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DISSERTAÇÃO DE MESTRADO

OTIMIZAÇÃO DE ENSAIOS MOLECULARES PARA O DIGNÓSTICO DA COVID-19

KAROLINE ALMEIDA FELIX DE SOUSA

Salvador – Bahia
2022

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa para a obtenção do grau de Mestre**.**

Orientador: Prof. Dr. Bruno Solano de Freitas Souza
Coorientadora: Profa. Dra. Carolina Kymie Vasques Nonaka

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KAROLINE ALMEIDA FELIX DE SOUSA

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COMISSÃO EXAMINADORA

Dra. Marta Giovanetti
Pesquisadora IOC/FIOCRUZ

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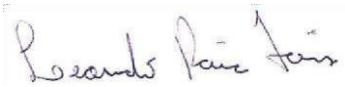

Dr. Leonardo Paiva Farias
Pesquisador IGM/FIOCRUZ

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RESUMO

INTRODUÇÃO: Para o controle da pandemia de SARS-CoV-2 a testagem em massa se revelou fundamental. No entanto, sua implementação possui alguns gargalos, especialmente em populações de baixo índice socioeconômico, já que a metodologia padrão-ouro para detecção do SARS-CoV-2 é o RT-PCR em amostras de swab nasofaríngeo, exigindo infraestrutura dedicada à coleta, laboratórios sofisticados e equipe especializada. Portanto, a necessidade de testes rápidos, simples e precisos para diagnosticar a infecção por SARS-CoV-2 é de extrema importância, bem como a validação destes ensaios em outras alternativas de amostras de mais fácil obtenção, como a saliva. Os sistemas utilizando CRISPR para o diagnóstico de COVID-19 tem se apresentado como promissores, já que apresentam alta sensibilidade, rapidez de detecção e não requerem laboratórios especializados. **OBJETIVO**: Otimizar métodos moleculares para a detecção do SARS-CoV-2 em amostras de saliva e validar um protótipo de teste molecular "point of care" baseado na tecnologia CRISPR/Cas12. **METODOLOGIA**: Trata-se de um estudo de corte transversal envolvendo 103 participantes, no período de março a maio de 2021, submetidos à coleta de amostras pareadas de swab nasofaríngeo e saliva. Para a validação do RT-PCR em amostras de saliva, foram utilizados três kits comerciais e diferentes protocolos pré-analíticos envolvendo a etapa de extração e condições de armazenamento/estabilidade das amostras. Para o teste rápido com CRISPR/Cas12 foram avaliadas 29 amostras no período de janeiro a fevereiro de 2022 e desenhados três de grupos de primers para a reação de amplificação isotérmica (LAMP) e os respectivos gRNAs com primers fluorescentes para a detecção do complexo RNA-proteína através da clivagem no fluxo lateral. **RESULTADOS**: Observou-se 100% e 89,1% de concordância comparando amostras de saliva em Allplex^{TM} e TaqPath^{TM} com RT-PCR em amostra de swab, respectivamente. A análise de estabilidade da saliva demonstrou capacidade de detecção do SARS-CoV-2 em amostras armazenadas por 30 dias a -80^{o}C. O ensaio CRISPR/Cas12 mostrou 100% de concordância entre os resultados obtidos por RT- PCR, com limite de detecção de 15,6 PFU. Não foi observada reação cruzada com outros vírus respiratórios. O método RT-PCR apresentou uma sensibilidade de aproximadamente 8 cópias de RNA/µL e o CRISPR/Cas12 de 84 cópias de RNA/µL. **CONCLUSÃO**: A saliva é uma amostra sensível para o diagnóstico da COVID-19. Nosso estudo mostra evidências de que é possível identificar indivíduos com infecção assintomática sem a necessidade de extração de RNA e solução estabilizadora de RNA, tornando assim o diagnóstico de saliva promissor para testes diretos na rotina laboratorial. O ensaio CRISPR/Cas12 oferece uma alternativa promissora mais rápida para a detecção de SARS-CoV-2, mas novos estudos são necessários para que este método seja validado.

Palavras-Chave: Saliva. COVID-19. CRISPR. Diagnóstico.

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ABSTRACT

INTRODUCTION: For the control of the SARS-CoV-2, pandemic mass testing proved to be crucial. However, its implementation has some bottlenecks, especially in low socioeconomic populations, since the gold standard methodology for SARS-CoV-2 detection is RT-PCR in nasopharyngeal swab samples, requiring dedicated collection infrastructure, sophisticated laboratories, and specialized staff. Therefore, the need for rapid, simple and accuracy tests to diagnose SARS-CoV-2 infection is of extreme importance, as well as the validation of the assays in other more easily obtained samples alternatives, such as saliva. Systems using CRISPR for the diagnosis of COVID-19 have shown promise, as they have high sensitivity, rapid detection, and do not require specialized laboratories **OBJECTIVE**: Optimize molecular methods for the detection of SARS-CoV-2 in saliva samples and validate a prototype "point of care" molecular test based on CRISPR/Cas12 technology. **METHODS**: This is a cross- sectional study involving 103 participants from March to May 2021, submitted to the collection of paired samples of nasopharyngeal swab and saliva. For the validation of RT-PCR in saliva samples, three commercial kits and different pre-analytical protocols involving the extraction step and storage/stability conditions of the samples were used. For the rapid test with CRISPR/Cas12, 29 samples were evaluated from January to February 2022 and three groups of primers were designed for the isothermal amplification reaction (LAMP) and the respective gRNAs with fluorescent primers for the detection of the RNA-protein complex by lateral flow cleavage. **RESULTS**: We observed 100% and 89.1% concordance in saliva samples comparing Allplex™ and TaqPath™ with RT- PCR on swab sample, respectively. Saliva stability analysis demonstrated the capability of detecting SARS-CoV-2 in samples stored for 30 days at-80ºC. The CRISPR/Cas12 assay showed 100% concordance between the results obtained by RT-PCR, with a detection limit of 15.6 PFU. No cross-reaction with other respiratory viruses was observed. The RT-PCR method showed a sensitivity of approximately 8 RNA copies/µL and the CRISPR/Cas12 of 84 RNA copies/µL. **CONCLUSIONS**: Saliva is a sensitive sample for the diagnosis of COVID-19. Our study shows evidence that it is possible to identify asymptomatic infectious individuals without the need for RNA extraction and RNA stabilizing solution, thus making saliva diagnostics promising for direct testing in the laboratory routine. The CRISPR/Cas12 assay offers a promising faster alternative for the detection of SARS-CoV-2, but further studies are necessary to validate this method.

Keywords: Saliva. COVID-19. CRISPR. Diagnosis.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ACE-2	Angiostensin-Converting Enzyme 2
Cas12a	CRISPR associated protein 12a
CDC	Centers for Disease Control and Prevention
COVID-19	Coronavírus Disease 2019
CRISPR	Clustered Regularly Interspaced Shortcr RNA
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
gRNA	Guide RNA
HIV	Human Immunodeficiency Virus
IgG	Imunoglobulina G
IgM	Imunoglobulina M
LAMP	Loop-Mediated Isothermal Amplification
LoD	Limit of Detection
MERS	Middle East Respiratory Syndrome
OMS	Organização Mundial de Saúde
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
RNA	Ribonucleic Acid
RT-LAMP	Reverse Transcription Loop-Mediated Isothermal AmplificationRT-
PCR	Reverse Transcription Polymerase Chain Reaction
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavírus 2
SDRA	Síndrome do desconforto respiratório agudo
ssRNA	Single-Stranded RNA

SUMÁRIO

1 **INTRODUÇÃO**

O SARS-CoV-2 agente causador da doença do coronavírus 2019 (COVID-19), provocou um surto no final de 2019 em Wuhan, na China, que foi seguido por uma rápida disseminação global levando os sistemas de saúde pública ao colapso devido a grave morbimortalidade respiratória nos indivíduos infectados. Em março de 2020 a Organização Mundial de Saúde (OMS) caracterizou a COVID-19 como uma pandemia(“WHO,” 2020).

Mesmo após várias iniciativas, ainda não se conhece a origem do SARS-CoV- 2, mas investigações genômicas sugerem uma possível origem de morcegos, com um hospedeiro intermediário ainda não identificado e possivelmente associado a materiais contaminados no mercado de frutos do mar em Wuhan, onde foram rastreados os primeiros casos (JIANG; DU; SHI, 2020). O SARS-CoV-2 é um vírus de RNA de fita simples positiva envelopado da família dos Coronaviridae. Sua transmissão é por contato direto com pessoas infectadas através de gotículas respiratórias. Os sintomas mais comuns são febre e tosse, com cerca de 80% dos casos apresentando quadro clínico assintomático ou brando (MALLAH et al., 2021). Entretanto, 20% dos casos desenvolvem a forma grave da doença, síndrome do desconforto respiratório agudo (SDRA), associando à pneumonia grave e insuficiênciarespiratória (RUANet al., 2020).

O swab nasofaríngeo é o principal meio de coleta de amostra para o diagnóstico molecular do SARS-CoV-2 por RT-PCR. Entretanto, uma série de obstáculos se apresentavam para a sua implementação em larga escala, incluindo a necessidade de infraestrutura e procedimentos de biossegurança específicos, a resistência da população por conta do desconforto associado ao procedimento de coleta, além do risco de exposição e transmissão viral para profissional que realiza o procedimento (AZZI et al., 2021). Como alternativa, a saliva conquistou atenção cientifica para o seu uso como amostra diagnóstica devido a sua facilidade de coleta pelo próprio paciente,consequentemente reduzindo o risco detransmissão para equipe de coleta.

Durante o surto de SARS em 2003, foi relatada a presença do vírus em gotículas de saliva. Destacando como fonte de infecção e uma amostra potencial para o diagnóstico (WANG et al., 2004). Estudos mostram a detecção de SARS-CoV-2 na saliva por RT-PCR em pacientes com diferentes gravidades da doença e com uma maior sensibilidade quando relacionados as amostras de swab nasofaríngeo (TEO etal.,2021; TO et al., 2020). Desta forma, empregar o uso da saliva pode proporcionar um procedimento rápido, seguro e adequado para o diagnóstico de COVID-19.

A reação em cadeia da polimerase (RT-PCR) é o padrão ouro no diagnóstico da COVID-19. Contudo, a técnica requer uso de laboratórios equipados com demora em seus resultados, reduzindo a perspectiva do diagnóstico rápido. A identificação deácido nucleicos baseada na tecnologia CRISPR / Cas surgiu nos últimos anos como uma promessa no desenvolvimento de métodos de

diagnostico molecular simples e rápidos, além de apresentar alta sensibilidade e especificidade (LI et al., 2019).

Para conter o avanço do SARS-CoV-2 em todo o mundo preconiza-se uma melhor estratégia de testagem e ferramentas diagnósticas. Portanto, torna-se necessário o uso de ensaios ultrassensíveis, rápidos e que não necessitem de um altograu de conhecimento técnicoe equipamento caros para o diagnóstico do COVID-19. Desta forma, esse projeto buscou avaliar alternativas para o diagnóstico molecular da COVID-19 através da utilização de amostras de saliva em reações de RT-PCR, além do desenvolvimento de um protóripo de teste molecular "point of care" baseado na tecnologia CRISPR-Cas12.

2 **REVISÃO DE LITERATURA**

2.1 O VÍRUS SARS-CoV-2

2.1.1 **Histórico e Epidemiologia**

No final de 2019 o escritório da OMS na China foi notificado sobre um novo surto de pneumonia na cidade de Wuhan, a capital e maior cidade da província de Hubei, na China (ZHU; WEI; NIU, 2020). Grande maioria dos pacientes frequentavamregularmente o mercado atacadista de frutos do mar em Wuhan e a causa da doença ainda era desconhecida. Através do teste molecular de reação em cadeia da polimerase (PCR), o patógeno foi confirmado sendo um vírus (JEFFERSON RIBEIRODA SILVA et al., 2019).

Posteriormente, foi realizado o sequenciamento e o genoma viral não correspondia com nenhum vírus sequenciado anteriormente. Em janeiro de 2020, a doença causada pelo novo vírus foi denominada como COVID-19 e com base em testes filogenéticos o Comitê Internacional de Taxonomia de Vírus nomeou o novo vírus de SARS-CoV-2 (GORBALENYA et al., 2020).

SARS-CoV-2, causador da doença coranavirus 2019 (COVID-19), é relacionado ao SARS-CoV, um coronavírus que pertence à categoria de síndrome respiratória aguda grave (GORBALENYA et al., 2020). Até o momento, foram identificados sete coronavírus humanos (COMMITTEE, 2020; CUI; LI; SHI, 2019). O SARS-CoV-2 é membro da

Nidovirales, família Coronaviridae e sua subfamília é Orthocoronavirinae, que é subdivida em quatro gêneros, os que se originam de genes de aves e suínos, Gammacoronavirus e Deltacoronavirus e os que se originam de morcegos, os gêneros Alphacoronavirus e Betacoronavirus (CHEN; LIU; GUO, 2020; WOO et al., 2012).

Além dos SARS-CoVs, o MERS-CoV também é um coronavirus que infecta humanos. Ambos apresentam sintomas semelhantes, febre, falta de ar, tosse e pneumonia (ZAKI et al., 2012). De acordo com a OMS a taxa de mortalidade do MERSchegou a 35% e o principal hospedeiro de transmissão era os camelos. Na sociedade americana e europeia o efeito do MERS foi mínimo. Atualmente a infecção continua no Oriente Médio (MACKAY; ARDEN, 2015; WHO, 2022).

A pandemia do COVID-19 originou um impacto na mortalidade e morbidade, diferentemente das doenças anteriores associadas ao coronavírus (DONG; DU; GARDNER, 2020; ZHU et al., 2020). Devido a rápida disseminação, a OMS nomeou a sua rápida

disseminação como, a primeira pandemia do século 21 (E; H; L, 2020). O SARS-CoV-2 obteve uma grande propagação e alta letalidade por meio de viagensdecorrente da transmissão de indivíduos pré-sintomáticos ou assintomáticos de continente a continente (JT; K; GM, 2020; Y et al., 2020). Esses portadores viajantes facilitam a rápida disseminação (GANDHI; YOKOE; HAVLIR, 2020; WIT et al., 2016).

2.1.2 **Organização genômica e mecanismos de infecção**

O tamanho da partícula viral do SARS-CoV-2 é de aproximadamente 80 a 120 nm de diâmetro e suas espículas (spike) apresentam diâmetros de 9 a 12 nm (CUI; LI; SHI, 2019; WRAPP et al., 2020). Ele é um vírus de forma esférica, envelopado e não segmentado (XU et al., 2020). Cada virion consiste em um ssRNA com cauda 5′-cap e 3′-poly-A positivo.

