood feeding on domestic or wild vertebrates relative to humans is strongly dependent on the local host availability[16]. Importantly, *Ae. albopictus* can support the transmission of epidemic arboviruses (e.g., chikungunya, dengue, and Zika viruses) and possibly many other enzootic and zoonotic arboviruses[5,17–19]. In addition, experimental vector competence studies and viral genome detection or isolation in nature indicated that *Ae. albopictus* can transmit several enzootic arboviruses (e.g., La Crosse Virus, West Nile Virus, Eastern equine encephalomyelitis, Cache Valley Virus, Keystone Virus, Potosi Virus, Tensaw Virus, Chandipura, Jamestown Canyon, Orungo, Rift Valley, Ross River, Oropuche virus, Mayaro virus, and yellow fever virus (YFV)[20–35]). Its vector competence for several pathogens, its opportunistic feeding behavior, and its capacity to colonize urban, rural and natural habitats suggest that *Ae. albopictus* could be a bridge vector to allow pathogen transfers from animal to human compartments and vice versa[13,36].

Since its first detection in Brazil in the 1980s, *Ae. albopictus* has geographically expanded to approximately 60% of Brazilian municipalities[37], across urban, peri-urban and rural environments[38–40], and in some cases at the border of the Atlantic and Amazon forests[12,41–43]. The Brazilian Amazon forest harbors 187 different arboviruses, among which, 34 are of medical interest[44,45]. Of particular interest is sylvatic YFV, which causes cyclic epidemic waves with high lethality in Brazil. Following its spatial expansion to the Southeast region in late 2016, the YFV territory now largely overlaps with areas of high *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) and *Ae. albopictus* infestation, and this increases the risk of a reemergence of urban transmission throughout South America[37,46–48]. However, few studies have focused on *Ae. albopictus* colonization and dispersion in Brazil. Some studies inside forests were carried out as part of the entomological surveillance during yellow fever epizootics, or as part of ecological projects on native mosquitoes[42,49]. However, systematic investigations are needed to measure *Ae. albopictus* dispersal penetration into neotropical forests to understand its potential as a bridge vector in relation to the emergence risk of zoonotic arboviruses in bordering Brazilian cities and elsewhere. A higher risk occurs when mammalian diversity is elevated, as it is the case for the Atlantic and Cerrado forests, which are considered hotspots for biodiversity conservation[50].

Here we studied the capacity of *Ae. albopictus* to colonize and disperse into forested environments from the edge (i.e., modified environment) to deeper locations (i.e., more preserved environment) in three Brazilian biomes, the Amazon, Cerrado and Atlantic forests. We also analyzed mosquito blood meals to determine *Ae. albopictus* host feeding patterns and potential interactions with wild vertebrates.

Results

Ae. albopictus forest colonization

During the 2016 survey, the maximal distance of *Ae. albopictus* egg detection was 300 m from the forest edge, even when sampling was carried out up to 1000 m inside the forest. Specifically, in Adolpho Ducke-Amazonas, the total egg number per distance (five ovitraps per distance) rapidly decreased from 0 to 200 m from the edge (0 m: 1423, 100 m: 118, 200 m: 20 eggs) (Fig. 1). Similarly, at Morro dos Macacos-Goias and Pedra Branca-Rio de Janeiro, eggs were found up to 300 m (0 m: 65, 100 m: 25, 200 m: 21, 300 m: 13 at Morro dos Macacos, and 0 m: 638, 100 m: 2, 200 m: 1, 300 m: 7 at Pedra Branca). Overall, the number of eggs decreased significantly ($p < 0.00001$) with distance from the forest edge in all sampled areas (explained deviance of more than 70%) (Tables S2 and S4).

In the areas surveyed in 2017, mosquito eggs were detected up to the maximal sampling distance (300 m from the forest edge) at four sites: Belo Horizonte-Minas Gerais (0 m: 9, 100 m: 99), Simonésia-Minas Gerais (0 m: 5, 100 m: 55, 200 m: 23, 300 m: 109), Domingos Martins-Espirito Santo (0 m: 26, 100 m: 42, 200 m: 2, 300 m: 17), and Salvador-Bahia (0 m: 236, 100 m: 117, 200 m: 4, 300 m: 64). Although technical problems prevented egg counting in Casimiro de Abreu-Rio de Janeiro and Maricá-Rio de Janeiro, positive ovitraps were detected at all distances (Table S2). Eggs from Serra- Espirito Santo were destroyed during transport.

In Adolpho Ducke and Salvador, all hatched eggs belonged to *Ae. albopictus*. In Pedra Branca, 1.6% of all hatched eggs collected at 0 m corresponded to *Ae. aegypti*, and in Belo Horizonte, 10% of the eggs collected at 100 meters were from *Haemagogus leucocelaenus*. All hatched eggs collected in Maricá, Casimiro de Abreu and those collected at 200 m in Simonésia and at 300 m in Domingos Martins belonged to sylvatic mosquito species (Table S2).

Ae. albopictus forest dispersal

In Adolpho Ducke, BG-Sentinel traps collected 1,232 *Ae. albopictus* adults (768 females and 464 males) with a

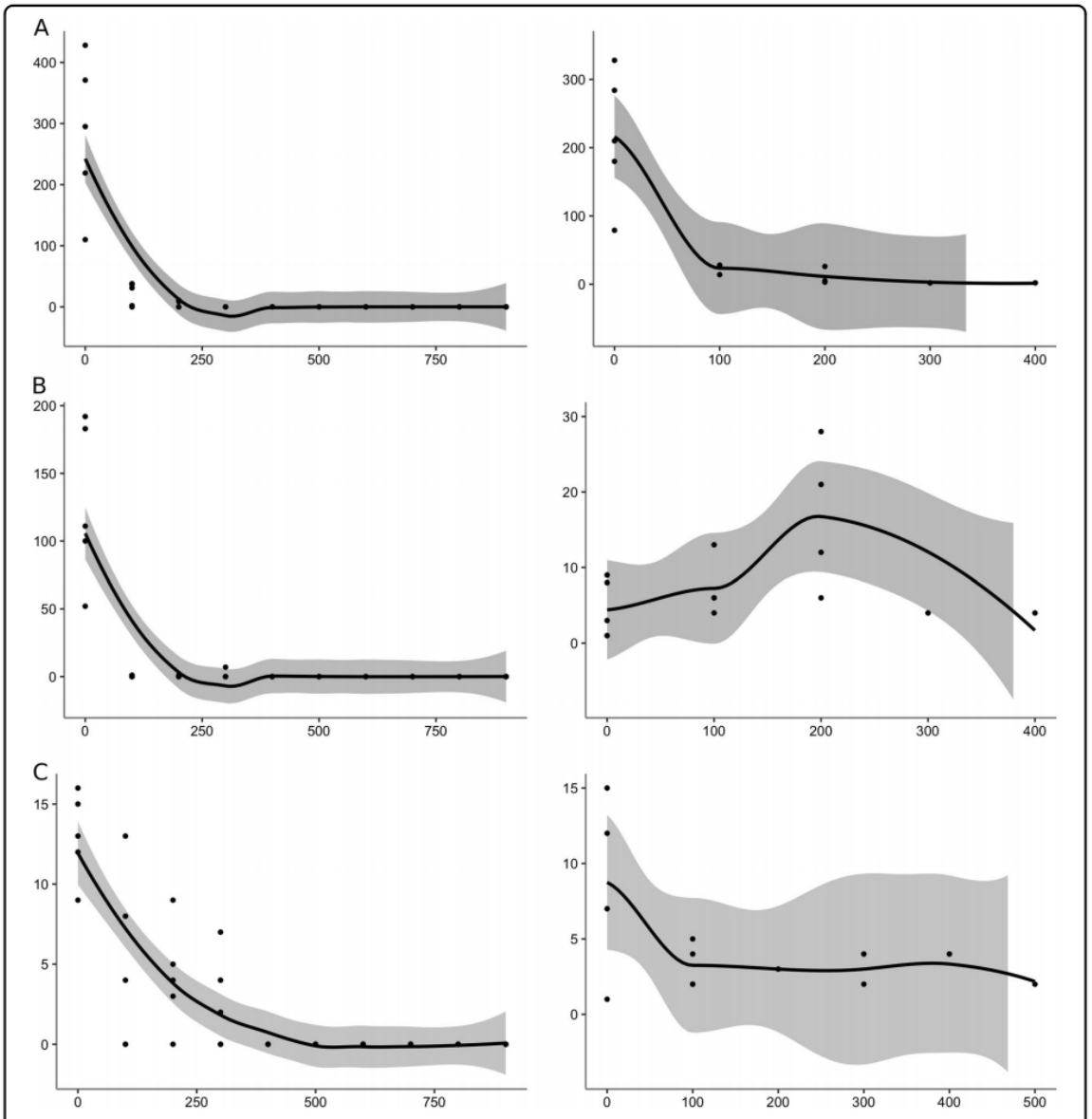

Fig. 1 Charts in ggplot with LOESS smoothing showing the total number of eggs (left panels) and *Ae. albopictus* females (right panels) on the y-axis as a function of the distance (in meters) from the forest edge (x-axis) at the (**a**) Adolpho Ducke, (**b**) Pedra Branca, and (**c**) Morro dos Macacos sampling sites. The shaded area represents the 95% confidence interval of the mean value (solid line). Statistical details are provided in Table S4

mean number of specimens per trap and per day (MN/T/D) of 7 (SD = 8.35). At Morro dos Macacos and Pedra Branca, 63 (59 females and 4 males) and 139 (129 females and 10 males) adult *Ae. albopictus* specimens were collected, respectively (MN/T/D: 1.43 ± 0.81 and 2.07 ± 1.68, respectively). At these three sites, *Ae. albopictus* adults were sampled from the edge to 500 meters into the forest. At Morro dos Macacos, 49% of all *Ae. albopictus* specimens were trapped at 0 meter, with two females trapped at 500 m. At Adolpho Ducke, 89% of females were collected at 0 meter, with two females collected at 400 m. At Pedra Branca, 48% of females were sampled at 200 m, with seven specimens collected at 400 m.

Along of the Adolpho Ducke transect, the abundance of *Ae. albopictus* females was significantly ($p < 0.00001$) associated with distance, decreasing from 0 meters up to

400 m, with an explained deviance of 30%. Conversely, in Pedra Branca, *Ae. albopictus* abundance increased up to 200 m, and then decreased up to 400 m. Nevertheless, the distance explained the presence of adults in the forest ($p < 0.00001$; explained deviance of 55.20%). In Morro dos Macacos, the number of *Ae. albopictus* significantly ($p < 0.00001$) decreased with distance and the distance explained the presence of adults in the forest (explained deviance of 48.32%) (Fig. 1 and online Tables S3 and S4 for statistical details).

In 2017, adult *Ae. albopictus* specimens were trapped as far as 300 m (limit of trap deployment) at all sites, with the exception of Casimiro de Abreu and Maricá in the Atlantic forest. The total number of adults caught across the 300 m transects varied from two females in Casimiro de Abreu to 43 females in Salvador. Similarly, only females were trapped in Belo Horizonte ($n = 12$), Domingos Martins ($n = 5$), Maricá ($n = 7$) and Serra ($n = 3$). At Simonésia, 28 specimens were collected (20 females and 8 males) (Table S3).

Ae. albopictus blood meal analysis

Among all the trapped *Ae. albopictus* females, 66 were engorged with blood ($n = 61$ in Adolpho Ducke, $n = 1$ in Morro dos Macacos, and $n = 4$ in Pedra Branca). Most of them (91%) were collected at the forest edge (online Table S5) and very few were collected from deeper in the forest (five at 100 m in Adolpho Ducke; one at 200 m in Pedra Branca). Molecular analyses indicated that most blood meals (98%) were taken from mammals, mainly humans (71%), dogs (21%), brown rats (*Rattus norvegicus*, 3%) and greater round-eared bats (*Tonatia bidens*, 3%) (Fig. 2, online Table S5). Only one mosquito was found to have fed on a bird (great antshrike, *Taraba major*, 1.5%). No engorged *Ae. albopictus* were captured in the seven additional sampling sites.

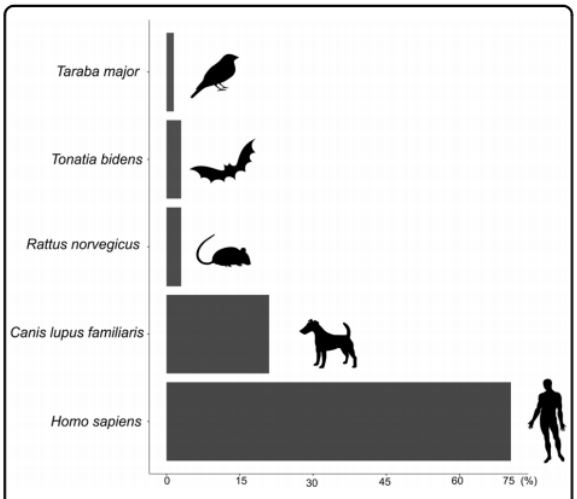

Fig. 2 Horizontal bar chart in ggplot showing the number of vertebrate species found in the blood meals of *Ae. albopictus* specimens. The bar graph value was calculated using data from 66 *Ae. albopictus* specimens analyzed without distinction of area or collection distance from the forest edge

Discussion

Here, we studied the establishment of *Ae. albopictus* at the edge of and inside Brazilian forests as well as its feeding habits to understand the potential risk of this invading mosquito transferring zoonotic arboviruses to humans. Its abundance, opportunistic trophic behavior and vector competence for several viruses contributes to its potential role in the spill-over of zoonotic pathogens from sylvatic hosts to humans, or to domestic animals and then potentially to humans[13,15].

We observed that forest colonization by *Ae. albopictus* rapidly decreased with distance from the edge (200–300 m inside the forest). Together with previous findings, these result confirm that *Ae. albopictus* population density declines as they penetrate into the forest[10,51]. Although *Ae. albopictus* was captured at all surveyed forests both before and during the YFV outbreak, its dispersal seems to be somewhat limited because adults were not sampled beyond 500 m from the forest edge. The number of trapped adults usually decreased with the distance from the edge of the forest, with the exception of Pedra Branca, where the abundance of *Ae. albopictus* females increased up to 200 m and then sharply decreased up to 400 m, probably due to the presence of human visitors between 0–200 m at this reserve (personal observation). The presence of humans in these areas could represent a blood source for feeding, as well as a source of breeding/oviposition sites (i.e., plastic containers left behind). *Aedes albopictus* colonization and dispersal occurs deeper into Brazilian secondary forests, as described previously in a one-year study at Represa do Cigano in Rio de Janeiro where the eggs of wild and rubidium chloride-marked females were detected up to 1000 m inside the anthropized area of the Tijuca Forest[12,41].

The blood meal identifications showed a clear pattern of mammalian host preference (especially humans), as reported in several previous field studies[13–15,52]. In addition to humans, blood meals were found to be from domestic or commensal hosts (dogs and brown rats) and, less frequently, from wild hosts (two bats and one bird), confirming the opportunistic feeding behavior of *Ae. albopictus*[5,13,14]. The greater round-eared bat is an omnivorous species that is usually found in hollow trees at low heights and hunts in clearings (modified environments and forest edges). The great antshrike is a bird that lives in clearings and low density vegetation at the forest edge[53,54]. These results highlight that *Ae.*

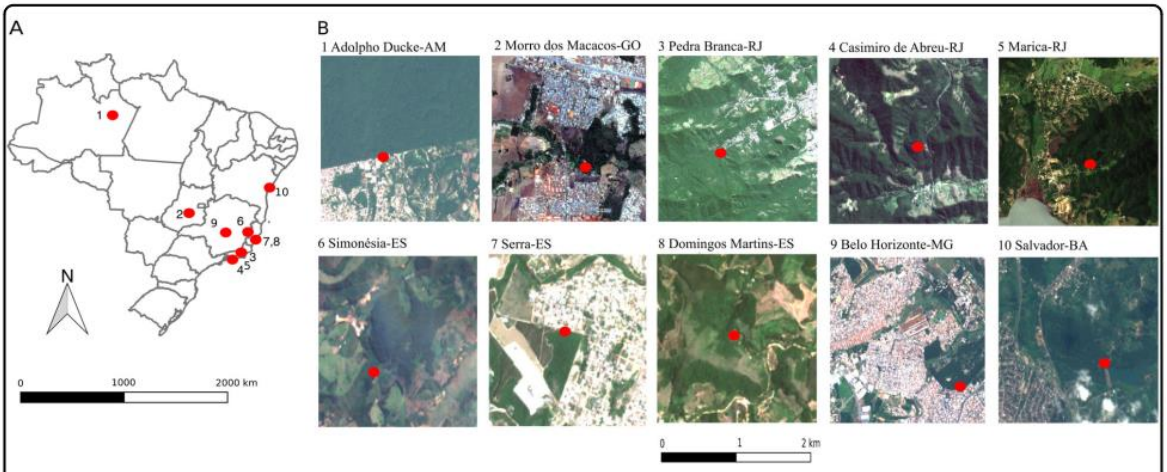

Fig. 3 *Ae. albopictus* sampling sites at ten urban-forest interfaces in Brazil. a Localization of the ten sites in Brazil; **b** Satellite images showing each sampling site as a red dot. Satellite images were downloaded from https://earthexplorer.usgs.gov and the maps were drawn using Qgis 2.18.14

albopictus feeds mostly on humans and domestic animals but also on wildlife at the edge of the forest and suggests host-seeking by flying at low heights. Additional studies should investigate the potential host feeding patterns of *Ae. albopictus* on wildlife living inside forest patches.

Our study and previous data[12,41] suggest that, in Brazil, *Ae. albopictus* can occupy the few hundred meters beyond the forest edge, but then its density progressively decreases with distance from the edge. This could be explained by a reduction in the availability of its key resources, which are common at the forest edge, such as suitable breeding sites and host abundance. Therefore, the degree of forest fragmentation and anthropization could be a relevant factor that modulates *Ae. albopictus* colonization and dispersal. Alternatively, *Ae. albopictus* populations could be maladapted to sylvatic environments (i.e., the species has lost its ancestral biological trait). The presence of competitors, predators or parasites in the natural larval habitats in neotropical forests could also explain this observation.

Although the forest edge could be considered an 'emergence area' where zoonotic diseases can spill-over from zoonotic reservoirs to humans via *Ae. albopictus*, the present results suggest that the risk of transfer from wildlife is less likely than from domestic commensal hosts. This must be confirmed by additional studies, particularly on the interactions between *Ae. albopictus* and monkeys that are YFV reservoirs. Natural YFV infections in the *Ae. albopictus* specimens captured in the Cerrado biome of Brazil during the 2016–2018 YFV outbreak were recently confirmed[48], suggesting that such interactions occurred. Moreover, it was previously shown that some Brazilian *Ae. albopictus* populations, including those living at the sites surveyed here (Manaus, Goiania and Rio de Janeiro), are competent to support the transmission of several YFV strains[26,55]. Furthermore, in vivo experiments have demonstrated that YFV can rapidly adapt to this vector species[56]. In this study, *Ae. albopictus* females were trapped up to 300 m in five of the seven surveyed forests where active enzootic YFV transmission was reported in 2017, as well as at the forest edge of all the surveyed forests. Our data suggests an ecological overlap between *Ae. albopictus* and YFV reservoirs. It is crucial that future studies better evaluate the propensity of this mosquito to bite monkeys that live in the canopy, thus helping the transfer of sylvatic YFV to humans and the emergence of a rural/intermediate cycle in Brazil, or the reemergence of the urban cycle.

Taken together, these results highlight the potential role of *Ae. albopictus* as a bridge vector for zoonotic diseases in the human-animal interface at the edge of Brazilian forests. Its potential participation in the spill-over of dozens of zoonotic arboviruses harbored in Brazilian forests from sylvatic or ubiquitous hosts directly to humans, from wildlife to commensal and domestic hosts, and between humans in the ecotone and natural and modified environments, as well as rural areas, deserves to be investigated. For instance, capuchins, marmosets, opossums and other commensal and ubiquitous mammals that live and find shelter in low vegetation and/or on the ground at the forest edge are amplifiers of arboviruses with great epidemic potential (e.g., YFV, Mayaro virus), and their territory overlaps with that of *Ae. albopictus* in Brazil. Understanding the mechanisms of disease emergence will allow for the development of early detection

and control programs to reduce disease incidences and economic burdens.