O genoma do coronavírus varia de 27 a 30 quilobases e codificam mais de cinco ORFs (CHAN et al., 2020). A principal é a ORF1a que ocupa a maior parte da extremidade 5’ e é seguida pela ORF1b. Eles são clivados em 16 proteínas não estruturais (Nsps). Na extremidade 3’ estão presentes genes que codificam proteínas estruturais da membrana (M), envelope (E), espícula (S) e nucleocapsídeo (N). Todas elassão características comuns de todos coronavírus. Embora o SARS-CoV-2 não tenha o gene da hemaglutinina-esterase (HE) que é encontrado em alguns betacoronavírus(CHAN et al., 2020).

A menor proteína encontrada no coronavírus é a proteína E, variando entre 8 e 10 quilodalton. Ela atua na construção de viroporinas, transportando de ions de Ca 2+, favorecendo a inserção de partículas virais nas células hospedeiras (LIAO; TAM; LIU, 2006; PHAM et al., 2017; ZHANG et al., 2014).

A proteína E evidencia funções e características comuns de outras CoVs. A inexistência da proteína pode modificar a morfologia e o tropismo do coronavírus (DEDIEGO et al., 2007). A proteína apresenta três domínios, uma cauda amino hidrofílico curta, um domínio C-terminal ativo e um domínio transmembranar hidrofóbico grande (SCHOEMAN; FIELDING, 2019).

A proteína M é a mais presente na partícula viral, formando o envelope viral (NEUMAN et al., 2011). Ela atua como uma proteína de membrana multispanning favorecendo a formação do vírus a partir da comunicação proteína-proteína (BIANCHI et al., 2020). É descrito na literatura que a proteína M agrupa sinais de empacotamentodo genoma através do seu contato com o RNA (HAAN; VENNEMA; ROTTIER, 2000). Pesquisadores relatam que a proteína favorece a patogênese, relatando sua interaçãocom o fator nuclear

kappa B (NF-kB) e com a Ciclo-oxigenase2 (COX-2) contribuindo com a resposta inflamatória do hospedeiro (FANG et al., 2007).

A entrada do vírus nas células é mediada pela proteína S, (BELOUZARD et al.,2012) que também está envolvida na resposta imune do hospedeiro. Essa proteína trimérica é composta pela subunidade S1 que age como um antígeno de superfície e a subunidade S2. A subunidade S1 contém dois domínios: o domínio N-terminal (NTD) e domínio C-terminal (CTD), que age como domínio de ligação ao receptor (RBD) (KE et al., 2020). Já foram descritas algumas mutações no RBD, resultando em um aumento no equilíbrio da estrutura da proteína S e enfraquecendo a ligação de anticorpos. É evidenciado que a subunidade S2 apresenta 99% de semelhança com as dos SARS-CoV humano e presentes em dois morcegos (SL-CoV ZXC21 e ZC45).Subunidade S2 é composta por um domínio transmembranar (TM), domínio citoplasmático (CP) e aminoácidos de fusão conservada (FP) (KADAM et al., 2021).

A proteína N é a proteína mais abundante na célula infectada, sua função é o empacotamento da molécula de RNA. Estudos evidenciam que a proteína N contribui na modulação da maquinaria celular do hospedeiro, colaborando com funções regulatórias, facilitando a multiplicação e disseminação do vírus através da apoptose celular durante seu ciclo de vida viral (ABABOU; LADBURY, 2007; MCBRIDE; ZYL; FIELDING, 2014).

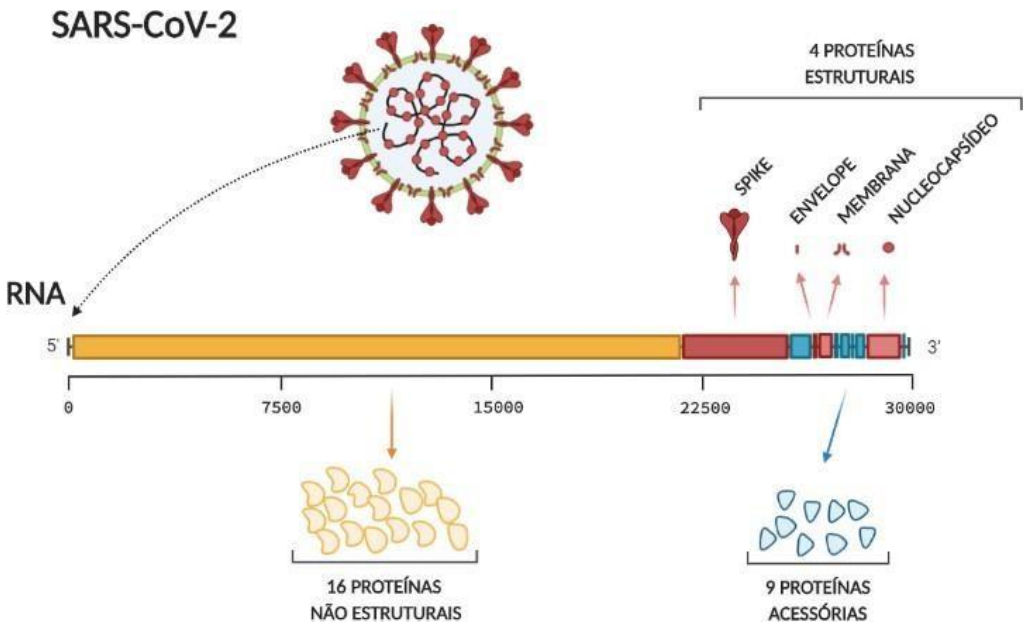

Figura 1 – Estrutura do genoma do SARS-CoV-2. Os genes estruturais presentes no terminal 3' codificam as proteínas estruturais, incluindo o pico S (Spike), envelope (E), membrana (M) e nucleocapsídeo que são características comuns para todos os coronavírus. No terminal 5' encontramos as proteínas não estruturais, que desempenham funções na replicação do RNA viral na elaboração de partículas novas. As 9 proteínas acessórias localizadas no terminal 3' ajudam a fuga das novas partículas, impedindo a sinalização celular para o sistema imunológico.
Fonte:

Já é relatado na literatura a afinidade da proteína S com o receptor ACE-2, e vários estudos demonstraram a entrada do vírus SARS-CoV-2 nas células após interação com os receptores ACE-2 (MATHEWSON et al., 2008; ZHOU et al., 2020). Merece destaque a alta afinidade da ligação da proteína S do SARS-CoV-2 com o receptor, estimada em cerca de 10 vezes maior se comparada à proteína S do SARS-CoV, sugerindo assim uma explicação para a maior transmissibilidade do SARS-CoV-2 (ANGELETTI et al., 2020).

Além do papel fundamental do ACE-2 na entrada celular do vírus, a protease serina-2 transmembrana (TMPRSS2) desempenha um papel importante para a absorção do vírus na membrana da célula e consequentemente a sua entrada na célula hospedeira (Figura 2) (SUNGNAK et al., 2020). Ainda que a TMPRSS2 seja importante na infecção viral, existem outras proteases que desempenham o mesmo papel, a catepsina L e B, podem agir como o sucessor ao TMPRSS2 (SALIAN et al., 2021).

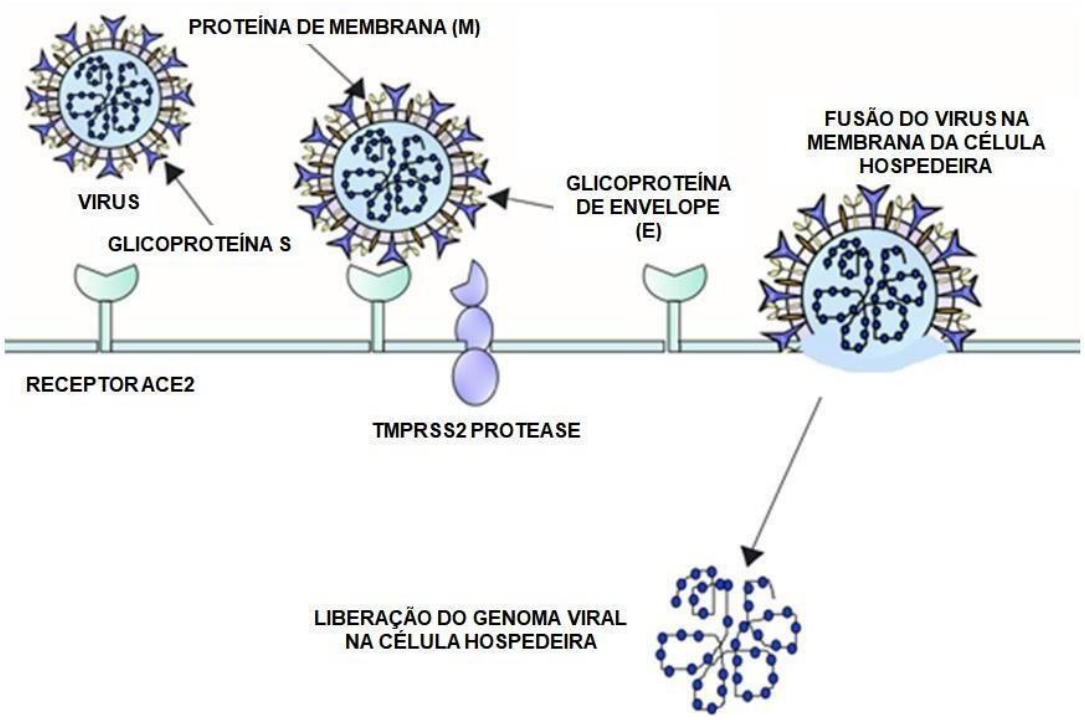

Figura 2 – Entrada do vírus na célula hospedeira. O domínio de ligação do receptor (RBD) da proteína *spike* (S) do vírus se liga ao receptor ACE2 da célula hospedeira. A proteína S é preparada pela protease TMPRSS2 para suceder a fusão viral e liberação do genoma viral na célula hospedeira
Fonte: (Adaptado de SALIAN et al., 2021).

2.1.3 **Transmissão e patologia**

A via de transmissão principal do SARS-CoV-2 é por gotículas respiratórias. A transmissão pode ocorrer de pessoas infectadas sintomáticas e assintomáticas. Um estudo

revelou que até 59% da transmissão pode ser atribuída a pessoas assintomáticas (JOHANSSON et al., 2021). A transmissão por contato em superfícies potencialmente contaminadas era uma preocupação no início da pandemia. Entretanto, dados recentes apontam que embora o vírus possa permanecer em superfícies, o cultivo nessas superfícies no laboratório não obteve êxito (MEDICINE, 2020).

Anteriormente, relatavam que a transmissão aérea do SARS-CoV-2 seria improvável, contudo, estudos relatam tosse seca e espirros de pacientes com COVID-19 provocam gotículas entre 0,6 e 100 μm e a presença de microgotículas infectantes suspensas no ar são suficientes para expor os indivíduos a contaminação (MACHHI et al., 2020). Por meio da fala e respiração, indivíduos assintomáticos podem produzir microgotículas (1 μm) que podem transportar partícula viral viável (SALIAN et al.,2021).

Contudo, medidas não farmacológicas de contenção, como o uso de máscaras e o distanciamento de 2 metros entre pessoas, podem diminuir significativamente a transmissão. Em 2003 o uso de máscaras faciais reduziu a disseminação de SARS-CoV na população. Estudam anteriores revelam que o uso de máscaras de algodão pode filtrar de 40 a 60% de partícula de NaCl (185nm). Destacando que máscaras médicas e de algodão não apresentam diferenças significativas quando usadas em locais fechados para conter partículas virais (TABATABAEIZADEH, 2021).

Pesquisadores relatam a possibilidade de transmissão materno-fetal intrauterina com base nos resultados obtidos de anticorpos IgM e IgG em recém- nascidos imediatamente após o nascimento (DHAMA et al., 2020a). O SARS-CoV-2 pode causar parto prematuro, aborto espontâneo e recentemente estudos mostraram que casos de parto prematuro e suas consequências podem associados ao vírus (CHEN et al., 2020).

A maior parte dos vírus encontram abordagens para invadir células e causar infecção em humanos. O SARS-CoV-2 invade as células através da enzima conversora de angiotensina 2 (ACE2), essa proteína é expressa em níveis elevados no coração, rins, testículos, tireoide, tecido adiposo e no intestino delgado (SINGH et al., 2020). Enquanto no baço, cérebro, vasos sanguíneos e medula óssea ela apresenta níveis mais baixos de expressão (SINGH et al., 2020). Entretanto, o trato respiratório é o alvo principal do SARS-CoV-2 causando inflamação grave nos pulmões (DHAMA et al., 2020).

2.2 DIAGNÓSTICO

Com base em outras infecções respiratórias a OMS recomendou o uso da reação em

cadeia da polimerase-transcriptase reversa em tempo real (RT-PCR) como protocolo padrão para o diagnóstico do SARS-CoV-2 (CORMAN et al., 2020). A reação é realizada com primers direcionados na identificação dos genes presentes no vírus, nucleocapsídeo (N), proteina Spike (S), envelope (E) e ORF1ab.

O RT-PCR em amostras de swab nasofaríngeo é considerado o padrão-ouro para a detecção do SARS-CoV-2. Trata-se de um teste sensível e específico e utilizado para detecção de diferentes patógenos. Este ensaio tem a capacidade de detectar e medir quantidades mínimas de ácidos nucleicos, como 1 cópia RNA viral / μL (1.000 copias / mL) (VOGELS et al., 2020). Estudos clínicos mostram que quando a carga viral é menor que um milhão de copias / mL, o risco de transmissão é baixo devido a presença de poucas partículas virais (SCOLA et al., 2020; WÖLFEL et al., 2020).

O indicador de amplificação detectável é conhecido como Ct (do inglês *cycle threshold*). Ele determina o ciclo em que o nível de fluorescência detectado na amostracruza o limiar estabelecido para distinguir fluorescência basal e amplificação de material genético. Estudos relatam que sequencias não especificas podem ocorrer após a leitura de mais de 28 ciclos, devido a inativação da Taq polimerase (SULE; OLUWAYELU, 2020).