Materials and methods

Study areas

Surveys were undertaken before (from January to May 2016) and during the 2017–2018 severe YFV outbreak (from February to June 2017). In 2016, mosquitoes were sampled in three urban-forest interfaces located in the cities of Manaus (Adolpho Ducke site, 3°00′12.78″S; 59° 55′37.86″W, January 7 to March 3), Goiania (Morro dos Macacos site, 6°40′16.32″S; 49°22′49.93″W, April 17 to May 27), and Rio de Janeiro (Pedra Branca site, 22°56′ 6.57″S; 43°26′42.19″W, from March 4 to April 16) (Fig. 3 and online Table S1). In 2017, we extended our survey by including seven supplementary urban-forest interfaces located in four Brazilian states where YFV was circulating. In addition to increasing the number of study sites to have a broader and more generalizable view of the *Ae. albopictus* colonization in Brazilian forests, the aim was also to assess the potential ecological overlap between *Ae. albopictus* and sylvatic YFV. The additional sites were Belo Horizonte (19°51′59.29″; 44°0′43.51, from February 10 to 13), Simonésia (19°55′ 12.06″; 41°54′20.23″", from February 16 to 18) in the Cerrado biome, Domingos Martins (20°17′12.48″; 40°50′ 14.35″, from February 21 to 23), Serra (20°6′46.89″; 40° 11′12.53″, from March 14 to 17), Casimiro de Abreu (22°26′33.31″; 42°12'30.34″, from March 28 to 31), Maricá (22°55′24.44″; 42°42′27.88″, from May 4 to 8) and Salvador (12°49′50.34″; 38°27′18.77″, from June 6 to 8) in the Atlantic forest (Fig. 3).

Study design

The field entomological survey was undertaken according to an anthropization gradient from the edge to the interior of the forest. At the three long-term surveyed sites (Adolpho Ducke, Morro dos Macacos, and Pedra Branca), the mosquito sampling design was based on a grid of five parallel lines spaced 25 m apart and intersected perpendicularly by a grid of ten parallel lines spaced 100 m apart, extending from the edge (0 meters) to 900 m inside the forest (online Figure S1). Field work was organized into two sampling phases with two different methods to differentiate between *Ae. albopictus* colonization (first sampling phase) and dispersion (second sampling phase). The term "colonization" refers to the capacity to exploit forest environments for suitable oviposition sites. The term "dispersion" was used to measure the abundance of host-seeking female mosquitoes in the forest. The first sampling phase (10 consecutive days) was conducted by deploying 50 ovitraps (one at each intersection between parallel and perpendicular lines) made up of a dark plastic cup with 300 ml of hay infusion and one wooden paddle for mosquito egg collection[57]. Every 5 days, paddles were replaced and the recovered paddles were transported to the laboratory for egg counting on day 10. In the laboratory, paddles were immersed in water for egg hatching, larval breeding and identification of emerging adults using appropriate morphological taxonomic keys[58]. The second sampling phase (7 consecutive days) was carried out using 15 BG-Sentinel traps (Biogents) baited with a combination of BG-lure and dry ice as source of CO_2 to capture host-seeking adult mosquitoes at different distances up to 500 meters inside the forest. The number of BG-Sentinel traps per distance varied due to operational limitations. Traps operated beginning at 8:00am on day 1 until 5:00pm on day 7 and were monitored every 24 h by replacing the collection bags containing mosquitoes and dry ice. We defined a "sampling cycle" as the 10 days of ovitrap monitoring followed by the 7 days of BG-Sentinel trap monitoring. Two sampling cycles were carried out in Adolpho Ducke and Morro dos Macacos and one was completed in Pedra Branca. The sampling method and approach used in the seven forest interfaces surveyed during the 2017 YFV outbreak were essentially the same, except that 12 BG-Sentinel traps were operated from 0 to 300 m from the edge of the forest for 5 days, considering the preliminary results of the 2016 sampling. Ovitraps were installed at the same points as the BG-Sentinel traps for 10 days.

Trophic behavior analysis

Blood-engorged *Ae. albopictus* females collected with the BG-Sentinel traps were used to identify the blood-feeding patterns in forest environments. DNA was extracted from abdomens containing blood and used for PCR amplification according to the protocol by Bitome-Essono et al.[59]. Specific primers were used to amplify a portion of the 16 S rRNA gene or to amplify a portion of the cytochrome B oxidase gene, if the first PCR test failed. Genomic DNA from a mosquito engorged with rabbit blood was used as positive control. DNase-free water was used as negative control. All PCR-amplified products (10 µl) were run on 2% agarose gels in TBE buffer and positive samples were sent to GENEWIZ for forward and reverse sequencing after purification. Consensus sequences were compared with existing sequences using the nucleotide BLAST database to determine the host species, according to Bitome-Essono et al.[59].

Statistical analysis

Data from the Adolpho Ducke, Pedra Branca and Morro dos Macacos forests were used to study (1) the relationship between the presence of eggs and the distance from the edge (colonization), and (2) the relationship between the presence of *Ae. albopictus* females and the distance from the edge (dispersion). After

completing the first exploratory data analysis, a negative binomial generalized linear model was developed. An offset variable was included to weigh for the adult mosquito sampling effort. Statistical analyses were performed using the R studio 3.3.1 tool in the R software, version 1.0.143. Data from the other seven study sites could not be analyzed due to the low number of sampled mosquitoes.

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Author contributions
TPDS, DR, FS, RLDO, and CP conceived of the study and designed the methodology. TPDS, FVSDA, and CP carried out the fieldwork and were assisted by RLDO, SLB, and MS. TPDS, FVSDA, and MSASN performed the morphological identification of mosquitoes and TPDS, DJ, and CP were responsible for the molecular identifications. Statistical analyzes were performed by TPDS and DR. TPDS, DR, FVSDA, RLDO, and CP wrote the manuscript. All authors edited, commented on the manuscript and accepted the final version.

Conflict of interest
The authors declare that they have no conflict of interest.

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References

1. Allen, T. et al. Global hotspots and correlates of emerging zoonotic diseases. *Nat. Commun.* **8**, 1–10 (2017).
2. Steiger, D. M., Ritchie, S. A. & Laurance, S. G. W. Mosquito communities and disease risk influenced by land use change and seasonality in the Australian tropics. *Parasites Vectors* **9**, 1–13 (2016).
3. Rochlin, I., Faraji, A., Ninivaggi, D. V., Barker, C. M. & Kilpatrick, A. M. Anthropogenic impacts on mosquito populations in North America over the past century. *Nat. Commun.* **7**, 1–14 (2016).
4. Steiger, D. M. et al. Effects of landscape disturbance on mosquito community composition in tropical Australia. *J. Vector Ecol.* **37**, 69–76 (2012).
5. Paupy, C., Delatte, H., Bagny, L., Corbel, V. & Fontenille, D. *Aedes albopictus*, an arbovirus vector: From the darkness to the light. *Microbes Infect.* **11**, 1177–1185 (2009).
6. Lambrechts, L., Scott, T. W. & Gubler, D. J. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS. Negl. Trop. Dis.* **4**, e646 (2010).
7. Juliano, S. A. & Lounibos, L. P. Ecology of invasive mosquitoes: effect on resident species and human health. *Ecol. Lett.* **8**, 558–574 (2005).
8. Hahn, M. B. et al. Reported distribution of *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus* in the United States, 1995-2016 (Diptera: Culicidae). *J. Med. Entomol.* **53**, 1169–1175 (2016).
9. Hahn, M. B. et al. Updated reported distribution of *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus* (Diptera: Culicidae) in the United States, 1995-2016. *J. Med. Entomol.* **54**, 1420–1424 (2017).
10. Hawley, W. A. The biology of *Aedes albopictus*. *J. Am. Mosq. Control. Assoc.* **4**, 2–39 (1988).
11. Gomes, A. C. et al. Microhabitats of *Aedes albopictus* (Skuse) in the Paraíba Valley region of the State of S. Paulo, Brazil. *Rev. Saude Publica* **26**, 108–118 (1992). Portuguese.
12. Lourenço-de-Oliveira, R., Castro, M. G., Braks, M. A. H. & Lounibos, L. P. The invasion of urban forest by dengue vectors in Rio de Janeiro. *J. Vector Ecol.* **29**, 94–100 (2004).
13. Delatte, H. et al. Blood-feeding behavior of *Aedes albopictus*, a vector of Chikungunya on La Réunion. *Vector-Borne Zoonotic Dis.* **10**, 249–258 (2010).
14. Faraji, A. et al. Comparative host feeding patterns of the Asian tiger mosquito, *Aedes albopictus*, in Urban and Suburban Northeastern USA and implications for disease transmission. *PLoS Negl. Trop. Dis.* **8**, e3037 (2014).
15. Kamgang, B. et al. Notes on the blood-feeding behavior of *Aedes albopictus* (Diptera: Culicidae) in Cameroon. *Parasites Vectors* **5**, 57 (2012).
16. Sivan, A., Shriram, A. N., Sunish, I. P. & Vidhya, P. T. Host-feeding pattern of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in heterogeneous landscapes of South Andaman, Andaman and Nicobar Islands, India. *Parasitol. Res.* **114**, 3539–3546 (2015).
17. Gratz, N. G. Critical review of *Aedes albopictus*. *Med. Vet. Entomol.* **18**, 215–227 (2004).
18. Grard, N. G. et al. Zika virus in Gabon (CentralAfrica)—2007: a new threat from *Aedes albopictus?*. *PLoS. Negl. Trop. Dis.* **8**, 1–6 (2014).
19. Vanlandingham, D. L., Higgs, S. & Huang, Y. J. S. *Aedes albopictus* (Diptera: Culicidae) and mosquito-borne viruses in the United States. *J. Med. Entomol.* **53**, 1024–1028 (2016).
20. Westby, K. M., Fritzen, C., Paulsen, D., Poindexter, S. & Moncayo, A. C. La Crosse Encephalitis virus infection in field-collected *Aedes albopictus*, *Aedes japonicus*, and *Aedes triseriatus* in Tennessee. *J. Am. Mosq. Control. Assoc.* **31**, 233–241 (2015).
21. Cupp, E. W. et al. West Nile virus infection in mosquitoes in the Mid-South USA, 2002–2005. *J. Med. Entomol.* **44**, 117–125 (2007).
22. Rezza, G. et al. Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* **370**, 1840–1846 (2007).
23. Weng, M. H., Lien, J. C., Wang, Y. M., Wu, H. L. & Chin, C. Susceptibility of three laboratory strains of *Aedes albopictus* (Diptera: Culicidae) to Japanese encephalitis virus from Taiwan. *J. Med. Entomol.* **34**, 745–747 (1997).
24. Wispelaere, M., Desprès, P. & Choumet, V. European *Aedes albopictus* and *Culex pipiens* are competent vectors for Japanese encephalitis virus. *PLoS. Negl. Trop. Dis.* **11**, 1–19 (2017).
25. Smith, G. C. & Francy, D. B. Laboratory studies of a Brazilian strain of *Aedes albopictus* as a potential vector of Mayaro and Oropouche viruses. *J. Am. Mosq. Control. Assoc.* **7**, 89–93 (1991).
26. Couto-Lima, D. et al. Potential risk of re-emergence of urban transmission of Yellow Fever virus in Brazil facilitated by competent *Aedes* populations. *Sci. Rep.* **7**, 1–12 (2017).
27. Johnson, B. et al. Vector competence of Brazilian *Aedes aegypti* and *Aedes abopictus* for a Brazilian yellow fever virus isolate. *Trans. R. Soc. Trop. Med. Hyg.* **96**, 611–613 (2002).
28. Farajollahi, A. et al. Field efficacy of BG-Sentinel and industry-standard traps for *Aedes albopictus* (Diptera: Culicidae) and West Nile virus surveillance. *J. Med. Entomol.* **46**, 919–925 (2009).
29. Mitchell, C. J. et al. Isolation of eastern equine encephalitis virus from *Aedes albopictus* in Florida. *Science* **257**, 526–527 (1992).
30. Mitchell, C. J., Haramis, L. D., Karabatsos, N., Smith, G. C. & Starwalt, V. J. Isolation of La Crosse, Cache Valley, and Potosi viruses from *Aedes* mosquitoes (Diptera: Culicidae) collected at used-tire sites in Illinois during 1994–1995. *J. Med. Entomol.* **35**, 573–577 (1998).

31. Armstrong, P. M. et al. Isolations of Cache Valley virus from *Aedes albopictus* (Diptera: Culicidae) in New Jersey and evaluation of its role as a regional arbovirus vector. *J. Med. Entomol.* **50**, 1310–1314 (2013).
32. Gerhardt, R. R. et al. First isolation of La Crosse virus from naturally infected *Aedes albopictus*. *Emerg. Infect. Dis.* **7**, 807–811 (2001).
33. Lambert, A. J. et al. La Crosse Virus in *Aedes albopictus* Mosquitoes, Texas, USA, 2009. *Emerg. Infect. Dis.* **16**, 856–858 (2010).
34. Harrison, B. A. et al. Isolation of potosi virus from *Aedes albopictus* in North Carolina. *J. Am. Mosq. Control. Assoc.* **11**, 225–229 (1995).
35. Flahault, A. et al. Chikungunya, La Réunion et Mayotte, 2005–2006: une épidémie sans histoire? *Sante Publique* **19**, 165–195 (2007).
36. Bataille, A. et al. Natural colonization and adaptation of a mosquito species in Galapagos and its implications for disease threats to endemic wildlife. *Proc. Natl. Acad. Sci. USA* **106**, 10230–10235 (2009).
37. Carvalho, R. G., Lourenço-de-Oliveira, R. & Braga, I. A. Updating the geographical distribution and frequency of *Aedes albopictus* in Brazil with remarks regarding its range in the Americas. *Mem. Inst. Oswaldo. Cruz.* **109**, 787–796 (2014).
38. Forattini, O. P. et al. Adults *Aedes albopictus* and *Ae. scapularis* behavior (Diptera: Culidae) in Southeastern Brazil. *Rev. Saude Publica* **34**, 461–467 (2000).
39. Braks, M. A. H., Honório, N., Lourenço-de-Oliveira, R., Juliano, S. & Lounibos, L. Convergent habitat segregation of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in southeastern Brazil and Florida. *J. Med. Entomol.* **40**, 785–794 (2003).
40. Lima-Camara, T., Honório, N. & Lourenço-de-Oliveira, R. Frequency and spatial distribution of *Aedes aegypti* and *Aedes albopictus* (Diptera, Culicidae) in Rio de Janeiro, Brazil. *Cad. Saude Publica* **22**, 2079–2084 (2006). Portuguese.
41. Maciel-de-Freitas, R., Neto, R. B., Gonçalves, J. M., Codeço, C. T. & Lourenço-de-Oliveira, R. Movement of dengue vectors between the human modified environment and an urban forest in Rio de Janeiro. *J. Med. Entomol.* **43**, 1112–1120 (2006).
42. Gomes, A. C. et al. *Aedes albopictus* appearance in epizootic areas of jungle Yellow Fever in South-Eastern and Southern regions of Brazil (Diptera: Culicidae). *Epidemiol. Serv. Saude* **17**, 71–76 (2008). Portuguese.
43. Segura Mde., N. et al. Occurrence of *Aedes albopictus* in the state of Pará, Brazil. *Rev. Saude Publica* **37**, 388–389 (2003). Portuguese.
44. Travassos da Rosa A. P., Vasconcelos P. F. C. & Travassos da Rosa J. F. S. (eds) *An Overview of Arbovirology in Brazil and Neighbouring Countries* (Instituto Evandro Chagas, Belém, 1998).
45. Vasconcelos, P. F. C. et al. Inadequate management of natural ecosystem in the Brazilian Amazon region results in the emergence and reemergence of arboviruses. *Cad. Saude Publica* **17**, 155–164 (2001).
46. Bonaldo, M. C. et al. Genome analysis of yellow fever virus of the ongoing outbreak in brazil reveals polymorphisms. *Mem. Inst. Oswaldo. Cruz.* **112**, 447–451 (2017).
47. Possas, C. et al. Yellow fever outbreak in Brazil: the puzzle of rapid viral spread and challenges for immunization [Submitted]. *Mem. Inst. Oswaldo Cruz.* https://doi.org/10.1590/0074-02760180278 (2018).
48. Possas, C., Martins, R. M., Lourenço-de-Oliveira, R. & Homma, A. Urgent call for action: Avoiding spread and re-urbanisation of yellow fever in Brazil. *Mem. Inst. Oswaldo. Cruz.* **113**, 1–2 (2018).
49. Alencar, J. et al. Flight height preference for oviposition of mosquito (Diptera: Culicidae) vectors of sylvatic yellow fever virus near the hydroelectric reservoir of Simplício, Minas Gerais, Brazil. *J. Med. Entomol.* **50**, 791–795 (2013).
50. Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B. & Kent, J. Biodiversity hotspots for conservation priorities. *Nature* **403**, 853–858 (2000).
51. Colless, D. H. Notes on the culicine mosquitoes of singapore. *Ann. Trop. Med. Parasitol.* **51**, 87–101 (1959).
52. Richards, S. L., Ponnusamy, L., Unnasch, T. R., Hassan, H. K. & Apperson, C. S. Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in relation to availability of human and domestic animals in suburban landscapes of central North Carolina. *J. Med. Entomol.* **43**, 543–551 (2006).
53. Esbérard, C. E. L. & Bergallo, H. G. Biological aspects of *Tonatia bidens* (Spix) in Rio de Janeiro State, southeastern Brazil (Mammalia, Chiroptera, Phyllostomidae). *Rev. Bras. Zool.* **21**, 253–259 (2004). Portuguese.
54. Lara, K. M., Pinho, J. B. & Silveira, R. M. L. Reproductive biology of *Taraba major* (Aves, Thamnophilidae) from the region of Pirizal, Porcao Norte Do Pantanal, Mato Grosso, Brazil. *Pap. Avulsos Zool.* **52**, 349–359 (2012).
55. Lourenço-de-Oliveira, R., Vazeille, M., Maria, A. N. A., Filippis, B. D. E. & Failloux, A. Large genetic differentiation and low variation in vector competence for dengue and yellow fever viruses of *Aedes albopictus* from Brazil, the United States, and the Cayman Islands. *Am. J. Trop. Med. Hyg.* **69**, 105–114 (2003).
56. Amraoui, F. et al. Experimental adaptation of the Yellow Fever virus to the mosquito *Aedes albopictus* and potential risk of urban epidemics in Brazil, South America. *Sci. Rep.* **8**, 14337 (2018).
57. Honório, N. et al. Dispersal of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in an urban endemic dengue area in the State of Rio de Janeiro, Brazil. *Mem. Inst. Oswaldo. Cruz.* **98**, 191–198 (2003).
58. Consoli, R. A. G. B. & Lourenço-de-Oliveira, R. (eds) *Principais Mosquitos de Importância Sanitária no Brasil* (Fiocruz, Rio de Janeiro, 1994).
59. Bitome-Essono, P. Y. et al. Tracking zoonotic pathogens using blood-sucking flies as "flying syringes". *eLife* **6**, e22069 (2017).

7.4 Anexo IV – Artigo: Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms.

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Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms

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The current yellow fever outbreak in Brazil is the most severe one in the country in recent times. It has rapidly spread to areas where YF virus (YFV) activity has not been observed for more than 70 years and vaccine coverage is almost null. Here, we sequenced the whole YFV genome of two naturally infected howler-monkeys (*Alouatta clamitans*) obtained from the Municipality of Domingos Martins, state of Espírito Santo, Brazil. These two ongoing-outbreak genome sequences are identical. They clustered in the 1E sub-clade (South America genotype I) along with the Brazilian and Venezuelan strains recently characterised from infections in humans and non-human primates that have been described in the last 20 years. However, we detected eight unique amino acid changes in the viral proteins, including the structural capsid protein (one change), and the components of the viral replicase complex, the NS3 (two changes) and NS5 (five changes) proteins, that could impact the capacity of viral infection in vertebrate and/or invertebrate hosts and spreading of the ongoing outbreak.

Key words: yellow fever virus - 2017 Brazil outbreak - amino acid changes

Yellow fever virus (YFV) is the prototype member of the genus *Flavivirus* and family Flaviviridae. It is an arbovirus transmitted by the bite of infected mosquitoes in Africa and Americas, causing a disease with a large spectrum of symptoms, from mild disease to severe and deadly haemorrhagic fever in humans and New World non-human primates (NHP) (Vasconcelos & Monath 2016). Two main YFV cycles are described: the urban cycle involving the domestic mosquito *Aedes* (*Stegomyia*) *aegypti*, currently restricted to Africa, and the wild cycle in which humans are essentially infected by epizooties-affected NHPs, having sylvatic arboreal tree-hole breeding mosquitoes as vectors (species of *Aedes*, in Africa, and of *Haemagogus* and *Sabethes*, in the Americas). A rural or intermediate cycle may also occur in zones of emergence recorded in Africa (Monath & Vasconcelos 2015).

YFV is a single-stranded, positive-sense RNA virus with a genome of approximately 11 kb. Seven lineages have been identified: five in Africa (West Africa I and II, East Africa, East/Central Africa and Angola), and two in the Americas (South America I and II) (Bryant et al. 2007). Phylogenetic analysis provided evidence that the YFV circulating in the Americas is derived from a Western African lineage ancestor that emerged in Africa and was imported into the American East coast from West Africa during the slave trade (Vasconcelos et al. 2004, Bryant et al. 2007, Nunes et al. 2012).