Foi relatado que os valores de Ct de 25 a 28 eram geralmente adequados; quando mais de 28 ciclos, a detecção de sequências amplificadas não especificamente pode ocorrer ou levar a resultados variáveis devido à inativação da Taq polimerase. Diferentes valores de Ct são utilizados para o diagnóstico da COVID- 19, valores abaixo de 40, geralmente são indicados como indicador de positividade para SARS-CoV-2 (VOGELS et al., 2020).

Apesar disso, considerando que o valor de Ct é inversamente proporcional à quantidade de material genético presente na amostra, estudos mostram que as amostras que apresentam valores de Ct variando entre 15 e 24 podem indicar maior carga viral, e valores de25 a 30 são consideradas com carga viral moderada, enquanto valores maiores que 33,33 podem ser de baixa carga viral ou negativas (SULE; OLUWAYELU, 2020; VOGELS et al., 2020).

O transporte indevido, congelamento e descongelamento, tempo de amostragem e diferentes protocolos agregados pode ocasionar nas diferenças nos valores de corte de Ct, aproximando do limite de detecção de RNA viral e apresentar resultados falso-negativos (HONG et al., 2020; PAN et al., 2020).

2.2.1 **LAMP**

Uma das ferramentas mais importantes para o diagnóstico de doenças e

desenvolvimentos na área da biotecnologia é a amplificação de ácido nucleicos (PARIDA et al., 2008). Em 2000, foi desenvolvido um novo método de amplificação que utiliza o calor para amplificar um milhão de copias de DNA em menos de uma hora. Esse método foi denominado de amplificação isotérmica mediada por loop (LAMP) (NOTOMI et al., 2000).

A reação de LAMP utiliza um conjunto de seis ou quatros primers diferentes, ligando-se a oito ou seis regiões diferentes na molécula alvo. O conjunto de primer envolvem dois primers internos (FIP e BIP), dois externos (F3 e B3) e primers de loop.Os primers FIP e BIP ligam-se a região F2c ou B2c no DNA alvo, seguido dos primers externos (F3 e B3) que hibridizam com a região F3c ou B3c, deslocando a fita complementar ligada ao FIP e BIP (NOTOMI et al., 2000). Resultando na formação de um DNA em forma de haltere, sequentemente, essa mesma estrutura de haltere se torna o princípio para a amplificação exponencial (Figura 3). Os iniciadores de loop podem ser adicionados para acelerar o processo de amplificação e sistemas baseados em ensaios de fluxo lateral (SILVA; PARDEE; PENA, 2020).

Para expandir o diagnóstico da técnica de LAMP, inúmeras melhorias foram aplicadas ao protocolo original, entre elas, inserir o LAMP de transcrição reversa (RT- LAMP). O RT-LAMP utiliza transcriptase reversa para produzir a partir do RNA o DNA complementar, usando o DNA polimerase para amplificação. Esta técnica permite que o experimento seja capaz de ser concluído em uma única etapa e com apenas uma temperatura constante (NOTOMI et al., 2000; MORI et al., 2013).

O diagnóstico do HIV é um exemplo do uso bem-sucedido do RT-LAMP. Em 2015, o uso do RT-LAMP se mostrou eficaz no diagnóstico do HIV. (OCWIEJA et al., 2015 ; ODARI et al., 2015). No ano seguinte, o RT-LAMP foi empregado para a detecção do vírus zika sintético (ZIKV), validando a atividade enzimática dupla, através do *Bst* 3.0 polimerase amplificando em condições semelhantes o DNA e o RNA (TIAN et al., 2016). RT-LAMP também foi usado para detectar patógenos de animais e plantas. Por exemplo, o vírus da Batai que causa defeitos congênitos em ruminantes (gado) e o vírus da mancha clorótica da folha da macieira. (PENG etal., 2017; LIU at al., 2016).

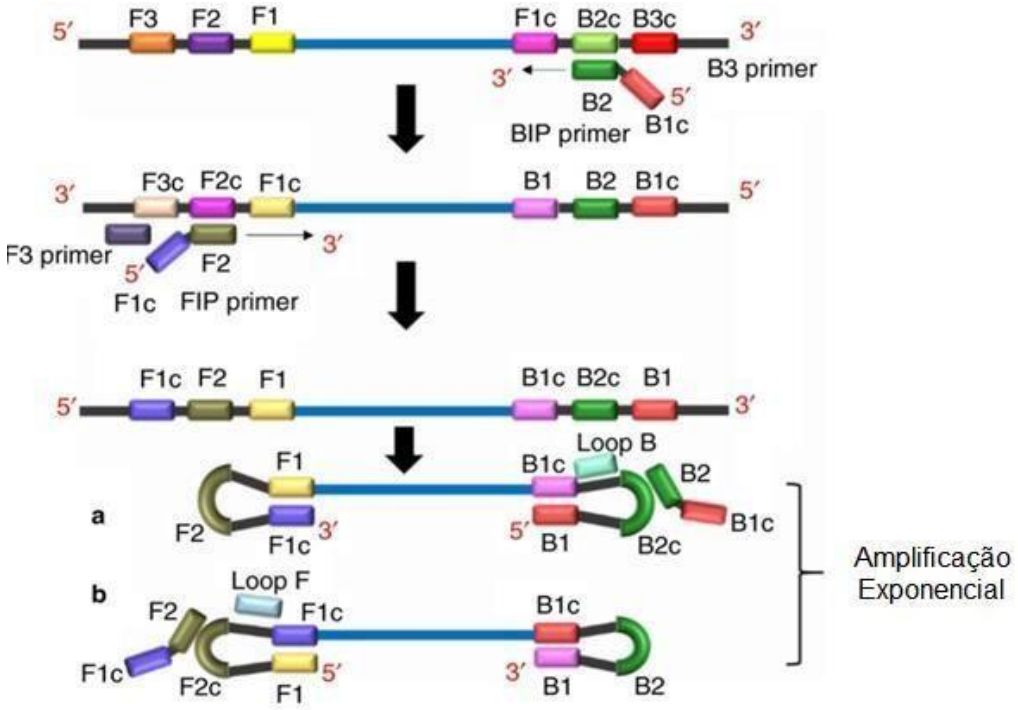

Figura 3 – Representação esquemática da reação de LAMP. A mistura envolvida na reação de LAMP envolve em dNTPs, polimerase Bst, corante de fluorescência, primers e molde de DNA
Fonte: (Adaptado de SILVA; PARDEE; PENA, 2020)

2.2.2 **CRISPR**

Em 1987, foi identificado pela primeira vez sequências repetidas incomuns em *E. coli* separadas por sequências não repetitivas (ISHINO et al., 1987). Ao longo dos anos foram encontradas sequências semelhantes, mas a função das sequências repetidas e interrompidas era desconhecida.

Em 2002 essas sequenciais foram denominadas de repetição palindrômica curta espaçada regularmente agrupada (CRISPR)**,** anos depois relataram uma relação funcional entre os genes, envolvia o metabolismo do DNA e a expressão genica, algumas bactérias apresentavam determinada sequência de DNA viral no locus CRISPR conhecidas por serem resistentes à infecção, apontando que assequencias poderia está relacionada ao sistema imune adaptativo em procariotos (BOLOTIN et al., 2005; JANSEN et al., 2002; MOJICA et al., 2005; POURCEL; SALVIGNOL; VERGNAUD, 2005). Apenas em 2007 foi publicado evidências experimentais sobre essa hipótese (BARRANGOU et al., 2007).

A edição do genoma é um processo que provoca modificações em uma sequência alvo, removendo, inserindo ou substituindo bases. Nos últimos anos, novas metodologias foram estudadas para editar genomas simples e complexos, revolucionando a pesquisa cientifica. O sistema CRISPR tem sido utilizado na pesquisa devido a sua alta sensibilidade, especificidade,

confiabilidade e facilidade deuso (LINO et al., 2018). O sistema CRISPR/ Cas se baseia no reconhecimento guiado por RNA da sequência parcialmente complementar no DNA, posteriormente introduzindo uma quebra no local reconhecido ou nas proximidades (TERNS, 2018).

As enzimas Cas de classe 2, são as mais estudadas, devido as suas atividades de nuclease que permitem a engenharia genômica e as técnicas de detecção de ácido nucleico (BARRANGOU; HORVATH, 2017). Entretanto, os tipos I — III e a sua funçãosão os mais estudados, enquanto os tipos IV — VI foram identificados mais recentemente (MAKAROVA; KOONIN, 2015). Pertencendo a classe 2, tipo II, a Cas9era considerada a melhor escolha na edição genica. Porém, existem limitações para a sua aplicação, seu sistema é extenso e dificulta a eficiência, introduz mutações inespecíficas que são semelhantes, mas não idênticas, inicia mutações aleatórias fora do alvo no genoma (ZHANG et al., 2015). Desse modo, modificações foram focados para melhorar a confiabilidade e eficiência do sistema. A Cas12 é regulada por um único RNA, identifica sequencias ricas em timidina (T), aumentando a especificidade de locais genômicos e diferentemente do Cas9, ela não requer um tracrRNA para realizar a sua função (ZETSCHE et al., 2015).

Técnicas baseadas em CRISPR atualmente têm potencial ou já estão em uso como uma opção de teste de ponto de atendimento no diagnóstico de patógenos. Desde a sua descoberta, o sistema foi aceito e utilizado para identificação de oncogenes e incluído à pesquisa do câncer (TIAN et al., 2019). A área de infecções virais é a mais explorada para sistemas de diagnóstico baseados em CRISPR. Vários cientistas desenvolveram métodos nomeados de DETECTR e SHERLOCK, que utilizam as famílias CRISPR-Cas12a e Cas13a, respectivamente (MUSTAFÁ; MAKHAWI, 2021; HILLARY et al., 2021). Essas técnicas são usadas para detectar SARS-CoV-2 de amostras de RNA extraídas de pacientes suspeitos. O ensaio DETECTR é baseado na clivagem de Cas12a, na presença do DNA alvo a Cas12a/crRNA forma um complexo de hibridização DNA-RNA alvo e leva à clivagem de ssDNA repórter (MUSTAFÁ; MAKHAWI, 2021).

2.3 DIAGNÓSTICO MOLECULAR EM AMOSTRAS DE SALIVA

A saliva humana é um fluido produzido pelas glândulas salivares. As glândulas parótidas, submandibulares e sublinguais são as principais glândulas salivares. A saliva consiste em 99% de água e 1% de moléculas orgânicas e as inorgânicas (MARTINA et al., 2020). A saliva facilita a mastigação e digestão, cria barreiras para alimentos ácidos, possuem

propriedades antimicrobianas, protege os dentes da carie inibindo a desmineralização (QIN; STEEL; FAZEL, 2017).

Nas últimas décadas, vem crescendo estudos que mostram que a saliva é útil para o diagnóstico precoce de vários tipos de câncer, evidenciando os anticorpos anti- p53 que são marcadores tumorais conhecidos e estão presentes na saliva (LAVECCHIA et al., 2000).

O Instituto Nacional de Pesquisa Odontológica e Crânio-facial (NIDCR), analisanovas ferramentas de diagnostico utilizando fluidos orais desde 2002 (LEE; WONG, 2009). Estudos mostram que a expressão de ACE2 é mais evidente em glândulas salivares do que nos pulmões, revelando que o SARS-CoV-2 podem ter as glândulassalivares como alvo potencial (XU et al., 2020).

A coleta de amostras de saliva não requer contato próximo com os pacientes, podendo ser realizadas fora dos hospitais de forma rápida, barata, fácil, não invasiva. A saliva não coagula e não é necessário profissionais de saúde treinados para a coleta(HARIKRISHNAN, 2020). Pesquisas recentes sugerem que amostras de saliva podem apresentar sensibilidade maior em comparação como amostras de swab nasofaríngeo para o diagnóstico de COVID-19 (SAHAJPAL et al., 2021; TEO et al., 2021).

3 **OBJETIVOS**

3.1 OBJETIVO GERAL

Otimizar o diagnóstico molecular da COVID-19 através da utilização de amostras de saliva em reações de RT-PCR e do estabelecimento de um protótipo de teste "point of care" baseado na tecnologia CRISPR-Cas12.

3.2 OBJETIVOS ESPECÍFICOS

- Validar a utilização de amostras de saliva no rastreamento da COVID-19 através de testes moleculares;
- Comparar a performance do RT-PCR em amostras pareadas de saliva e swab nasofaríngeo;
- Desenvolver um protótipo de um novo teste rápido molecular baseado na tecnologia CRISPR/Cas12

4 **ARTIGO 1**

O artigo descreve os resultados obtidos neste projeto de mestrado, buscando atender aos objetivos levantados de validar a utilização de amostras de saliva no rastreamento da COVID-19 e comparar a performance do RT-PCR em amostras pareadas de saliva e swab nasofaríngeo. No momento encontra-se submetido a revista internacional *Diagnostics.*

SARS-CoV-2 detection by RT-PCR in matched saliva and nasopharyngeal samples reveals high concordance in different commercial assays

Karoline Almeida Felix de Sousa, Carolina Kymie Vasques Nonaka, Renata Naves de Ávila Mendonça, Verena Neiva Mascarenhas, Thamires Gomes Lopes Weber, Ana Verena Almeida Mendes, Carlos Gustavo Régis, Ricardo Khouri, Bruno Solano de Freitas Souza and Clarissa Araújo Gurgel Rocha.

diagnostics

MDPI

Article

SARS-CoV-2 detection by RT-PCR in matched saliva and nasopharyngeal samples reveals high concordance in different commercial assays

Karoline Almeida Felix de Sousa[1,2], Carolina Kymie Vasques Nonaka[2,3], Renata Naves de Ávila Mendonça[3], Verena Neiva Mascarenhas[3], Thamires Gomes Lopes Weber[3], Carlos Gustavo Régis[1], Ana Verena Almeida Mendes[3], Ricardo Khouri[1,4], Bruno Solano de Freitas Souza[1,2,3,*] and Clarissa Araújo Gurgel Rocha[1,2,4,5,*]

[1] Gonçalo Moniz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Salvador, Brazil.
[2] Center for Biotechnology and Cell Therapy, D´Or Institute for Research and Education (IDOR), Salvador, Brazil.
[3] São Rafael Hospital, Salvador, Brazil.
[4] Faculty of Medicine, Federal University of Bahia, Salvador, Brazil.
[5] Faculty of Dentistry, Federal University of Bahia, Salvador, Brazil.
* E-mail: clarissa.gurgel@fiocruz.br; Tel: +557131762289 (CAGR); bruno.souza@fiocruz.br; Tel: +557132816970 (BSFS)

Abstract: Objective: Self-collected saliva samples could increase diagnostic efficiency, benefit healthcare workers, patient care, and infection control. This study evaluated the performance of self-collected saliva samples compared to nasopharyngeal swabs in three commercial kits for the qualitative detection SARS-CoV-2. Methods: Matched nasopharyngeal and saliva samples were collected from 103 patients with either asymptomatic or symptomatic COVID-19. Both samples were evaluated in three commercial kits (TaqCheck™, Allplex™ kits and TaqPath™). In order to evaluate sample stability, viral RNA extraction was performed on samples in the presence or absence of RNA shield. Storage conditions, including duration, temperature and stability after frozen-thawed were also evaluated. Results: All saliva samples showed 100% concordance with the nasopharyngeal swab results using TaqCheck™ and Allplex™ kits and 93% using TaqPath™ kit. No difference was seen in the samples that used the RNA shield compared to the group without the solution. The Ct values of the frozen-thawed samples after 30 days increased compared to the values on day 0 however kept consistent results. Conclusion: The high concordance of SARS-CoV-2 detection by RT-PCR in matched saliva and nasopharyngeal samples using different commercial assays reinforces that self-collected saliva samples are a non-invasive, rapid and reliable sample for diagnosing SARS-CoV-2 infection.

Keywords: COVID-19, saliva, diagnostic method, SARS-CoV-2, RT-PCR.

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1. Introduction

Implementing efficient testing strategies is crucial to ensure widespread molecular detection of SARS-CoV-2, monitor viral dissemination, and control outbreaks [1, 2]. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)-based detection of SARS-CoV-2 is the "gold standard" method for diagnosing COVID-19. Several RT-PCR kits have been developed based on the conserved regions of the SARS-CoV-2 genome [3-5]. Due to the high sensitivity and specificity, both upper and lower respiratory tract samples have been considered standards. However, collecting samples from the respiratory tract requires a dedicated

infrastructure, trained personnel and well-established procedures to avoid contamination of healthcare workers, which are not always available, especially in developing countries. Moreover, adherence to testing protocols requiring repeated nasopharyngeal (NP) swab collection may be low since this procedure may be associated with different levels of discomfort, cough, bleeding, rhinitis, sneeze, and/or vomit [6-9].

Saliva has emerged as a potential alternative to respiratory tract samples, with reported comparable levels of sensitivity and specificity to nasopharyngeal swabs [10]. Saliva sampling offers several advantages, requiring fewer material resources and infrastructure [11,12], being a non-invasive method, well-tolerated for children, the elderly, and people with disabilities [13]. In 2020, the RT-PCR assay for SARS-CoV-2 virus detection in saliva was approved by the U.S Food and Drug Administration (FDA) [14], and a recent study showed a high positive agreement (94%) comparing saliva by SalivaDirect with nasopharyngeal swabs by commercial RT-PCR kit [15]. A meta-analysis study demonstrated more than 90% concordance rate of saliva and nasopharyngeal swab, including high sensitivity and specificity [10].

Currently, The American Centers for Disease Control and Prevention and the European Centre for Disease Prevention and Control authorize the utilization of oral swabs or saliva as specimens for SARS-CoV-2 virus diagnosis. Saliva testing has already been well accepted for SARS-CoV-2 detection in several countries, including South Korea, Germany and Japan [16].

Despite the amount of evidence suggesting comparable performance between NP swabs to saliva samples, there is still a need to characterize whether patient profile, the severity of symptoms, and the time between disease onset and sample collection may affect the performance. In addition, practical questions regarding kit performance and pre-analytic factors that could affect sample stability have not been sufficiently explored. Here, we compared the diagnostic performance of three commercial kits for the qualitative detection of SARS-CoV-2 by RT-PCR in salivary samples with matched nasopharyngeal swab samples and correlated the patient's clinical data.

2. Materials and Methods

2.1. Patients and Sample Collection

This study was approved by the Institutional Review Board (CAAE: 38580920.6.3001.0040). All included patients were tested according to the clinical indications. A total of 103 symptomatic and asymptomatic patients, convenience cohort study, were recruited during admission in the emergency room at São Rafael Hospital (Salvador, Bahia, Brazil) from March 16th to May 6th 2021. The informed consent form had been obtained from each patient. Demographic data, comorbidities and symptoms were collected from electronic medical records.

Paired samples from those patients, saliva and Nasopharyngeal (NP) Swab, were collected for this study. In comparison, NP swab was obtained by a qualified technician and stored at −20°C until being processed as a service offered by São Rafael diagnostic laboratory. Approximately 5 mL of saliva was self-collected in a certified DNase and RNase conical polypropylene sterile graduated tube (50 mL). Subjects were queried to avoid eating and drinking 30 min before sampling. After being collected, the saliva samples were stored at -80° degrees until processing.

2.2. Sample Processing and RT-PCR for SARS-CoV-2

The samples were processed following the recommendations of a reference kit for SARS-CoV-2 detection in saliva samples, TaqCheck™ SARS-CoV-2 Fast PCR assay kit (ThermoFisher Scientific, Massachusetts, USA). Saliva samples were incubated with TweenTM20 Detergent (1%)/TBE Buffer (2X) (ThermoFisher

Scientific, Massachusetts, USA). A volume of 100 µL of saliva was incubated at 95 °C for 30 min; then vortexed until the sample appeared homogeneous and transferred to 100 µL of TBE / Tween mix (20 µL of TBE Buffer (10X) + 10 µL of Tween 20 Detergent (10%) + 70 µL of nuclease-free water).

For stability assessment, were randomly selected 28 positive samples and in the final volume of 1mL added 1:2 RNA Shield™ (Zymo Research, California, USA) and compared with 1 mL of the paired sample without RNA Shield™. Both samples were stored at -80°degrees for 30 days. Followed by viral RNA extraction using MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit (ThermoFisher Scientific, Massachusetts, EUA), according to the manufacturer's recommendations. The automated equipment KingFisher Sample Purification Systems was used (ThermoFisher Scientific, Massachusetts, EUA). Likewise, were randomly selected 30 positive samples and stored without RNA Shield™ at -80°C for 30 days, followed by processing with TBE / Tween mix.

The kits Allplex™ SARS-CoV-2 Assay (Seegene®, South Korea), TaqPath™ COVID-19 CE-IVD RT-PCR kit (ThermoFisher Scientific, Massachusetts, USA) and TaqCheck™ SARS-CoV-2 Fast PCR assay kit (ThermoFisher Scientific) were evaluated, according to the study design (Figure 1). All protocols were performed using the Applied Biosystems™ 7500 Fast (ThermoFisher Scientific) and software v2.3 for further analysis.

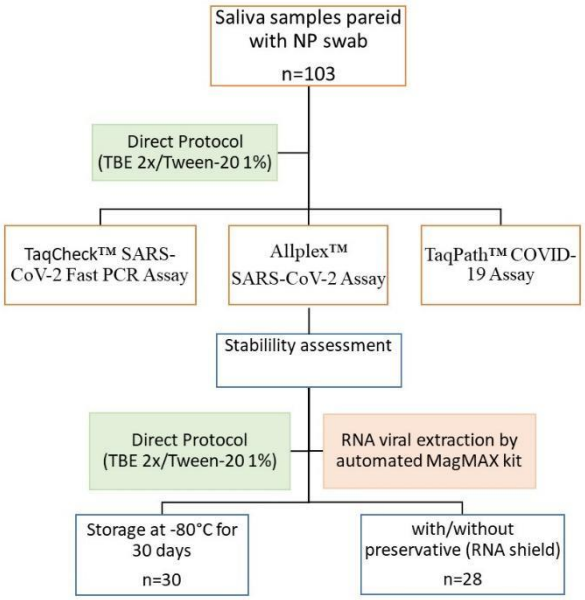

Figure 1. Flowchart of the study design.

2.3. Statistical analysis

The results obtained with saliva samples were compared with nasopharyngeal swab samples. The N gene amplification by RT-PCR was used to compare the Cts of the SARS-CoV-2 detection kits. The kappa coefficient was used to assess the agreement of the results. All statistical tests were done using GraphPad Prism version 9.0 software (GraphPad, La Jolla, CA, USA)

3. Results

In this study, 103 paired samples (NP swab and saliva) were obtained from patients admitted to the emergency room of a private hospital (São Rafael Hospi-

tal, Salvador, Bahia, Brazil) referring flu symptoms (n= 78) or asymptomatic subjects reporting close contact to COVID-19 patients in the past days (n= 25). Symptomatic patients were submitted to sample collection in a median of 5 days from illness onset. Among symptomatic patients, 56.4% (n= 44) tested positive for SARS-CoV-2. Cough (65.2%), headache (52.2%) and fever (45.7%) were the most frequent symptoms reported by patients. Other clinical symptoms and demographic data are described in Table 1.

Table 1. Clinical and demographic characteristics of the subjects (n=103) and results of the detection of SARS-CoV-2 in nasopharyngeal swab by RT-PCR.

	Detection of SARS-CoV-2	
	Positive (n = 46)	Negative (n = 57)
Age (median)	40 (18-79)	42 (18-79)
Male	29 (63.04%)	24 (42.10%)
Symptomatic	44 (99.65%)	34 (59.65%)
Fever	21 (45.65%)	11 (19.29%)
Cough	30 (65.21%)	16 (28.07%)
Sore throat	10 (21.73%)	10 (17.54%)
Dyspnea	15 (32.60%)	6 (10.52%)
Headache	24 (52.17%)	19 (33.33%)
Myalgia	20 (43.47%)	13 (22.80%)
Anosmia	7 (15.21%)	1 (1.75%)
Diarrhea	2 (4.34%)	10 (17.54%)
Vomiting	3 (6.52%)	4 (7.01%)

3.1. Comparative performance of self-collected saliva samples in different protocols

After saliva collection, we evaluated an extraction-free protocol using Tween™-20 Detergent (1%)/TBE Buffer, followed by detection using two commercially available kits for molecular diagnosis of SARS-CoV-2 by RT-PCR (Allplex™ (Seegene) and TaqPath™ (ThermoFisher). The samples were also processed with a commercial kit TaqCheck™ (ThermoFisher) validated for saliva samples and compared to NP samples. Allplex™ (Seegene) and TaqPath™ (ThermoFisher) have been validated for NP samples, being routinely utilized in diagnostics by the clinical laboratory of São Rafael Hospital, but had not been validated for saliva samples by the manufacturers at the time of present study initiation. We observed 1.0 and 0.9 of values in the kappa coefficient corresponding to an excellent agreement (n=46 positive, n=57 negative) when comparing the Allplex™ (Seegene) and the TaqPath™ (ThermoFisher) with the TaqCheck™ (ThermoFisher) in saliva samples (Table 2).

Table 2. Comparison between the three commercial kits for detection of SARS-CoV-2 in saliva samples (n=103) by RT-PCR using Cohen's kappa coefficient.

SARS-CoV-2 detection kit	Kappa	I.C 95%
TaqCheck (saliva) vs Allplex (NP)	1.0	1.0-1.0
TaqCheck (saliva) vs Allplex (saliva)	1.0	1.0-1.0
TaqCheck (saliva) vs TaqPath (saliva)	0.923	0.849-0.997
Allplex (NP) vs Allplex (saliva)	1.0	1.0-1.0
Allplex (NP) vs TaqPath (saliva)	0.923	0.849-0.997

We observed the same kappa coefficients (n=46 positive, n=57 negative) when comparing the Allplex™ (Seegene) and TaqPath™ (ThermoFisher) in saliva samples with the NP swab. We also evaluated the possible influence of time between sample collection and symptom onset on test sensitivity using Ct values (Figure 2). NP samples presented lower median Ct values than saliva samples, reaching statistical significance for samples collected ten days after symptom onset.

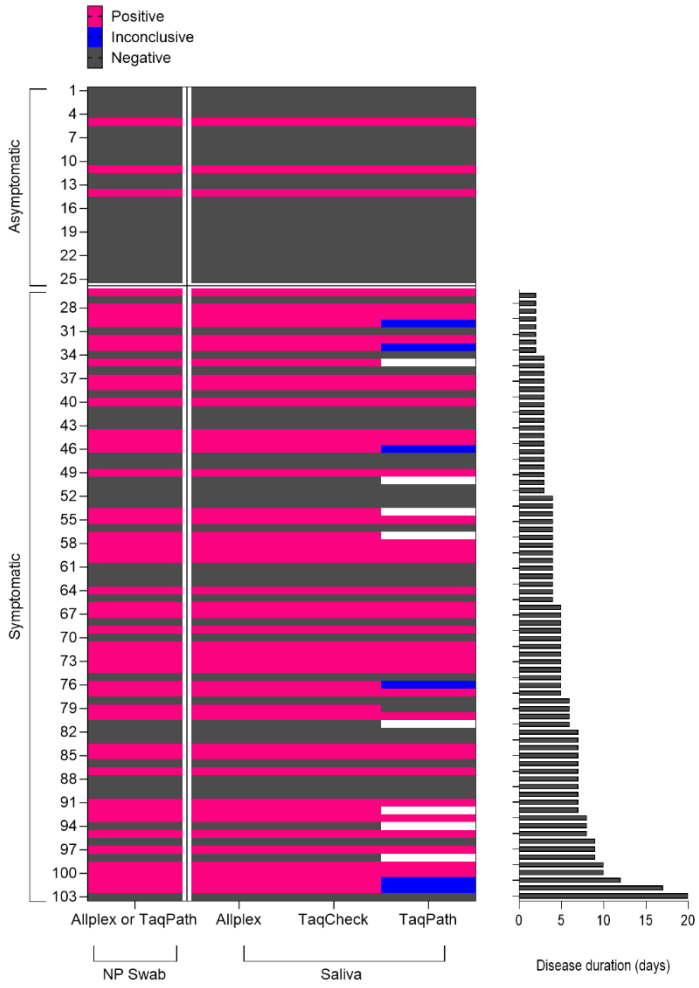

Figure 2. Heat map showing the RT-PCR results for detecting SARS-CoV-2 in paired NP swab and saliva samples, obtained from asymptomatic patients (contactors or not) and

symptomatic patients with collections performed after different days of symptoms. Positive tests (pink), negatives (black), inconclusive (blue) and not tested (white).

3.2 Evaluation of the stability of stored saliva samples

Next, we evaluated the stability of randomly selected 30 samples with positive results stored at -80°C for 30 days without RNA stabilizing solution. The results were 100% in agreement after 30 days since we detected SARS-CoV-2 with Ct < 40 in all samples with Allplex™ (Seegene), with median Ct = 27.8 in stored samples and Ct = 25.0 in fresh samples.