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The South American I is the most frequent genotype recorded in Brazil (Nunes et al. 2012, Monath & Vasconcelos 2015). Five lineages have been recognised in the South American genotype I, namely, 1A to 1E, which were associated with epidemics recorded during the cyclic expansions and retractions of YFV circulation in Brazil and other tropical American countries (Vasconcelos et al. 2004, de Souza et al. 2010). Since the turn of the century, the lineages 1D and 1E have been found in Brazil. However, since 2008, only YF viruses from lineage 1E have been detected in Brazil (de Souza et al. 2010, Nunes et al. 2012).

The most severe YFV epidemic recorded in Brazil in the recent decades has been reported since late 2016. Until the 10th epidemiological week of 2017, 1,558 cumulative cases with 137 confirmed YFV deaths were reported (COES 2017). Most importantly, this epidemic has rapidly and alarmingly spread eastward, reaching the most populated Brazilian regions where vaccine coverage is minor. Epizooties in NHPs and humans cases have been diagnosed in states considered YFV-free territories for almost 70 years.

Here, we present the complete genome sequence of two YFV samples collected during the current Brazilian epidemic along with a comparative analysis of recent YFV genome sequences characterised as belonging to the South American genotype I.

Blood samples were obtained from one recently dead and one dying howler-monkey (*Alouatta clamitans*) found on the Velho Rio farm (20º 17' 08" S 40º 50' 15" W), in Areinha, district of Ponto Alto, Municipality of Domingos Martins, state of Espírito Santo, Brazil, on

February 20th and 22nd, 2017, respectively. Following centrifugation (2,000 *g* for 10 min), plasma samples were immediately frozen and transported to the laboratory in N_2. Next, plasma samples were screened through reverse transcriptase polymerase chain reaction (RT-PCR), for which RNA was extracted from 140 µL of plasma using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. RNA was eluted in 60 µL of AVE buffer and stored at -80ºC until use. Viral RNA was reverse transcribed using the High Capacity System (Applied Biosystems) with random hexamers according to the manufacturer's recommendations. The reverse transcription reaction was carried out at 25ºC for 10 min, 37ºC for 120 min and 85ºC for 5 min. Further, the viral RNA was amplified by conventional PCR using PCR Master Mix (Promega), carried out at 95ºC for 2 min, followed by 30 cycles at 95ºC for 1 min, 58ºC for 1 min and 72ºC for 50 s, and then an extension at 72°C for 5 min. The set of primers utilised in this procedure were 5'-CTGTGTGCTAATTGAGGTGCATTG-3' and 5'-ATGTCATCAGGCTCTTCTCT- 3'. The YFV infection of the monkeys was confirmed by the specific detection of a single amplicon with the expected YFV amplicon size of 650 bp (Fig. 1).

To sequence of the full-length YFV genomes from the positive plasma monkey samples, 12 PCR amplicons were obtained (Supplementary data, Table). Viral RNA was reverse transcribed using the Superscript III First-Strand Synthesis System (Invitrogen) with random hexamers. Alternatively, we generated the first strand cDNA with the reverse primer P11R encoding the 3'UTR end (5′-AGTGGTTTTGTGTTTGTCA-3') and further processed it with YF12F and YF12R for the synthesis of the second strand of cDNA. The cDNA was amplified by conventional PCR using GoTaq Green Master Mix (Promega) according to the manufacturer's instructions. The thermocycling program in a Veriti 96-well thermocycler (Applied Biosystems) was used to amplify regions (1) to (11): 1 cycle at 95ºC for 5 min; 30 cycles at 95ºC for 40 s, at 50ºC for 40 s, and at 72ºC for 2 min; and finally 1 cycle at 72ºC for 10 min followed by incubation at 4ºC. For region (12), we applied 1 cycle at 95ºC for 5 min; 40 cycles at 70ºC for 40 s, 65ºC or 70ºC at 40 s, 72ºC at 50 s; and 1 cycle at 72ºC for 10 min and hold of 4ºC. Aliquots (3 µL of 50 µL) of amplified products were detected by electrophoresis on a 1% agarose gel, visualised by ethidium bromide staining and UV illumination, and purified with QIAquick PCR Purification Kit (QIAGEN). The amplicons were nucleotides that were directly sequenced without molecular cloning. Nucleotide sequencing reactions were performed using the ABI BigDye terminator V3.1 Ready Reaction Cycle Sequencing Mixture (Applied Biosystems) according to manufacturer's recommendations. Nucleotide sequence was determined by capillary electrophoresis at the sequencing facility of Fiocruz-RJ (RPT01A - *Sequenciamento de DNA* - RJ). Raw sequence data were aligned and edited using the SeqMan module of LaserGene (DNASTAR Inc.).

The complete genome sequences of both YF viruses were deposited in the GenBank database under the following accession numbers: KY885000 for strain ES-504/BRA/2017 and KY885001 for strain ES-505/BRA/2017. When we compared these genomes, they displayed 100% identity. The evolutionary relationships of these two YFV strains from the ongoing outbreak with the modern YF sequences, primarily from South American genotype I, was established by phylogenetic analysis. Initially, we selected a set of sequences of the prM/E junction fragment using the Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The 666-bp sequence consists of the last 108 nucleotides of the prM gene, including the entire 225 nucleotides of the M gene, and the first 333 nucleotides of the E gene. Nucleotide sequences were aligned using the CLUSTAL W program (Thompson et al. 1994) with selected YF viral sequences available at the GenBank database. A phylogenetic tree was generated by the Neighbour-joining method (Saitou & Nei 1987) using a matrix of genetic distances established under the Kimura-two parameter model (Kimura 1980), by means of the MEGA7 program (Kumar et al. 2016). The robustness of each node was assessed by bootstrap resampling (2,000 replicates) (Felsenstein 1985). The homologous region (prM/E) of a dengue virus strain available at the GenBank database (PaH881/88; Accession number: AF349753) was used as an outgroup. The Asibi prototype yellow fever strain (Accession number: AY640589) and the vaccine strain 17DD-Brazil (Accession number: DQ100292) were also incorporated into the analysis.

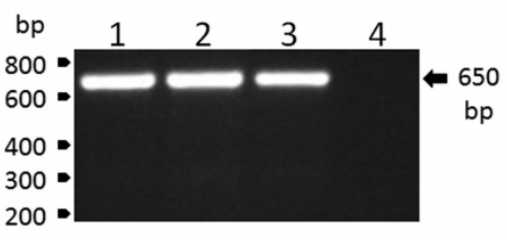

Fig. 1: detection of genomic RNA of yellow fever virus (YFV) by reverse transcriptase polymerase chain reaction (RT-PCR) analysis in plasma samples of howler monkeys from Velho Rio farm, in Areinha, Espírito Santo, Brazil. The numbered lanes refer to (1) positive control of the reaction that was performed with the YFV strain BeAn754036 obtained from insect C6/36 cell cultures; (2) YF RNA (strain ES-504/BRA/2017) and (3) YF RNA (strain ES-505/BRA/2017) that were extracted from howler-monkey plasma samples; and (4) a negative control of amplification. The size marker migration is indicated on the left of the figures, and the size of YFV amplicon is on the right.

The South American YF sequences formed two major clusters: the South America I and the South America II genotypes, supported by 97% and 98% bootstrap values, respectively (Fig. 2). The South America genotype I clade is further divided into sub-clades as described by Vasconcelos et al. (2004) and de Souza et al. (2010). Sequence strains from ES-504/BRA/2017 (GenBank access number: KY885000) and ES-505/BRA/2017 (GenBank access number: KY885000) belonged to the South America genotype I, and grouped within the 1E sub-clade in conjunction with other modern strains detected in Brazil (years: 2002, 2004, 2008) and Venezuela (years: 1998, 2005-2007, 2010). The recent Brazilian and

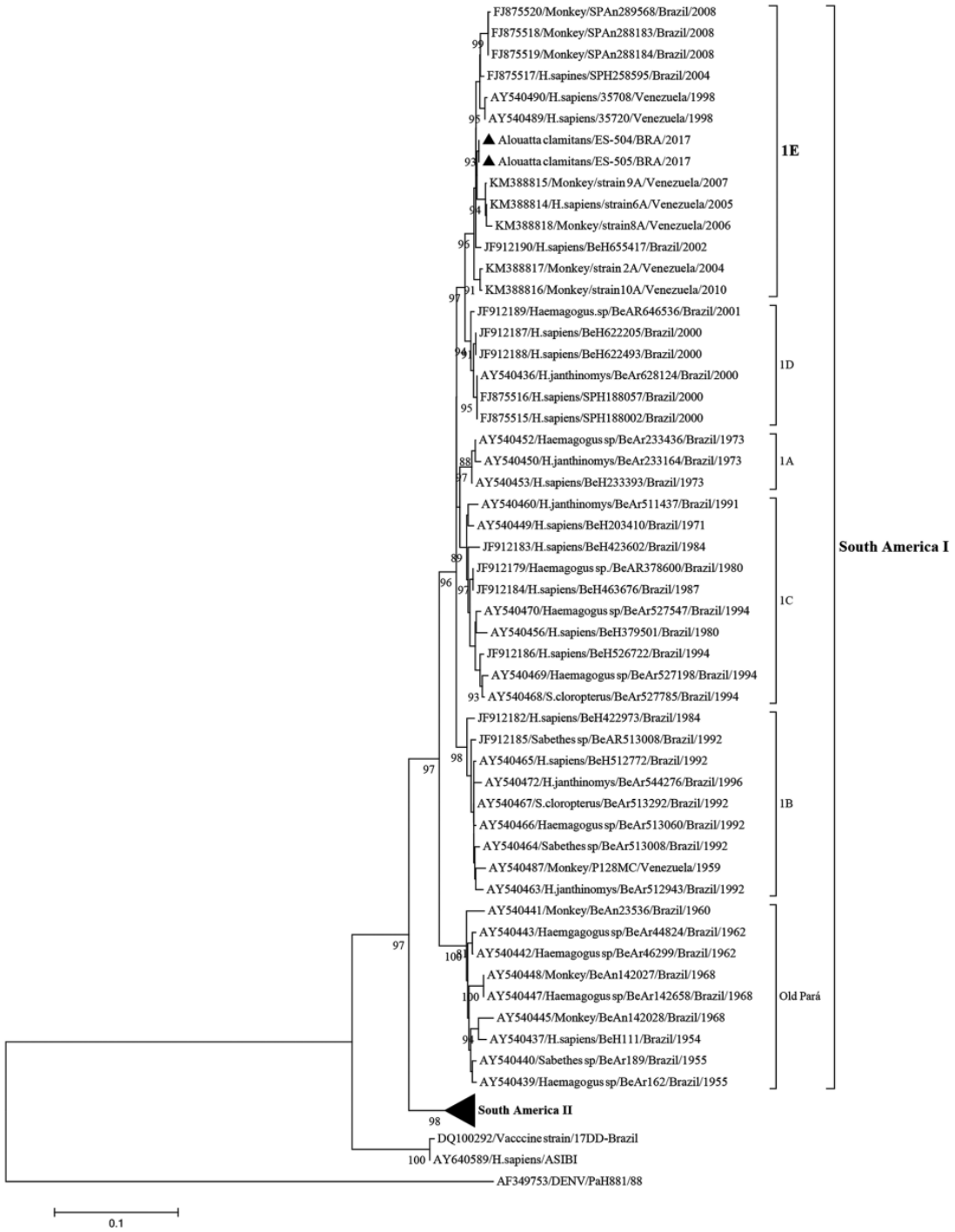

Fig. 2: phylogenetic analysis based on the prM/E junction region of yellow fever virus (YFV) strains analysed in the current study and 71 YFV sequences retrieved from the National Centre for Biotechnology Information (NCBI). Only bootstrap values up to 80% are shown. YFV genotypes are shown at the right side of the figure. The scale bar at the bottom represents 0.1 substitutions per nucleotide position (nt. subst./site). YFV first described in the current study are marked with a filled triangle. Accession numbers of the strains belonging to the South America genotype II are AY161929, AY161931-32, AY161934-35, AY161941-42, AY161944-45, AY161947-48, AY161950-51, AY540433-35, AY540446, and AY540457.