The influence of RNA stabilization was evaluated using RNA shield with saliva and storage at -20°C for up to 30 days compared to paired samples without RNA shield and without purification step. A viral RNA purification step was performed only when RNA shield was added to the samples followed by Allplex™ (Seegene) SARS-CoV-2 detection kit. However, we observed a benefit when added RNA stabilizing solution. It was observed lower Ct value with RNA shield and RNA purification compared to without RNA shield and extraction-free protocol, with median Ct=18.4 and CT=27.2 respectively.

4. Discussion

In this study, we observed a 100% concordance RT-PCR of saliva samples for SARS-CoV-2 detection compared to nasopharyngeal swab using Allplex™ SARS-CoV-2 detection kit and TaqCheck™ SARS-CoV-2 Fast PCR kit. Our results agree with other studies that support saliva as an alternative sample for COVID-19 diagnosis [17]. In addition to the similar sensitivity, the lower cost, simplicity of self-collection, and increased patient comfort make the saliva more advantageous than the nasopharyngeal swab. Furthermore, we demonstrate that both RNA extraction-free protocols can be used with Allplex™ SARS-CoV-2 detection kit.

Most people infected with SARS-CoV-2 develop a mild to moderate respiratory illness. The risk of severe illness increases for older adults and those with certain underlying medical conditions [18]. The prevalence of fever, cough and headache were frequently observed in both positive (COVID-19 detected) and negative (COVID-19 not detected) evaluated groups. All individuals included in the cohort study were mild or subclinical cases. Interestingly, our study saw the presence of viral RNA in asymptomatic patients. SARS-CoV-2 testing as contact tracing are strategies to identify people infected with SARS-CoV-2 [19]. Concordant with our finding, it was demonstrated that peak positivity in asymptomatic RT-PCR occurs between 1 and 3 days after infection [20]. Saliva was a reliable source for diagnosing COVID-19 by detecting viral RNA in symptomatic and asymptomatic individuals despite showing less sensitivity than NP swabs.

Although the RT-PCR was considered the gold standard method for SARS-CoV-2 detection, it is important to note that RT-PCR may have better and worse performance among COVID-19 diagnostic assays. In the early stage of the pandemic, a study evaluated some primer-probe sets in SARS-CoV-2 RT-PCR diagnostic assays and demonstrated a difference in sensitivity between them [21]. Another interfering condition already described is the probability of detecting SARS-CoV-2 will vary throughout the infection [22]. In agreement with the literature, we observe one hundred per cent agreement between NP swab and saliva specimens using Allplex™ SARS-CoV-2 assay or TaqCheck™ SARS-CoV-2 Fast PCR. Despite using different prime-probe sets, all protocols presented a lower Ct at the onset of symptoms and higher Ct over time in both samples.

As a limitation of this study, individuals with severe symptoms and pediatric were not included. The NP swab was processed within 12 hours as part of the laboratory's routine. Saliva was frozen for at least one week, which can be considered a sensitivity interferer. We did not include the serological confirmation of COVID-19 in all cases.

5. Conclusions

In conclusion, saliva is a sensitive sample for the diagnosis of COVID-19. Our study shows that it is possible to identify asymptomatic infectious individuals without the need for RNA extraction and RNA stabilizing solution, thus making saliva diagnosis promising for direct testing in the laboratory routine.

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Institutional Review Board Statement:

This study was approved by the Institutional Review Board (CAAE: 38580920.6.3001.0040)

Informed Consent Statement:

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Johanna N, Citrawijaya H, Wangge G. Mass screening vs lockdown vs combination of both to control COVID-19: A systematic review. Journal of Public. Health Research. **2020**, 9, 523-531.
2. Woo PCY, Lau SKP, Lam CSF, Lau CCY, Tsang AKL, Lau JHN, et al. Discovery of Seven Novel Mammalian and Avian Coronaviruses in the Genus Deltacoronavirus Supports Bat Coronaviruses as the Gene Source of Alphacoronavirus and Betacoronavirus and Avian Coronaviruses as the Gene Source of Gammacoronavirus and Deltacoronavirus. Journal of Virology. **2012**, 86, 3995-4008.
3. Granato PA, Kimball SR, Alkins BR, Cross DC, Unz MM. Comparative evaluation of the Thermo fisher TaqPathTM COVID-19 combo kit with the Cepheid Xpert® Xpress SARS-CoV-2 assay for detecting SARS-CoV-2 in nasopharyngeal specimens. BMC Infectious Diseases. **2021**, 21, 1–6.
4. Farfour E, Lesprit P, Visseaux B, Pascreau T, Jolly E, Houhou N, et al. The Allplex 2019-nCoV (Seegene) assay: which performances are for SARS-CoV-2 infection diagnosis?. European Journal of Clinical Microbiology & Infectious Diseases. **2020**, 39, 1997–2000.
5. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro surveillance. **2020**, 25.
6. Kapoor P, Chowdhry A, Kharbanda OP, Bablani Popli D, Gautam K, Saini V. Exploring salivary diagnostics in COVID-19: a scoping review and research suggestions. BDJ Open. **2021**, 7.
7. Kevadiya BD, Machhi J, Herskovitz J, Oleynikov MD, Blomberg WR, Bajwa N, et al. Diagnostics for SARS-CoV-2 infections. Nature Materials. **2021**, 20, 593–605.
8. Vilela ACS, Costa NL. The COVID-19 and Saliva Paradox. Journal of Oral and Maxillofacial Surgery. **2020**, 78, 2105.
9. Fernandes LL, Pacheco VB, Borges L, Athwal HK, Paula Eduardo F, Bezinelli L, et al. Saliva in the Diagnosis of COVID-19: A Review and New Research Directions. J Dent Res. **2020**, 99, 1435–43.
10. Butler-Laporte G, Lawandi A, Schiller I, Yao M, Dendukuri N, McDonald EG, et al. Comparison of Saliva and Nasopharyngeal Swab Nucleic Acid Amplification Testing for Detection of SARS-CoV-2. JAMA Internal Medicine. **2021**, 181, 353 – 360.
11. Teo AKJ, Choudhury Y, Tan IB, Cher CY, Chew SH, Wan ZY, et al. Saliva is more sensitive than nasopharyngeal or nasal swabs for diagnosis of asymptomatic and mild COVID-19 infection. Scientific Reports. **2021**, 11.
12. Liu L, Wei Q, Alvarez X, Wang H, Du Y, Zhu H, et al. Epithelial Cells Lining Salivary Gland Ducts Are Early Target Cells of Severe Acute Respiratory Syndrome Coronavirus Infection in the Upper Respiratory Tracts of Rhesus Macaques. Journal of Virology. **2011**, 85, 4025–4030.
13. Ning B, Yu T, Zhang S, Huang Z, Tian D, Lin Z, et al. A smartphone-read ultrasensitive and quantitative saliva test for COVID-19. Science Advances. **2021**, 7.
14. Food and Drug Administration. FDA Authorizes First Diagnostic Test Using At-Home Collection of Saliva Specimens. **2020**. [cited 8 May 2020] Available from:https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-diagnostic-test-using-home-collection-saliva.
15. Vogels CBF, Watkins AE, Harden CA, Brackney DE, Shafer J, Wang J, et al. SalivaDirect: A simplified and flexible platform to enhance SARS-CoV-2 testing capacity. Med **2021**, 2, 263-280.
16. Oba J, Taniguchi H, Sato M, Takamatsu R, Morikawa S, Nakagawa T, et al. RT-PCR Screening Tests for SARS-CoV-2 with Saliva Samples in Asymptomatic People: Strategy to Maintain Social and Economic Activities while Reducing the Risk of Spreading the Virus. The Keio Journal of Medicine. **2021**, 70, 35–43.
17. Chen L, Zhao J, Peng J, Li X, Deng X, Geng Z, et al. Detection of SARS-CoV-2 in saliva and characterization of oral symptoms in COVID-19 patients. Cell Proliferation. **2020**, 53.
18. Coronavirus Disease (COVID-19) Situation Reports. [cited May 2022]. Available from: https://www.o.int/emergencies/diseases/novel-coronavirus-2019/situation-reports.
19. Mina MJ, Andersen KG. COVID-19 testing: One size does not fit all. Science. **2021**, 371, 126–127.
20. Hellewell J, Russell TW, Beale R, Kelly G, Houlihan C, Nastouli E, et al. Estimating the effectiveness of routine asymptomatic PCR testing at different frequencies for the detection of SARS-CoV-2 infections. BMC Medicine. **2021**, 19.
21. Vogels CBF, Brito AF, Wyllie AL, Fauver JR, Ott IM, Kalinich CC, et al. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT–qPCR primer–probe sets. Nature Microbiology. **2020**, 5, 1299–1305.
22. Lippi G, Simundic A-M, Plebani, M. Potential preanalytical and analytical vulnerabilities in the laboratory diagnosis of coronavirus disease 2019 (COVID-19). Clinical Chemistry and Laboratory Medicine (CCLM). **2020**, 58, 1070–1076.

5 **ARTIGO 2**

O artigo descreve os resultados obtidos neste projeto de mestrado, buscando atenderao objetivo de desenvolver um protótipo de um novo teste rápido molecular baseado na tecnologia CRISPR/Cas12. No momento encontra-se em fase final de formatação para a submissão na revista internacional *Scientific Reports*.

Rapid detection of SARS-CoV-2 based on the LAMP assay associated with the CRISPR-Cas12a system

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Rapid detection of SARS-CoV-2 based on the LAMP assay associated with the CRISPR-Cas12a system

Karoline Almeida Felix de Sousa[1], Carolina Kymie Vasques Nonaka[2,3], Carlos Gustavo Reis[1], Ricardo Khouri[1,4], Clarissa Araújo Gurgel Rocha[1,2,4,5]& Bruno Solano de Freitas Souza[1-4*]

[1] Gonçalo Moniz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Salvador, Brazil.
[2] Center for Biotechnology and Cell Therapy, São Rafael Hospital, Salvador, Brazil.
[3] São Rafael Hospital, Salvador, Brazil.
[4] Faculty of Medicine, Federal University of Bahia, Salvador, Brazil.
[5] Faculty of Dentistry, Federal University of Bahia, Salvador, Brazil.

[*]Correspondence should be addressed to Dr. Bruno Solano de Freitas Souza, PhD, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Candeal, Salvador, Bahia, Brazil. CEP: 40296-710. Phone: +557131762260; Fax: +557131762289. E-mail address: bruno.solano@fiocruz.br.

ABSTRACT

Introduction: The global COVID-19 pandemic has challenged the public health system worldwide for the past two years. Mass testing has proven to be critical for the control of SARS-CoV-2 spread, however, its implementation has faced some obstacles, especially in low-income countries. The gold standard method for SARS-CoV-2 detection is RT-PCR, requiring dedicated infrastructure for collection, specialized staff, and sophisticated laboratories. Therefore, the need for rapid, simple, and accurate tests to diagnose SARS-CoV-2 infection is of extreme importance. Systems using CRISPR for the diagnosis of COVID-19 have shown promise, with reported high sensitivity, rapid detection, and do not require specialized molecular biology laboratories. **Methods:** To validate a prototype of CRISPR-based SARS-CoV-2 detection test, we evaluated the test performance in 29 nasopharyngeal samples, from which 23 samples were obtained from cases suspected of COVID-19 collected at the emergency department of a reference hospital in Brazil, and 6 samples with known results positive for H3N2 or respiratory syncytial virus and negative for SARS-CoV-2. Two reference sample with known SARS-CoV-2 RNA concentration (3,000 RNA copies/ml) or viral titer determined by plaque assay (10^5 PFU/ml) were used to calculate the limit of detection (LoD). The ORF1ab gene of SARS-CoV-2 was amplified by isothermal amplification reaction (LAMP) and the human RP gene was used as control. Subsequently, the results were read on the Gemini XPS fluorescence microplate reader. **Results:** The RT-LAMP-CRISPR/Cas12 assay showed 100%

concordance between the results obtained by RT-PCR. The RT-PCR methodology using the commercial kit presented a detection limit of 0.01 PFU/mL and the CRISPR/Cas12 methodology was effective at detecting a limit of 15.6 PFU/mL. The RT-PCR method presented a sensitivity of approximately 8 RNA copies/µL (dilution point 10^{-1}) and CRISPR/Cas12 at 84 RNA copies/µL (dilution point 10^{0}). **Conclusion:** The RT-LAMP-CRISPR/Cas12a assay offered a promising rapid alternative for the detection of SARS-CoV-2.

Keywords: COVID-19, RT-LAMP, CRISPR, Cas12a, SARS-CoV-2, RT-PCR

1. Introduction

In late 2019, the first cases of pneumonia caused by SARS-CoV-2 were reported by the in Wuhan, China [1]. The virus spread rapidly around the world and in March 2020 the WHO declared a state of pandemic [2]. By January 2022, more than 280 million infections and more of 5 million deaths had been reported [3]. Studies report that the probability of infection after contactwith an infected people is up to 83% [1].

Accordingly, screening plans have been implemented to accurately identify SARS-CoV-2 infected patients, thus reducing the risk of contagion [4]. Nucleic acid amplification is the most indicated for the detection of SARS-CoV-2, as it detects the virus in the first days of infection [5]. However, the present gold standard detection assays using RT-PCR require the use of laboratories equipped, transport of samples to central laboratories with 4-6 h delay were for the release of their results, reducing the prospect of rapid diagnosis [6].

The devastating effect of SARS-CoV-2 worldwide demonstrates the need for the implementation of easy diagnostic techniques to be applied in remote areas, airports, and in low-income countries with higher risks of SARS-CoV-2 transmission, and the use of rapid, portable, and low-cost equipment to control viral spread in the population.

Approaches have been used in order to accelerate diagnostic testing for COVID-19, reverse transcription loop-mediated isothermal amplification (RT-LAMP), detecting RNA virus in a single reaction at only a single temperature, presenting a rapid alternative to PCR-based methods [7,8]. However, this assay has low specificity, making it difficult to use for the diagnosis of SARS-CoV-2 [9,10].

The U.S. Food and Drug Administration (FDA) has approved the detection of viral RNA based on CRISPR technology. This system is promising in the diagnosis of SARS-CoV-2 because of its high specificity and sensitivity in detecting nucleic acid through a single Cas

protein [11,12]. Therefore, we report here the development of a rapid assay using CRISPR-Cas12a-based RT-LAMP for the detection of SARS-CoV-2 RNA.

2. Methods

2.1 Ethical statement and Samples

A total of 29 clinical samples (nasopharyngeal swabs), including 13 positive and 16 negative samples, were collected at São Rafael Hospital (Salvador, Bahia, Brazil) from Feb to March 2022. Written consent was obtained from all enrolled patients and the local Ethics Committee (IRB) of the São Rafael Hospital approved this study (CAAE: 38580920.6.3001.0040). Samples were submitted to RNA extraction using kit viral RNA 93 MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit (ThermoFisher Scientific, 94 Massachusetts, EUA). The detection for SARS-CoV-2 virus was performed using kit Allplex™ SARS-CoV-2 (Seegene®, Corea do Sul), according to the manufacturer's recommendations and the thermal cycler ABI 7500 FAST (Applied Biosystem, EUA). To establish the Limit of Detection (LoD) of Cas12a, was used 1:10 serial dilution in nuclease-free water of titration SARS-CoV-2 (1.5 x 10^5 PFU), provided by the virology laboratory of the Federal University of Minas Gerais and reference sample with 3.000 RNA copies/mL (AccuPlex SARS-CoV-2 reference, Sera Care).

2.2 Nucleic acid preparation

SARS-CoV-2 genome sequences were obtained from the GISAID website (www.gisaid.org) and lined up to obtain a consensus sequence. Possible compatible sites for the construction of guide RNA were located throughout the sequence, subsequently; oligonucleotides with regions of the N gene and the ORF1ab gene were designed using PrimerExplorer v.5 (https://primerexplorer.jp/e/) according to instructions in the guide published by the site developers. Selected regions were then used in "nucleotide versus nucleotide" searches on the website https://blast.ncbi.nlm.nih.gov/Blast.cgi to check for homology with sequences of coronaviruses that infect humans. For control, the RNAse P gene was used as a target as previously described [9]. All the primers and RNAgs used in this study are listed in S1 and S2 Tables.

2.3 Cas12a detection reactions

Fragments of ORF1ab, N gene of SARS-CoV-2 and RNAse P gene were synthesized and amplified using the RT-LAMP assay, following the instructions of the manufacturer New England Biolabs Inc (https://www.neb.com/protocols/2014/10/09/typical-rt-lamp-protocol). The reactions were performed individually for the detection of separately genes, using MgSO 4 and dNTP mix at final concentrations of 8mM and 1.4mM respectively. 7 µl of RNA was used, heating the final reaction at 65°C for 30 min.

The optimized trans cleavage assays were performed as described previously [9,10]. We used approximately 30 nM of LbCas12a (NEB) and incubated for 10 min with 40 nM gRNA in addition to NEBuffer 2.1 at 37 °C. Subsequently, 2 µl of amplicon was added and after the RNA-protein complex formation, 100 nM of the fluorescent reporter 6-FAMBHQ was added to a black 384-well plate and readings were performed in the Gemini XPS fluorescence microplate.

2.4 Statistical analysis

Prism Software (GraphPad, La Jolla, CA, USA) was used to analyze all the data. Data regarding Ct values and fluorescence levels from RT-PCR and RT-LAMP/CRISPR-CAS12a, respectively, were analyzed by Pearson correlation (r).

3. Results

First, we evaluated the performance of different primer sets and SARS-CoV-2 targets in RT-LAMP reactions. After evaluation and standardization of the oligonucleotides, fragments from the ORF1ab gene (G2) (S1 Table) of SARS-CoV-2 showed satisfactory amplification by LAMP. Then, we tested RNA extracted from 23 respiratory swab samples collected from patients with suspected COVID-19 and 6 samples negative for SARS-CoV-2 and positive for H3N2 or respiratory syncytial virus using the RT-LAMP-CRISPR/Cas12a assay with fluorescence-based reads.

SARS-CoV-2 was detected in 13 patient swabs and no cross-reaction with other respiratory viruses was observed. We observed 100% concordance of the RT-LAMP-CRISPR/Cas12a assay results compared to the gold standard RT-PCR protocol (Figure 1). The RP gene was used as an internal control for the RT-LAMP-CRISPR/Cas12a assay.

Subsequently, we compared the analytical limit of detection (LoD) of the LAMP-

CRISPR/Cas12a assay and RT-PCR. Serial dilution of titration SARS-CoV-2 RNA (1.5 x 10^5 PFU/mL) was performed, the RT-PCR methodology using the commercial kit presented a detection limit of 0.15 PFU/mL (diluition 10^{-6}) and the RT-LAMP-CRISPR/Cas12a assay was able to detect a limit of 15.6 PFU/mL (diluition 10^{-4}) (Figure 2).

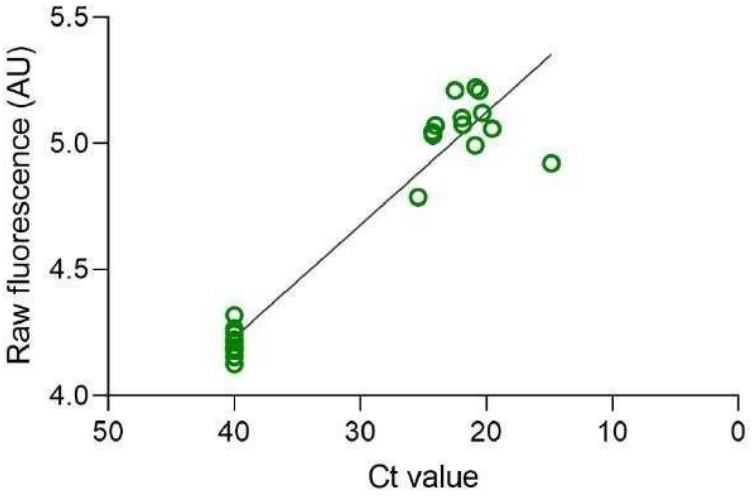

Figure 1. Correlation of the Ct values obtained by RT-PCR and fluorescence level obtained from the LAMP/CRISPR assay in 23 samples. X-axis Ct values (RT-PCR) and Y-axis raw fluorescence values (RT-LAMP-CRISPR/Cas12a). R^2 = 0,9176.

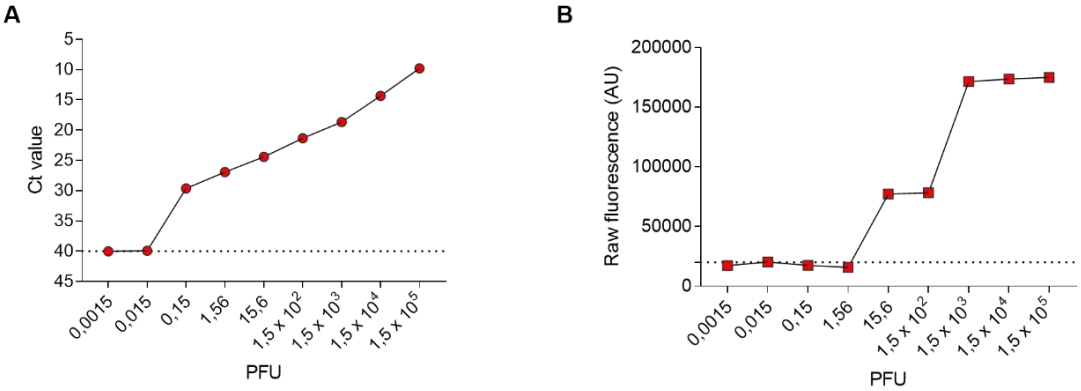

Figure 2. Comparison of the limit of quantification of titration SARS-CoV-2 virus (point 10^0 = 1.5 x 10^5 PFU) between methodologies. (A) RT-PCR using a commercial kit (detected < Ct 40, triplicate by spot). (B) RT-LAMP-CRISPR/Cas12a protocol (detected > 20000 AU, quintuplicate by spot).

To determine the sensitivity of RT-LAMP-CRISPR/Cas12a, we used a reference sample with 3,000 RNA copies/mL (AccuPlex SARS-CoV-2 reference, Sera Care). The RT-PCR

method demonstrated a sensitivity of approximately 8 RNA copies/µL (point 10^{-1}) and the LAMP-CRISPR-Cas12 to 84 RNA copies/µL (point 10^{0}), as illustrated in Figure 3.

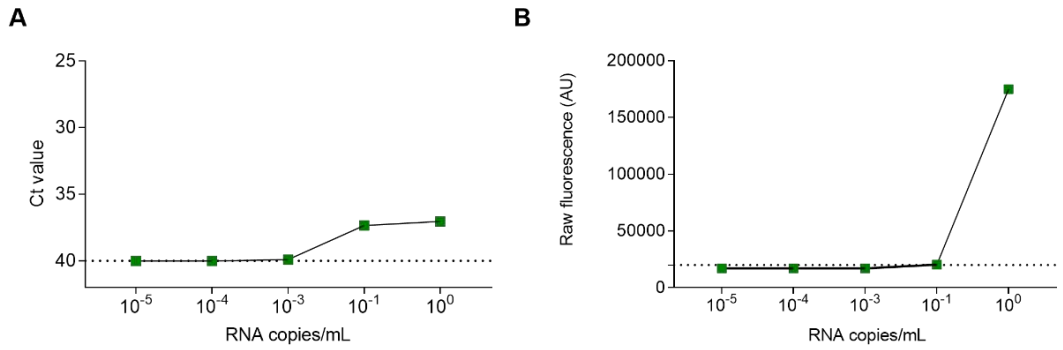

Figure 3. Determination of the sensitivity of the (A) RT-PCR (B) RT-LAMP-CRISPR/Cas12a assays, in viral RNA copies/mL values.

4. Discussion

During the pandemic, a critical mechanism for delaying SARS-CoV-2 dissemination was to implement testing for the detection and isolation of cases as early as possible [13]. The RT-LAMP (Reverse transcription loop-mediated isothermal amplification) assay reported here is a fast, simple assay, avoiding the need for thermal cycling and the use of complex laboratory infrastructure. It could easily be used at critical points of COVID-19 transmission to simplify the diagnostic process.

Some studies have highlighted the use of the LAMP technique in detecting coronavirus infections in patient samples [14,15]. RT-LAMP-based assay demonstrated almost 90% sensitivity and high consistency compared to RT-PCR-based diagnostic methods [16]. The time required for this assay was about one hour, considerably less than for RT-PCR. The assay in this RT-LAMP-CRISPR/Cas12a study was performed in only 30 min. According to Hong Thi and collaborators, the RT-LAMP assay demonstrated higher sensitivity than conventional RT-PCR, with a detection limit of 0.01 PFU in clinical samples [17].

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has become important in molecular biology experiments in recent years. Reformulating the diagnoses of the present moment. Numerous CRISPR-based techniques are currently in use or have the potential to be an option for diagnosing SARS-CoV-2. Between them, SHERLOCK, and other lateral flow-based diagnostic kits competing in competition for diagnostic accuracy with RT-PCR-

based diagnostics [18].

This study demonstrated an alternative for sensitive detection of SARS-CoV-2 by rapid LAMP assay together with the CRISPR-Cas12a system. The methodology and results of this study demonstrated the ease of use, effectiveness, efficiency, accuracy, and satisfaction in the use of the technology using RT-LAMP-associated Cas12a through florescence detection in thediagnosis of SARS-CoV-2. This methodology could also be applied to a lateral-flow strip-based detection system. Studies reported that the use of the lateral flow strip showed a sensitivity of more than 90% compared with the gold standard RT-PCR test [19]. Finally, although the RT- LAMP/CRISPR-based method described herein display advantages in terms of protocol duration and no requirements of molecular biology infrastructure, the test sensitivity is significantly inferior to RT-PCR, requiring further development. The LoD is situated within the range of commercial RT-PCR, and may be sufficient for detection of SARS-CoV-2 in most clinical samples [20]. In the future, the detection described here can be odified to target other respiratory pathogens by optimizing the development of rapid, targeted assays.

S1 Table. Primers used for RT-LAMP-only detection

Oligo name	**Sequence (5' – 3')**
F3 SARS-CoV G1- Orf1ab gene	GGAAAGGTTATGGCTGTAGT
FIP SARS-CoV G1- Orf1ab gene	GCAAACCCGTTTAAAAACGATTGTGTGATCAACTCCGCGAAC
FL SARS-CoV G1- Orf1ab gene	GCATCAGCTGACTGAAGCATG
B3 SARS-CoV G1- Orf1ab gene	TCTTGGAAGCGACAACAA
BIP SARS-CoV G1- Orf1ab gene	CGGCACAGGCACTAGTACTGAAAACCAGCTACTTTATCATTGT
BL SARS-CoV G1- Orf1ab gene	GTCGTATACAGGGCTTTTGACA
F3 SARS-CoV G2- Orf1ab gene	CCGCAAGGTTCTTCTTCGTA
FIP SARS-CoV G2- Orf1ab gene	CCAAGCTCGTCGCCTAAGTCAAAAGGAGCTGGTGGCCATAG
FL SARS-CoV G2- Orf1ab gene	TGACTTTAGATCGGCGCCGTAA
B3 SARS-CoV G2- Orf1ab gene	CAGGGCCACAGAAGTTGTT
BIP SARS-CoV G2- Orf1ab gene	AGCAGTGGTGTTACCCGTGAACCGACATAGCGAGTGTATGCC
BL SARS-CoV G2- Orf1ab gene	ATGCGTGAGCTTAACGGAGG
F3 SARS-CoV G3- N gene	TCGCAATGGCTTGTCTTGT
FIP SARS-CoV G3- N gene	ACCACATGGAACGCGTACGCGCTTGATGTGGCTCAGCT
FL SARS-CoV G3- N gene	GCAAACAGTCTGAAAGAAGCAATGA
B3 SARS-CoV G3- N gene	TCTTGGAAGCGACAACAA
BIP SARS-CoV G3- N gene	CGGCACAGGCACTAGTACTGAAAACCAGCTACTTTATCATTGT
BL SARS-CoV G3- N gene	GTCGTATACAGGGCTTTTGACA

F3 RNase P POP7	TTGATGAGCTGGAGCCA
B3 RNase P POP7	CACCCTCAATGCAGAGTC
FIP RNase P POP7	GTGTGACCCTGAAGACTCGGTTTTAGCCACTGACTCGGATC
BIP RNase P POP7	CCTCCGTGATATGGCTCTTCGTTTTTTTCTTACATGGCTCTGGTC
LF RNase P POP7	ATGTGGATGGCTGAGTTGTT
LB RNase P POP7	CATGCTGAGTACTGGACCTC

S2 Table. gRNA and reporters used for CRISPR-Cas12a-based SARS-CoV-2 detection.