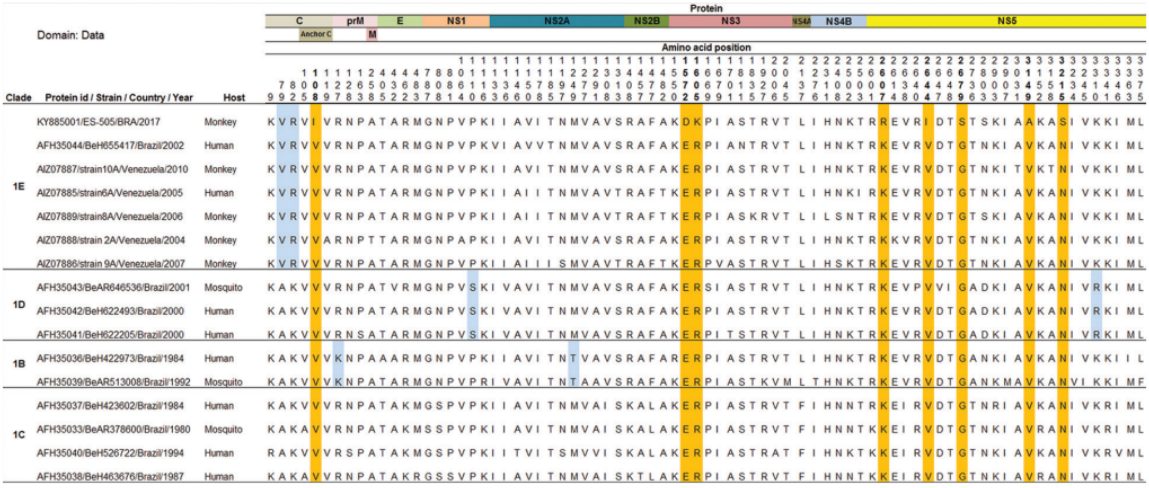

Fig. 3: amino acid (aa) differences revealed by the alignment of the precursor polyproteins of 16 Brazilian and Venezuelan yellow fever (YF) viruses detected since 1980. On the left of the alignment data, the identification of clades and yellow fever virus (YFV) sequences are supplied. On the top of the alignment, the YF viral proteins positions are indicated along with the aa positions of aa differences. The set of aa residues highlighted in blue indicate a related-clade pattern. The orange-highlighted aa indicate the position of the current YF sequences compared to the other YF sequences. For simplicity, only the ES-505/BRA/2017 strain sequence data were included in this figure.

Venezuelan strains that were characterised from infections in humans and NHPs, also clustered in the 1E subclade (South America genotype I). Auguste et al. (2015) suggested that Brazil is the major source of YFV introduction into Venezuela. However, our data suggest that the most recent Brazilian YFV strains would have originated from a Venezuelan YFV strain, since the oldest strains in the E1 sub-clade were isolated in Venezuela in 1998 (Fig. 2). The acquisition in phylogenetic studies of additional complete YF genomes from ancestral and present circulating strains from humans, NHPs and mosquitos became necessary.

The comparison of the YFV precursor polyproteins obtained from complete genome sequences with those detected in Brazil and Venezuela since 1980 demonstrated eight unique and semi-conservative amino acid (aa) changes in the C, NS3 and NS5 proteins (Fig. 3). These changes map to the following polyprotein positions: (1) 108 for isoleucine (C protein); (2) 1572 for aspartic acid and 1605 for lysine (NS3 region); and (3) 2607 for arginine, 2644 for isoleucine, 2679 for serine, 3149 for alanine and 3215 for serine (NS5 protein). Interestingly, seven out of eight aa changes are located in the two important proteins of the viral replicase complex-NS3 and NS5 - and are perhaps associated with some selective advantage for viral fitness reflecting the ability of the virus to infect vertebrate and/or invertebrate hosts and spread the infection.

However, it remains to be determined whether these specific aa changes are unique to the strains belonging to the ongoing outbreak. Alternatively, they, or at least some of them, could occur in some ancestral sequences that have not been sequenced so far. Hence, there are relatively very few complete YFV genomes from the Americas available at the GenBank database. On the other hand, this matter will be better clarified with the elucidation of the genomes of other circulating YF viruses in the current outbreak from infected mosquitos, NHPs and human biological samples. A wider understanding of the molecular epidemiology and evolution of YFV and their potential association with viral spreading and infectivity is of utmost relevance to determine the ancestral and modern YFV strains.

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AUTHORS' CONTRIBUTION

MCB and RLO - Conceived the study; FVSA - carried out the collection of biological specimens; RMM, AFB and MGC - carried out viral RNA extraction from the biological specimens and the diagnosis by RT-PCR; AACS - performed rapid viral RNA extraction and genome sequencing; MCB, AACS and MMG - analysed the genome sequences; MMG - performed phylogenetic analysis; and RLO, MCB and MMG - prepared the manuscript. All authors critically read and approved the final version of the manuscript.

REFERENCES

Auguste AJ, Lemey P, Bergren NA, Giambalvo D, Moncada M, Morón D, et al. Enzootic transmission of yellow fever virus, Venezuela. Emerg Infect Dis. 2015; 21(1): 99-102.

Bryant JE, Holmes EC, Barrett AD. Out of Africa: a molecular perspective on the introduction of yellow fever virus into the Americas. PLoS Pathog. 2007; 3(5): e75.

COES - Febre Amarela. Informe 32/2017. 2017. Available from: http://portalarquivos.saude.gov.br/images/pdf/2017/marco/16/COES-FEBRE-AMARELA---INFORME-32---Atualiza----o-em-16mar2017---s-13horas.pdf.

de Souza RP, Foster PG, Sallum MA, Coimbra TL, Maeda AY, Silveira VR, et al. Detection of a new yellow fever virus lineage within the South American genotype I in Brazil. J Med Virol. 2010; 82(1): 175-85.

Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985; 39(4): 783-91.

Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16(2): 111-20.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33(7): 1870-4.

Monath TP, Vasconcelos PF. Yellow fever. J Clin Virol. 2015; 64: 160-73.

Nunes MR, Palacios G, Cardoso JF, Martins LC, Sousa Jr EC, de Lima CP, et al. Genomic and phylogenetic characterization of Brazilian yellow fever virus strains. J Virol. 2012; 86(24): 13263-71.

Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4(4): 406-25.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994; 22(22): 4673-80.

Vasconcelos PF, Bryant JE, da Rosa TP, Tesh RB, Rodrigues SG, Barrett AD. Genetic divergence and dispersal of yellow fever virus, Brazil. Emerg Infect Dis. 2004; 10(9): 1578-84.

Vasconcelos PF, Monath TP. Yellow fever remains a potential threat to public health. Vector Borne Zoonotic Dis. 2016; 16(8): 566-67.

7.5 **Anexo V –** Licenças e autorizações

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
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Número: 41837-4	Data da Emissão: 25/08/2015 17:57	Data para Revalidação*: 23/09/2016

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Dados do titular

Nome: Danilo Simonini Teixeira	CPF: 922.004.351-34
Título do Projeto: Qual o risco da re-emergencia da Febre Amarela no estado do Rio de Janeiro	
Nome da Instituição : FUNDAÇÃO OSWALDO CRUZ	CNPJ: 33.781.055/0001-35

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Determinaçao das áreas de coleta	11/2014	11/2014
2	Revisao bibliograficas de presença/ausencia de PNHs em areas focais para o estudo	11/2014	12/2014
3	Determinacao de presença/ausência de PNHs nos CETAS e de vetores nas regioes selecionadas	12/2014	12/2014
4	Localizaçao de áreas (CETAS) com populacoes de primatas	12/2014	12/2014
5	Submissao do projeto para aquisiçao de licença de captura/ Comite de Ética	12/2014	12/2014
6	Captura e coleta de materias biológicos de PNHs e captura de vetores nas areas focais	02/2015	07/2015
7	Análise laboratorial	04/2015	12/2015
8	Resultados e Conclusão	09/2015	12/2015

Observações e ressalvas

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Outras ressalvas

1	1- O acesso a Rebio fica condicionado a apresentação da autorização da pesquisa; 2- O pesquisador deverá encaminhar por e-mail o cronograma dos trabalhos de campo contendo (relação dos participantes, local, data e hora para o email rebio.tingua@icmbio.gov.br; 3 - Cópia dos relatórios e publicações resultantes da atividade de pesquisa devem ser encaminhadas à administração da UC.
2	1-LER O BOLETIM Nº19 DO PNI -MAMIFEROS DO PNI-SITE DO PNI-QUE FAZEMOS-BOLETIM-LA SERA ENCONTRADO A ESPÉCIES DE PRIMATAS DO PNI,OS NATIVOS E OS EXÓTICOS TAMBÉM CITAÇÕES SOBRE MORTALIDADE DE MACACOS ,UM HOSPEDEIRO DA FEBRE AMARELA.INFORMO QUE NÃO SERÁ PERMITIDO ABATE DE MAMÍFEROS,SENDO QUE O Callithris que é exotico podemos discutir com a equipe técnica.

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Número: 54707-1	Data da Emissão: 25/08/2016 18:28	Data para Revalidação*: 24/09/2017

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Dados do titular

Nome: RICARDO LOURENÇO DE OLIVEIRA	CPF: 289.349.207-00
Título do Projeto: Primatas como fontes de infecção de zoonoses no Rio de Janeiro?	
Nome da Instituição : FUNDAÇÃO OSWALDO CRUZ	CNPJ: 33.781.055/0001-35

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Expedições em campo para coleta de amostras in-situ	06/2016	06/2018
2	Coleta de amostras em CETAS	06/2016	12/2018
3	Diagnósticos e análises microscópicos, sorológicos e moleculares	06/2016	02/2019
4	Resultados e Conclusões	02/2019	06/2019

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2	Disponibilizar uma cópia digital dos resultados da pesquisa para o acervo da APA Petrópolis. Contendo a localização e data (georreferenciada) das espécies capturadas, coletadas e registradas dentro dos limites geográficos da APA Petrópolis. Em relação às espécies ameaçadas, em caso de captura ou registro da espécie Callithrix aurita, a equipe do PARNASO deverá ser comunicada imediatamente pelo e-mail: pesquisa.parnaso@icmbio.gov.br. Se for o caso, os profissionais em campo deverão estar preparados inclusive para o envio de amostras biológicas ao PARNASO/ICMBio ou instituição parceira.
3	ABATE/COLETA/ANESTESIA E ARMADILHA NOS PRIMATAS DO PNI NAO SERAOPERMITIDOS. O PESQUISADOR DEVERÁ MARCAR UMA REUNIÃO COM O SETOR DE PESQUISA ANTES DOS TREABLHOS-leoquilombo@gmaila.com
4	1- Observar o previsto na Cartilha do Pesquisador do PARNASO. 2- Sempre que possível, aproveitar a captura dos primatas para a obtenção do máximo de informações e amostras possíveis, resguardado o bem estar do animal, aproveitando parcerias com outros projetos.

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Dados do titular

Nome: RICARDO LOURENÇO DE OLIVEIRA	CPF: 289.349.207-00
Título do Projeto: Primatas como fontes de infecção de zoonoses no Rio de Janeiro?	
Nome da Instituição : FUNDAÇÃO OSWALDO CRUZ	CNPJ: 33.781.055/0001-35

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Expedições em campo para coleta de amostras in-situ	06/2016	06/2018
2	Coleta de amostras em CETAS	06/2016	12/2018
3	Diagnósticos e análises microscópicos, sorológicos e moleculares	06/2016	02/2019
4	Resultados e Conclusões	02/2019	06/2019

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	1- O acesso a Rebio fica condicionado a apresentação da autorização da pesquisa; 2- O pesquisador deverá encaminhar por e-mail o cronograma dos trabalhos de campo contendo (relação dos participantes, local, data e hora para o email rebio.tingua@icmbio.gov.br; 3 - Cópia dos relatórios e publicações resultantes da atividade de pesquisa devem ser encaminhadas à administração da UC.
2	Disponibilizar uma cópia digital dos resultados da pesquisa para o acervo da APA Petrópolis. Contendo a localização e data (georreferenciada) das espécies capturadas, coletadas e registradas dentro dos limites geográficos da APA Petrópolis. Em relação às espécies ameaçadas, em caso de captura ou registro da espécie Callithrix aurita, a equipe do PARNASO deverá ser comunicada imediatamente pelo e-mail: pesquisa.parnaso@icmbio.gov.br. Se for o caso, os profissionais em campo deverão estar preparados inclusive para o envio de amostras biológicas ao PARNASO/ICMBio ou instituição parceira.
3	ABATE/COLETA/ANESTESIA E ARMADILHA NOS PRIMATAS DO PNI NAO SERAOPERMITIDOS. O PESQUISADOR DEVERÁ MARCAR UMA REUNIÃO COM O SETOR DE PESQUISA ANTES DOS TREABLHOS-leoquilombo@gmaila.com
4	1- Observar o previsto na Cartilha do Pesquisador do PARNASO. 2- Sempre que possível, aproveitar a captura dos primatas para a obtenção do máximo de informações e amostras possíveis, resguardado o bem estar do animal, aproveitando parcerias com outros projetos.

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 84936571

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Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 54707-4	Data da Emissão: 26/09/2017 12:26	Data para Revalidação*: 26/10/2018

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: RICARDO LOURENÇO DE OLIVEIRA	CPF: 289.349.207-00
Título do Projeto: Primatas como fontes de infecção de zoonoses no Rio de Janeiro?	
Nome da Instituição : FUNDAÇÃO OSWALDO CRUZ	CNPJ: 33.781.055/0001-35

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Expedições em campo para coleta de amostras in-situ	06/2016	06/2018
2	Coleta de amostras em CETAS	06/2016	12/2018
3	Diagnósticos e análises microscópicos, sorológicos e moleculares	06/2016	02/2019
4	Resultados e Conclusões	02/2019	06/2019

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	1- O acesso a Rebio fica condicionado a apresentação da autorização da pesquisa; 2- O pesquisador deverá encaminhar por e-mail o cronograma dos trabalhos de campo contendo (relação dos participantes, local, data e hora para o email rebio.tingua@icmbio.gov.br; 3 - Cópia dos relatórios e publicações resultantes da atividade de pesquisa devem ser encaminhadas à administração da UC.
2	Disponibilizar uma cópia digital dos resultados da pesquisa para o acervo da APA Petrópolis. Contendo a localização e data (georreferenciada) das espécies capturadas, coletadas e registradas dentro dos limites geográficos da APA Petrópolis. Em relação às espécies ameaçadas, em caso de captura ou registro da espécie Callithrix aurita, a equipe do PARNASO deverá ser comunicada imediatamente pelo e-mail: pesquisa.parnaso@icmbio.gov.br. Se for o caso, os profissionais em campo deverão estar preparados inclusive para o envio de amostras biológicas ao PARNASO/ICMBio ou instituição parceira.
3	ABATE/COLETA/ANESTESIA E ARMADILHA NOS PRIMATAS DO PNI NÃO SERÃOPERMITIDOS. O PESQUISADOR DEVERÁ MARCAR UMA REUNIÃO COM O SETOR DE PESQUISA ANTES DOS TREABLHOS-leoquilombo@gmaila.com
4	1- Observar o previsto na Cartilha do Pesquisador do PARNASO. 2- Sempre que possível, aproveitar a captura dos primatas para a obtenção do máximo de informações e amostras possíveis, resguardado o bem estar do animal, aproveitando parcerias com outros projetos.

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 32473623

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Autorização para atividades com finalidade científica

Número: 54707-6	Data da Emissão: 30/01/2019 13:23:01	Data da Revalidação*: 30/01/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: RICARDO LOURENÇO DE OLIVEIRA	CPF: 289.349.207-00
Nome da Instituição: Fundação Oswaldo Cruz	CNPJ: 33.781.055/0001-35

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Expedições em campo para coleta de amostras in-situ	06/2016	06/2018
2	Diagnósticos e análises microscópicos, sorológicos e moleculares	06/2016	02/2019
3	Resultados e Conclusões	02/2019	06/2019
4	Coleta de amostras em CETAS	06/2016	12/2018
5	Expedições em campo para coleta e monitoramento da circulação da Febre Amarela	10/2018	12/2019

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Filipe Vieira Santos de Abreu	Biólogo	080.429.536-07	Brasileira
2	Marcelo Quintela Gomes	Técnico	021.868.947-00	Brasileira
3	DANILO SIMONINI TEIXEIRA	Veterinário	922.004.351-34	Brasileira
4	Edmilson dos Santos	Biólogo	497.722.360-87	Brasileira
5	Pollyanna Cardoso Araújo	Veterinária	023.638.051-60	Brasileira
6	Waldemir Paixão Vargas	Biólogo	751.645.237-87	Brasileira
7	Rafaella Moraes de Miranda	Bióloga	003.782.012-55	Brasileira

GOVERNO DO ESTADO DO RIO DE JANEIRO
SECRETARIA DE ESTADO DO AMBIENTE – SEA
INSTITUTO ESTADUAL DO AMBIENTE - INEA

AUTORIZAÇÃO DE PESQUISA CIENTÍFICA INEA Nº 012/2016

AUTORIZAÇÃO PARA PESQUISA CIENTÍFICA EM UNIDADE DE CONSERVAÇÃO

O Diretor de Biodiversidade e Áreas Protegidas do Instituto Estadual do Ambiente – INEA, no uso de suas atribuições legais, considerando a Portaria IEF/RJ/PR nº 227 de 18/12/2007 e considerando, ainda, o que consta no procedimento administrativo E-07/002.13371/2015, **AUTORIZA** o pesquisador **RICARDO LOURENÇO DE OLIVEIRA**, vinculado a Fundação Oswaldo Cruz, e sua equipe, Filipe Vieira Santos de Abreu, Waldemir Paixão Vargas, Danilo Simonini Teixeira, Edmilson dos Santos, Marcelo Quintela Gomes, Monique Albuquerque Motta, a obter dados no Parque Estadual do Desengano – PED, Parque Estadual da Pedra Branca – PEPB, Parque Estadual dos Três Picos – PETP, Parque Estadual do Mendanha – PEM, Parque Estadual da Pedra Selada – PEPS, Parque Estadual da Ilha Grande – PEIG, Reserva Biológica Estadual de Araras – RBA, Área de Proteção Ambiental do Alto Iguaçu – APA do Alto Iguaçu, Área de Proteção Ambiental de Macaé de Cima – APA de Macaé de Cima, Área de Proteção Ambiental da Bacia do Rio Macacu – APA da Bacia do Rio Macacu e Área de Proteção Ambiental do Rio Guandu – APA Guandu, com vistas à execução do projeto de pesquisa **"Primatas como fontes de infecção de zoonoses no Rio de Janeiro"**, devendo ser observadas as condições discriminadas no verso deste documento e ainda aquelas previstas na Portaria supracitada.

A presente autorização tem validade de **02 (dois) anos** a partir da data de sua assinatura.

Rio de Janeiro, 07 de fevereiro de 2016.