Orf1ab-gene gRNA	UAAUUUCUACUAAGUGUAGUAAAGAAAACUGGAGGAACACUAAAC	gRNA for the CRISPR-Cas12a system
RNaseP POP7 gRNA	UAAUUUCUACUAAGUGUAGAUAAUUACUUGGGUGUGACCCU	
6-FAMBHQ	/56-FAM/TTTTTTTTTTT/3BHQ_1/	For fluorescence assay
6-FAMBIOTIN	/56-FAM/TTATTATT/3Bio/	For lateral flow assay

References

1. Chan JFW, Yuan S, Kok KH, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. The Lancet. 2020;395(10223):514-523. doi:10.1016/S0140-6736(20)30154-9.
2. World Health Organization Virtual Press Conference on COVID-19, 11 March 2020. [(accessed on 2 January 2022)]. Available online: https://www.who.int/docs/default-source/%0Acoronaviruse/transcripts/who-audioemergencies-coronavirus-press-conference-fulland- fnal-11mar2020.pdf?sfvrsn=cb432bb3_2.
3. Our World in Data COVID-19 Data Explorer. [(accessed on 2 January 2022)]. Available online: https://ourworldindata.org/explorers/coronavirus-data-explorer.
4. Ji T, Liu Z, Wang G, et al. Detection of COVID-19: A review of the current literature and future perspectives. Biosens Bioelectron. 2020;166:112455. doi: 10.1016/j.bios.2020.112455.
5. Eis-Hübinger AM, Hönemann M, Wenzel JJ, et al. Ad hoc laboratory-based surveillance of SARS- CoV-2 by real-time RT-PCR using minipools of RNA prepared from routine respiratory samples. Journal of Clinical Virology. 2020;127:104381. doi:10.1016/j.jcv.2020.104381.
6. Morshed M.G, Lee MK, Jorgensen D, et al. Molecular methods used in clinical laboratory: prospects and pitfalls. Immunol Med Microbiol. 2007;49:184-91. doi: 10.1111/j.1574-

695X.2006.00191.x.

7. Niemz A, Ferguson T.M, Boyle D.S. Point-of-care nucleic acid testing for infectious diseases.Trends Biotechnol. 2011;29:240-50. doi: 10.1016/j.tibtech.2011.01.007.
8. Mori Y, Notomi T. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost- effective diagnostic method for infectious diseases. J Infect Chemother. 2009;15:62- 69. doi: 10.1007/s10156-009-0669-9.
9. Chou PH, Lin YC, Teng PH, et al. Real-time target-specific detection of loop-mediated isothermalamplification for white spot syndrome virus using fluorescence energy transfer-based probes. J VirolMethods. 2011;173:67-74. doi: 10.1016/j.jviromet.2011.01.009. Epub 2011 Jan 21.
10. Nagai K, Horita N, Yamamoto M, et al. Diagnostic test accuracy of loop-mediated isothermal amplification assay for Mycobacterium tuberculosis: systematic review and meta-analysis. Scientific Reports. 2016;6:39090. doi: 10.1038/srep39090.
11. Aman R, Mahas A, Mahfouz M. Nucleic Acid Detection Using CRISPR/Cas BiosensingTechnologies. Synth Biol. 2020;9:1226-1233. doi: 10.1021/acssynbio.9b00507.
12. Li Y, Li S, Wang J, et al. CRISPR/Cas Systems towards Next-Generation Biosensing. TrendsBiotechnol. 2019;37:730-743. doi: 10.1016/j.tibtech.2018.12.005.
13. World Health Organization COVID-19 Strategy update, 14 April 2020. [(accessed on 15 April 2022)]. Available online: https://www.who.int/publications/m/item/covid-19- strategy-update.
14. Pyrc K, Milewska A, Potempa J. Development of loop-mediated isothermal amplification assay for detection of human coronavirus-NL63. Journal of Virological Methods. 2011;175(1):133-136.doi:10.1016/j.jviromet.2011.04.024.
15. Poon LLM, Leung CSW, Tashiro M, et al. Rapid Detection of the Severe Acute Respiratory Syndrome (SARS) Coronavirus by a Loop-Mediated Isothermal Amplification Assay. Clinical Chemistry. 2004;50(6):1050-1052. doi:10.1373/clinchem.2004.032011.
16. Hu X, Deng Q, Li J, et al. Development and Clinical Application of a Rapid and Sensitive Loop- Mediated Isothermal Amplification Test for SARS-CoV-2 Infection. mSphere. 2020;5(4). doi:10.1128/mSphere.00808-20.
17. Thai HTC, Le MQ, Vuong CD, et al. Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Method for Rapid Detection of Severe Acute Respiratory Syndrome Coronavirus. Journal of Clinical Microbiology. 2004;42(5):1956- 1961. doi:10.1128/JCM.42.5.1956-1961.2004.
18. Broughton JP, Deng X, Yu G, et al. CRISPR–Cas12-based detection of SARS-CoV-2.

Nature Biotechnology 2020 38:7. 2020;38(7):870-874. doi:10.1038/s41587-020-0513-4.

19. Merino P, Guinea J, Muñoz-Gallego I, et al. Multicenter evaluation of the Panbio™ COVID-19 rapid antigen-detection test for the diagnosis of SARS-CoV-2 infection. Clinical Microbiology and Infection. 2021;27(5):758-761. doi:10.1016/j.cmi.2021.02.001.
20. Mostafa H.H., Hardick J., Morehead E., et al. Comparison of the Analytical Sensitivity of Seven Commonly Used Commercial SARS-CoV-2 Automated Molecular Assays. J. Clin. Virol. 2020;130:104578. doi: 10.1016/j.jcv.2020.104578.

6 **DISCUSSÃO**

Neste estudo, apresentamos evidências do uso clínico de amostras de saliva no diagnóstico molecular de COVID-19, bem como buscamos desenvolver um protótipo de teste molecular rápido baseado na tecnologia CRISPR/Cas12. A amostrade saliva proporciona ao paciente uma coleta facial e prática através da autocoleta, deixando de lado a necessidade de mobilização de uma equipe de coleta de swabs nasofaríngeos. Demonstramos que a saliva detecta SARS-CoV-2, alcançando 100% de concordância com o swab nasofaringeo. Nossos resultados estão de acordo com dados publicados reafirmando a utilidade da saliva como uma amostra alternativa paratriagem e diagnóstico de COVID-19 (LEUNG et al., 2021; TO et al., 2020; WONG et al., 2020). Trabalhos publicados anteriormente, mostram que a sensibilidade do RT- PCR na análise de amostras de saliva para COVID-19 foram de 70 a 100%, em comparação com o amostras de swabs de garganta e nasofaringe (PASOMSUB et al., 2021; WYLLIE et al., 2020). A diferença na sensibilidade podem ser esclarecidos em consequência de que secreções nasais e orofaríngeas, possuírem carga viral é potencialmente maior (LEUNG et al., 2021; WONG et al., 2020), nos antecedentes clínicos dos indivíduos e no momento da amostragem em cada estudo ou no volume coletado de amostras, valores maiores que 8 mL de saliva para teste (WYLLIE et al.,2020) .

A diferença no volume salivar pode afetar a carga viral na saliva e estar associada à diferença na sensibilidade diagnóstica. Estudos demostram que em pacientes graves a saliva se torna mais promissora para detecção de SARS-CoV-2 (CHEN et al., 2020). Outros trabalhos demostram que carga viral do SARS-CoV-2 na saliva apresenta tendência de diminuir desde o início dos sintomas (TO et al., 2020), as amostras de saliva devem ser coletadas durante a fase inicial do início dos sintomas para aumentar a sensibilidade. Entretanto, no presente estudo, não observamos diferenças significativas na gravidade da doença ou sintomas clínicos entre os pacientes em cuja saliva o RNA viral foi e não foi detectado. Todos os indivíduos no estudo apresentaram casos leves ou subclínicos. Nosso estudo tambémobservou a presença de RNA viral em pacientes assintomáticos. Esses achados sustentam estudos anteriores revelando a presença de RNA viral na saliva de pacientes sintomáticos e assintomáticos (KOJIMA et al., 2021). Portanto, nossos resultados demostram que a saliva, é uma fonte confiável e prática para a triagem e diagnóstico de COVID-19.

Demonstramos também o monitoramos a estabilidade das amostras de saliva e

observamos que a amostra permaneceu estável, se armazenada em freezer -80°C, por até 30 dias. Nosso resultado vai de acordo com estudos anteriores demostrando a estabilidade das amostras de saliva positivas para SARS-CoV-2 (WILLIAMS et al., 2021). O SARS-CoV-2 pode permanecer estável na saliva, devida a proteínas virais que evitam a morte celular (GOH et al., 2020). Essa descoberta é promissora para a utilização de amostras de saliva em áreas remotas que pode levar dias até o transporte para os centros de diagnóstico. Entretanto, apenas dos dados favoráveis a estabilidade da amostra, é recomendável a utilização de geladeiras e freezer e o processamento rápido da amostra para o posteriormente possam ser tomadas medidas, limitando assim a transmissão da doença.

Curiosamente, o uso de diferentes kits de RT-PCR no presente estudo resultou em diferentes resultados de Ct em amostras positivas na saliva. Portanto, apresentando uma atenção importante para laboratórios clínicos, onde protocolos laboratoriais mais sensíveis devem ser implantados. Como limitação do estudo com ouso de saliva, incluímos pacientes assintomáticos ou aqueles que tinham doença leve. Os resultados não podem ser comparados em outras populações, como por exemplo, pacientes pediátricos, no qual é de extrema necessidade de coleta alternativos de amostras (LUDVIGSSON, 2020; SANTOS et al., 2020). Amostras de saliva foram congeladas por até uma semana, enquanto as amostras de swab nasofaringeo foram processadas até 12 horas depois da coleta. Considerando assim um interferente de sensibilidade no estudo.

Diante da atual situação pandêmica, a necessidade de equipamentos específicos, diagnóstico demorados e a disponibilidade limitada de centros de diagnóstico, torna-se complexo para o controle da disseminação do SARS-CoV-2. Háuma necessidade urgente de equipamentos de baixo custo, portáteis, rápidos, e técnicas de diagnóstico fáceis de usar que podem ser aplicáveis em áreas remotas com recursos limitados que correm maior risco de transmissão (VANDENBERG et al., 2020; PHUA et al., 2020). Aqui, mostramos que a detecção do SARS-CoV-2 com CRISPR-Cas12a oferece resposta promissora para testes rápidos no local de atendimento.

A amplificação isotérmica mediada por transcriptase reversa (RT-LAMP) é uma técnica de amplificação de ácido nucleico rápida, barata e prática. No entanto, essa tem baixa especificidade, podendo aumentar as chances de uma falso-positivo (CHOU et al., 2011; NEGAI et al., 2016). Com isso, estudos associam o LAMP ao sistema CRISPR/Cas, a ferramenta de edição de genoma mais popular (MUSTAFÁ; MAKHAWI, 2021). Estudos mostram que o ensaio baseado em CRISPR-Cas13 (SHERLOCK) (GOOTENBERG et al.,

2018; MYHRVOLD et al., 2018) e CRISPR- Cas12a (DETECTR) (CHEN et al., 2018) apresentam vantagens para o diagnóstico molecular tradicional, como por exemplo a reação em cadeia da polimerase (PCR). Durante a pandemia do SARS-CoV-2 a Food and Drug Administration (FDA) aprovouo primeiro kit de detecção de COVID-19 baseado em CRISPR, fornecendo resultados em aproximadamente uma hora (BROUGHTON et al., 2020). Apresentando uma sensibilidade na faixa de 75-100%, dos métodos de detecção de SARS-CoV-2 baseados em RT-LAMP CRISPR. Nossos resultados demonstraram uma concordância de 100% de amostras avaliadas no ensaio RT-LAMP CRISPR em relação ao padrão-ouro PCR.

A sensibilidade demostra o potencial do teste de identificar corretamente umindivíduo com uma doença. Enquanto, a especificidade é potencial do teste para identificar com precisão pessoas que não têm a doença (SWIFT; HEALE; TWYCROSS, 2020). Com isso, testamos 6 amostras negativas para SARS-CoV-2 e positiva para outros patógenos respiratórios, nosso teste não demonstrou reação cruzada com SARS-CoV-2. Esses resultados demonstram que o ensaio é altamente específico para SARS-CoV-2. A alta sensibilidade do teste pode ser atribuído, ao alvo do nosso teste é o gene ORF1ab e estudos relatam que os crRNAs direcionadosaos genes ORF1ab e N do SARS-CoV-2 apresentam incompatibilidades com outros coronavírus, sendo assim, demostrando uma alta especificidade do ensaio que utilizam esses alvos, uma vez que os crRNAs apresentam sensibilidade a mutações de nucleotídeo único (LI et al., 2018).

Estudos realizados demonstram que o limite de detecção mais baixo (0,03 cópias/µL) de SARS-CoV-2 foi através de concentração mediada por esferas magnéticas de RNAs, 30 vezes menor que o do teste RT-PCR aprovado pelo CDC (JOUNG et al., 2020). O LoD é definido como o menor número de cópias de RNA viralque podem ser detectadas de forma confiável com uma determinada amostra. Encontramos no nosso ensaio RT-LAMP-CRISPR/Cas12a, um limite de detecção de 84 cópias de RNA/µL, dentro dos limites esperados pelo CDC.

Várias estratégias para melhorar a sensibilidade e especificidade da detecção de SARS-CoV-2 baseada em RT-LAMP CRISPR são estudadas, entre elas o uso do ensaio de fluxo lateral (LFA) a técnica de leitura colorimétrica mais popular (BROUGHTON et al., 2020). Esta estratégia é promissora e poderá ser utilizada nas próximas etapas para facilitar a acessibilidade do teste.

7 **CONCLUSÃO**

O diagnóstico é essencial para controlar a pandemia de COVID-19. A implementação de qualquer tipo de amostragem clínica para diagnóstico deve considerar os riscos para os profissionais de saúde, a facilidade da coleta e principalmente a sensibilidade dos ensaios. Os resultados deste estudo sugerem que o diagnóstico de SARS-CoV-2 baseado em saliva é promissor, podendo ser uma opção de amostra alternativa para o diagnóstico de COVID-19.

A rápida disseminação do SARS-CoV-2 ressalta a importância do desenvolvimento de técnicas de Point-of-Care, como amplificação isotérmica e técnicas baseadas em CRISPR para que possam ser usadas para testar populações e isolar pessoas infectadas, principalmente em países que não possuem recursos e tecnicos com habilidades suficientes. O ensaio de detecção RT-LAMP- CRSPR/Cas12a relatado aqui pode atender à necessidade de um teste que forneça resultados rápidos, sendo promissor para o diagnóstico do SARS-CoV-2.