Paulo Schiavo
Diretor de Biodiversidade e Áreas Protegidas

GOVERNO DO ESTADO DO RIO DE JANEIRO
SECRETARIA DE ESTADO DO AMBIENTE – SEA
INSTITUTO ESTADUAL DO AMBIENTE - INEA

AUTORIZAÇÃO DE PESQUISA CIENTÍFICA INEA N° 019/2018

AUTORIZAÇÃO PARA PESQUISA CIENTÍFICA EM UNIDADE DE CONSERVAÇÃO

O Diretor de Biodiversidade e Áreas Protegidas do Instituto Estadual do Ambiente – INEA, no uso de suas atribuições legais, considerando a Portaria IEF/RJ/PR nº 227 de 18/12/2007 e considerando, ainda, o que consta no procedimento administrativo E-07/002.13371/2015, **AUTORIZA** o pesquisador **RICARDO LOURENÇO DE OLIVEIRA**, vinculado a Fundação Oswaldo Cruz, e sua equipe, Filipe Vieira Santos de Abreu, Waldemir Paixão Vargas, Danilo Simonini Teixeira, Edmilson dos Santos, Marcelo Quintela Gomes, Monique Albuquerque Motta, Taissa Pereira dos Santos, Renato Carvalho de Andrade, Mauro Menezes Muniz, Marcelo Celestino dos Santos a obter dados no Parque Estadual do Desengano – PED, Parque Estadual da Pedra Branca – PEPB, Parque Estadual dos Três Picos – PETP, Parque Estadual do Mendanha – PEM, Parque Estadual da Pedra Selada – PEPS, Parque Estadual da Ilha Grande – PEIG, Reserva Biológica Estadual de Araras – RBA, Área de Proteção Ambiental do Alto Iguaçu – APA do Alto Iguaçu, Área de Proteção Ambiental de Macaé de Cima – APA de Macaé de Cima, Área de Proteção Ambiental da Bacia do Rio Macacu – APA da Bacia do Rio Macacu e Área de Proteção Ambiental do Rio Guandu – APA Guandu, com vistas à execução do projeto de pesquisa **"Primatas como fontes de infecção de zoonoses no Rio de Janeiro"**, devendo ser observadas as condições discriminadas no verso deste documento e ainda aquelas previstas na Portaria supracitada.

A presente autorização tem validade de **02 (dois) anos** a partir da data de sua assinatura.

Rio de Janeiro, 19 de abril de 2018.

Paulo Schiavo
Diretor de Biodiversidade e Áreas Protegidas

GOVERNO DO Rio de Janeiro
PERTO DE VOCÊ
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Autorização para atividades com finalidade científica

Número: 52472-1	Data da Emissão: 26/01/2016 14:47	Data para Revalidação*: 24/02/2017

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Filipe Vieira Santos de Abreu	CPF: 080.429.536-07
Título do Projeto: Qual o risco de reemergência de febre amarela silvestre no Rio de Janeiro?	
Nome da Instituição : FUNDAÇÃO OSWALDO CRUZ	CNPJ: 33.781.055/0001-35

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta nas fronteiras com Minas Gerais e São Paulo	02/2016	02/2019
2	Coleta na região serrana do Rio de janeiro	02/2016	02/2019
3	Concomitantemente à análise de vetores, serão analisados os primatas não humanos (sisbio nº 41837-3)	02/2016	06/2019
4	Criação dos imaturos em laboratório	03/2016	06/2019
5	Isolamento viral das amostras	06/2016	01/2019
6	Testes de competência vetorial	08/2016	12/2018

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	Está autorizada a manutenção temporária até 24 meses. Para períodos superiores a esse deve ser solicitada uma autorização junto ao órgão ambiental estadual ou ao IBAMA.
2	1)Parna de Itatiaia: Sugerimos a leitura do boletim de pesquisa nº19 – "Mamíferos de médio e grande porte no PNI" – Izar Aximoff – no trabalho tem uma citação sobre febre amarela nos primatas do Parque. 2)Parna Serra dos Órgãos: Observar o previsto na Cartilha do Pesquisador do PARNASO. 3)Rebio do Tinguá: O acesso a Rebio fica condicionado a apresentação da autorização da pesquisa; O pesquisador deverá encaminhar por e-mail o cronograma dos trabalhos de campo contendo (relação dos participantes, local, data e hora para o email rebio.tingua@icmbio.gov.br. 4)APA de Guapi-Mirim: Comunicar com antecedência as atividades de coleta na UC.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Tacilane Divina Cardoso	Técnica / bolsista Cculi	115.485.777-82	222472664 Detran-RJ	Brasileira

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Código de autenticação: 27648581

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Autorização para atividades com finalidade científica

Número: 52472-2	Data da Emissão: 29/06/2017 17:23	Data para Revalidação*: 29/07/2018

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Filipe Vieira Santos de Abreu	CPF: 080.429.536-07
Título do Projeto: Qual o risco de reemergência de febre amarela silvestre no Rio de Janeiro?	
Nome da Instituição : FUNDAÇÃO OSWALDO CRUZ	CNPJ: 33.781.055/0001-35

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta nas fronteiras com Minas Gerais e São Paulo	02/2016	02/2019
2	Coleta na região serrana do Rio de janeiro	02/2016	02/2019
3	Concomitantemente à análise de vetores, serão analisados os primatas não humanos (sisbio nº 41837-3)	02/2016	06/2019
4	Criação dos imaturos em laboratório	03/2016	06/2019
5	Isolamento viral das amostras	06/2016	01/2019
6	Testes de competência vetorial	08/2016	12/2018

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
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8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	Está autorizada a manutenção temporária até 24 meses. Para períodos superiores a esse deve ser solicitada uma autorização junto ao órgão ambiental estadual ou ao IBAMA.
2	1)Parna de Itatiaia: Sugerimos a leitura do boletim de pesquisa nº19 – "Mamíferos de médio e grande porte no PNI" – Izar Aximoff – no trabalho tem uma citação sobre febre amarela nos primatas do Parque. 2)Parna Serra dos Órgãos: Observar o previsto na Cartilha do Pesquisador do PARNASO. 3)Rebio do Tinguá: O acesso a Rebio fica condicionado a apresentação da autorização da pesquisa; O pesquisador deverá encaminhar por e-mail o cronograma dos trabalhos de campo contendo (relação dos participantes, local, data e hora para o email rebio.tingua@icmbio.gov.br. 4)APA de Guapi-Mirim: Comunicar com antecedência as atividades de coleta na UC.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Tacilane Divina Cardoso	Técnica / bolsista Cculi	115.485.777-82	222472664 Detran-RJ	Brasileira

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Instituto Oswaldo Cruz
Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA L-004/2015

Certificamos que o protocolo (CEUA/IOC-004/2014), intitulado "Estudo da dinâmica dos processos endêmicos-epidêmicos da febre amarela em áreas de recém transmissão e indenes da região Sul e Sudeste do Brasil, através de inquérito soro-epidemiológico em primatas não humanos", sob a responsabilidade de **RITA MARIA RIBEIRO NOGUEIRA** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

Esta licença tem validade até 31/12/2018 e inclui o uso total de:

Alouatta sp, Sapajus sp, Callithrix sp. (PRIMATA NÃO HUMANO):
- total 50 animais adultos.

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 10 de abril de 2015.

Flávio Alves Lara
Coordenador Adjunto da CEUA/Instituto Oswaldo Cruz
Fundação Oswaldo Cruz

FIOCRUZ-Fundação Oswaldo Cruz/IOC-Instituto Oswaldo Cruz
Av. Brasil, 4365 - Manguinhos - Rio de Janeiro - RJ - Brasil
CEP: 21040-360 Tel: (21) 2562-1056

Instituto Oswaldo Cruz

Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA L-037/2016

Certificamos que o protocolo (CEUA/IOC-029/2016), intitulado "Primatas como fontes de infecção de zoonoses no Rio de Janeiro", sob a responsabilidade de **RICARDO LOURENÇO DE OLIVEIRA** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

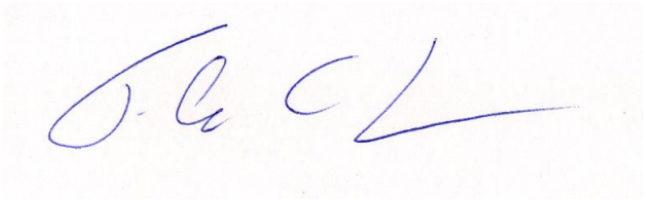
Esta licença tem validade até 31/07/2020 e inclui o uso total de:

Primatas não humanos, cepa:

Alouatta sp.
Cebus sp.
Callithrix sp.
Callicebus sp. – aproximadamente 80 animais

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 24 de agosto de 2016.



Flávio Alves Lara

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7.6 Anexo VI – Galeria de Fotos

Prancha 1: Coleta de mosquitos adultos (a) Armadilha BGsentinel em transecto florestal em Domingos Martisn (ES); (b) Armadilha de Shannon em Miguel Pereira (RJ); (c) Aspiração em residência onde registrou-se de óbito por febre amarela.

Prancha 02: Coleta e processamento dos mosquitos. (a) Captura através de Atração Humana Protegida e esclarecida; (b) Triagem dos mosquitos em campo; (c) identificação taxonômica em mesa fria no laboratório.

Prancha 3: Construção de plataformas para captura de bugio em dossel. (a) – (c) Parque Natural do Atalaia, Macaé, RJ; (d) Sumidouro, RJ.

Prancha 4: Armadilhas utilizadas para captura de primatas. (a) Tomahawk média sendo ignorada por *Callicebus nigrifrons* em Itamonte (MG); (b)Tomahawk pequena para captura de *Callithrix sp.* em Nova Iguaçú (RJ); (c) Armadilha manual grande instalada em dossel com dois *Sapajus nigritus* "fazendo um selfie".

Prancha 5: Exame de bugios em campo; (a) *A. g. clamitans* capturado com dardo em Miguel Pereira (RJ); (b) e (c) Necrópsia nas epizootias com suspeita de febre amarela em Domingos Martins (ES).

Prancha 6: Laboratórios de campo; (a) Exame de *C. nigrifons.* Itanhandu (MG); (b) Exame de *Callithrix sp.* em laboratório improvisado, Nova Iguaçú, (RJ); (c) Parque Nacional de Itatiaia (RJ); (d) Exame de *A. g. clamitans*, Teresópolis (RJ).

Prancha 7: Duas paisagens da febre amarela 2016-2019. (a) Casa com plantação de café no terreno e fragmento de mata ao fundo. Proprietário faleceu com diagnóstico de YFV, Simonésia (MG); (b) Paisagem com fragmentos de mata rodeados por pastagens em Carmo (RJ). Recolhemos e examinamos dois *A. g. clamitans* mortos, com resultados positivos para YFV, encontrados no fragmento que aparece em primeiro plano. A dispersão entre os fragmentos provavelmente se daria através do voo dos mosquitos vetores.

Prancha 8: Palestras, treinamentos e capacitação em serviço; (a) Ciclo de palestras para agentes de saúde e guarda-parques durante expedição no Parque Nacional Serra dos Órgãos, Teresópolis (RJ); (b) Capacitação em serviço da equipe de entomologia da FUNED, Belo Horizonte (MG); (c) Equipe do Ministério da Saúde, da Secretaria de Saúde do RJ e da Associação Mico Leão Dourado, durante investigação de YFV no Rio de Janeiro.