REFERÊNCIAS

ABABOU, A.; LADBURY, J. E. Survey of the year 2005: literature on applications of isothermal titration calorimetry. **Journal of Molecular Recognition**, v. 20, n. 1, p. 4–14, 1 jan.2007.

ANGELETTI, S. et al. COVID-2019: The role of the nsp2 and nsp3 in its pathogenesis.**Journal of Medical Virology**, v. 92, n. 6, p. 584–588, 1 jun. 2020.

AZZI, L. et al. Diagnostic Salivary Tests for SARS-CoV-2. **Journal of Dental Research**, v. 100, n. 2, p. 115, 1 fev. 2021.

BARRANGOU, R. et al. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. **Science**, v. 315, n. 5819, p. 1709–1712, 23 mar. 2007.

BARRANGOU, R.; HORVATH, P. A decade of discovery: CRISPR functions and applications. **Nature Microbiology 2017 2:7**, v. 2, n. 7, p. 1–9, 5 jun. 2017.

BELOUZARD, S. et al. Mechanisms of Coronavirus Cell Entry Mediated by the Viral Spike Protein. **Viruses**, v. 4, n. 6, p. 1011, 2012.

BIANCHI, M. et al. Sars-CoV-2 Envelope and Membrane Proteins: Differences from Closely Related Proteins Linked to Cross-species Transmission? 7 abr. 2020.

BOLOTIN, A. et al. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. **Microbiology**, v. 151, n. 8, p. 2551– 2561, 1 ago. 2005.

BRANDSMA, E. et al. Rapid, Sensitive, and Specific Severe Acute Respiratory Syndrome Coronavirus 2 Detection: A Multicenter Comparison Between Standard Quantitative Reverse-Transcriptase Polymerase Chain Reaction and CRISPR-Based DETECTR. **The Journal of Infectious Diseases**, v. 223, n. 2, p. 206–213, 3 fev. 2021.

BROUGHTON, J. P. et al. CRISPR–Cas12-based detection of SARS-CoV-2. **Nature Biotechnology 2020 38:7**, v. 38, n. 7, p. 870–874, 16 abr. 2020.

BHATTACHARYYA, R. P.; THAKKU, S. G.; HUNG, D. T. Harnessing CRISPR. Effectors for Infectious Disease Diagnostics. **ACS Infectious Diseases**, v. 4, n. 9, p. 1278–1282, 14 set. 2018.

CHAN, J. F.-W. et al. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. **Emerging Microbes & Infections**, v. 9, n. 1, p. 221, 1 jan. 2020.

CHEN, H. et al. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. **Lancet (London, England)**, v. 395, n. 10226, p. 809, 7 mar. 2020.

CHEN, J. S. et al. CRISPR-Cas12a target binding unleashes indiscriminate single- stranded DNase activity. **Science**, v. 360, n. 6387, p. 436–439, 27 abr. 2018.

CHEN, L. et al. Detection of SARS-CoV-2 in saliva and characterization of oral symptoms in COVID-19 patients. **Cell Proliferation**, v. 53, n. 12, 19 dez. 2020.

CHEN, Y.; LIU, Q.; GUO, D. Emerging coronaviruses: Genome structure, replication, and pathogenesis. **Journal of Medical Virology**, v. 92, n. 4, p. 418, 1 abr. 2020.

CHOU, P.-H. et al. Real-time target-specific detection of loop-mediated isothermal amplification for white spot syndrome virus using fluorescence energy transfer-based probes. **Journal of Virological Methods**, v. 173, n. 1, p. 67–74, abr. 2011.

COMMITTEE, I. C. ON T. OF V. E. The new scope of virus taxonomy: partitioning the virosphere into 15 hierarchical ranks. **Nature Microbiology**, v. 5, n. 5, p. 668, 1 maio 2020.

CORMAN, V. M. et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. **Eurosurveillance**, v. 25, n. 3, 23 jan. 2020.

CUI, J.; LI, F.; SHI, Z.-L. Origin and evolution of pathogenic coronaviruses. **Nature Reviews. Microbiology**, v. 17, n. 3, p. 181, 1 mar. 2019.

DEDIEGO, M. L. et al. A severe acute respiratory syndrome coronavirus that lacksthe e gene is attenuated in vitro and in vivo. **Journal of Virology**, v. 81, n. 4, p. 1701, 15 fev. 2007.

DHAMA, K. et al. Coronavirus disease 2019–COVID-19. **Clinical Microbiology Reviews**, v. 33, n. 4, p. 1–48, 1 out. 2020.

DONG, E.; DU, H.; GARDNER, L. An interactive web-based dashboard to track COVID-19 in real time. **The Lancet. Infectious Diseases**, v. 20, n. 5, p. 533, 1 maio 2020.

E, D.; H, D.; L, G. An interactive web-based dashboard to track COVID-19 in real time. **The Lancet. Infectious diseases**, v. 20, n. 5, p. 533–534, 1 maio 2020.

FANG, X. et al. The membrane protein of SARS-CoV suppresses NF-κB activation. **Journal of Medical Virology**, v. 79, n. 10, p. 1431, out. 2007.

FERREIRO-BARROS, C. C.; SUGAWARA, E. K.; SANCHES, L. R. Determination of a method for extraction of coenzyme Q10 in human plasma: optimization of the use of surfactants and others variables. **Einstein**. v. 10, n. 2, p. 203–211, 2012.

GANDHI, M.; YOKOE, D. S.; HAVLIR, D. V. Asymptomatic Transmission, the Achilles' Heel of Current Strategies to Control Covid-19. **The New England Journal of Medicine**, v. 382, n. 22, p. 2158–2160, 28 maio 2020.

GOH, G. K.-M. et al. Rigidity of the Outer Shell Predicted by a Protein Intrinsic Disorder Model Sheds Light on the COVID-19 (Wuhan-2019-nCoV) Infectivity. **Biomolecules**, v. 10, n.2, p. 331, 19 fev. 2020.

GOOTENBERG, J. S. et al. Multiplexed and portable nucleic acid detection platform with

Cas13, Cas12a, and Csm6. **Science**, v. 360, n. 6387, p. 439–444, 27 abr. 2018.
GORBALENYA, A. E. et al. The new scope of virus taxonomy: partitioning the virosphere into 15 hierarchical ranks. **Nat Microbiol**, v.5**,** p. 668–674. abr. 2020.

HAAN, C. A. M. DE; VENNEMA, H.; ROTTIER, P. J. M. Assembly of the Coronavirus Envelope: Homotypic Interactions between the M Proteins. **Journal of Virology**, v. 74, n. 11, p. 4967, jun. 2000.

HARIKRISHNAN, P. Saliva as a Potential Diagnostic Specimen for COVID-19 Testing. **The Journal of Craniofacial Surgery**, v. 31, n. 6, p. e653–e655, 1 set. 2020.

HILLARY, V. E.; IGNACIMUTHU, S.; CEASAR, S. A. Potential of CRISPR/Cas system in the diagnosis of COVID-19 infection. **Expert Review of Molecular Diagnostics**, v. 21, n. 11, p. 1179–1189, 2 nov. 2021.

HONG, K. H. et al. Guidelines for Laboratory Diagnosis of Coronavirus Disease 2019 (COVID-19) in Korea. **Annals of Laboratory Medicine**, v. 40, n. 5, p. 351–360, 1 set. 2020.

ISHINO, Y. et al. Nucleotide sequence of the iap gene, responsible for alkalinephosphatase isozyme conversion in Escherichia coli, and identification of the gene product. **Journal of Bacteriology**, v. 169, n. 12, p. 5429, 1987.

JANSEN, RUUD. et al. Identification of genes that are associated with DNA repeats in prokaryotes. **Molecular Microbiology**, v. 43, n. 6, p. 1565–1575, 1 mar. 2002.

JEFFERSON RIBEIRO DA SILVA, S. et al. Clinical and Laboratory Diagnosis of SARS-CoV-2, the Virus Causing COVID-19. 2019.

JIANG, S.; DU, L.; SHI, Z. An emerging coronavirus causing pneumonia outbreak in Wuhan, China: calling for developing therapeutic and prophylactic strategies. **Emerging Microbes & Infections**, v. 9, n. 1, p. 275, 1 jan. 2020.

JOHANSSON, M. A. et al. SARS-CoV-2 transmission from people without COVID- 19 Symptoms. **JAMA Network Open**, v. 4, n. 1, 1 jan. 2021.

JOUNG, J. et al. Detection of SARS-CoV-2 with SHERLOCK One-Pot Testing. **New England Journal of Medicine**, v. 383, n. 15, p. 1492–1494, 8 out. 2020.

JT, W.; K, L.; GM, L. Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. **Lancet (London, England)**, v. 395, n. 10225, p. 689–697, 29 fev. 2020.

KADAM, S. B. et al. SARS-CoV-2, the pandemic coronavirus: Molecular and structural insights. **Journal of Basic Microbiology**, v. 61, n. 3, p. 180, 1 mar. 2021.

KE, Z. et al. Structures and distributions of SARS-CoV-2 spike proteins on intact virions. **Nature**, v. 588, n. 7838, p. 498, 17 dez. 2020.

KOJIMA, N. et al. Self-Collected Oral Fluid and Nasal Swabs Demonstrate Comparable Sensitivity to Clinician Collected Nasopharyngeal Swabs for Coronavirus Disease 2019

Detection. **Clinical Infectious Diseases**, v. 73, n. 9, p. e3106–e3109, 2 nov. 2021.

LA VECCHIA, C. et al. Perceived risk of cancer in population samples from 5 European countries. **International Journal of Cancer**, v. 86, n. 5, p. 747–748, 2000.

LEE, DR. Y.-H.; WONG, DR. D. T. Saliva: An emerging biofluid for early detection of diseases. **American journal of dentistry**, v. 22, n. 4, p. 241, ago. 2009.

LEUNG, E. C. et al. Deep throat saliva as an alternative diagnostic specimen type for the detection of SARS-CoV-2. **Journal of Medical Virology**, v. 93, n. 1, p. 533–536, 14 jan. 2021.

LI, S.-Y. et al. CRISPR-Cas12a-assisted nucleic acid detection. **Cell Discovery**, v. 4, n. 1, p. 20, 24 dez. 2018.

LI, Y. et al. CRISPR/Cas Systems towards Next-Generation Biosensing. **Trends in Biotechnology**, v. 37, n. 7, p. 730–743, 1 jul. 2019.

LI, Z. et al. A chemical-enhanced system for CRISPR-Based nucleic acid detection. **Biosensors and Bioelectronics**, v. 192, p. 113493, nov. 2021.

LIAO, Y.; TAM, J. P.; LIU, D. X. Viroporin Activity of SARS-CoV E Protein. **The Nidoviruses**, v. 581, p. 199, 2006.

LINO, C. A. et al. Delivering CRISPR: a review of the challenges and approaches. **Drug Delivery**, v. 25, n. 1, p. 1234, 2018.

LIU, H. et al. Development of Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of Batai Virus in Cattle and Mosquitoes. **Vector-Borne and Zoonotic Diseases**, v. 16, n. 6, p. 415–422, jun. 2016.

LUDVIGSSON, J. F. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. **Acta Paediatrica**, v. 109, n. 6, p. 1088–1095, 14 jun. 2020.

MACHHI, J. et al. The Natural History, Pathobiology, and Clinical Manifestations of SARS-CoV-2 Infections. **Journal of Neuroimmune Pharmacology**, v. 15, n. 3, p. 1, 1 set. 2020.

MACKAY, I. M.; ARDEN, K. E. MERS coronavirus: diagnostics, epidemiology and transmission. **Virology Journal**, v. 12, n. 1, 22 dez. 2015.

MAKAROVA, K. S.; KOONIN, E. V. Annotation and Classification of CRISPR-Cas Systems. **Methods in molecular biology (Clifton, N.J.)**, v. 1311, p. 47, 2015.

MALLAH, S. I. et al. COVID-19: breaking down a global health crisis. **Annals of Clinical Microbiology and Antimicrobials**, v. 20, n. 1, p. 35, 1 dez. 2021.

MARTINA, E. et al. Saliva and Oral Diseases. **Journal of Clinical Medicine**, v. 9, n. 2,p. 466, 8 fev. 2020.

MATHEWSON, A. C. et al. Interaction of severe acute respiratory syndrome- coronavirus

and NL63 coronavirus spike proteins with angiotensin converting enzyme-2. **The Journal of General Virology**, v. 89, n. Pt 11, p. 2741, 2008.

MCBRIDE, R.; ZYL, M. VAN; FIELDING, B. C. The Coronavirus Nucleocapsid Is a Multifunctional Protein. **Viruses**, v. 6, n. 8, p. 2991, 7 ago. 2014.

MEDICINE, T. L. R. COVID-19 transmission—up in the air. **The Lancet. Respiratory Medicine**, v. 8, n. 12, p. 1159, 1 dez. 2020.

MOJICA, F. J. M. et al. Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. **Journal of Molecular Evolution 2005 60:2**,v. 60, n. 2, p. 174–182, fev. 2005.

MORI, Y.; KANDA, H.; NOTOMI, T. Loop-mediated isothermal amplification (LAMP): recent progress in research and development. **Journal of Infection and Chemotherapy**, v. 19, n. 3, p. 404–411, 2013.

MUSTAFA, M. I.; MAKHAWI, A. M. SHERLOCK and DETECTR: CRISPR-Cas Systems as Potential Rapid Diagnostic Tools for Emerging Infectious Diseases. **Journal of Clinical Microbiology**, v. 59, n. 3, 18 fev. 2021.

MYHRVOLD, C. et al. Field-deployable viral diagnostics using CRISPR-Cas13. **Science**, v. 360, n. 6387, p. 444–448, 27 abr. 2018.

NAGAI, K. et al. Diagnostic test accuracy of loop-mediated isothermal amplification assay for Mycobacterium tuberculosis: systematic review and meta-analysis. **Scientific Reports**, v. 6, n. 1, p. 39090, 13 dez. 2016.

NEUMAN, B. W. et al. A structural analysis of M protein in coronavirus assembly and morphology. **Journal of Structural Biology**, v. 174, n. 1, p. 11, abr. 2011.

NOTOMI, T. et al. Loop-mediated isothermal amplification of DNA. **Nucleic Acids Research**, v. 28, n. 12, p. e63, 2000.

OCWIEJA, K. E. et al. A Reverse Transcription Loop-Mediated Isothermal Amplification Assay Optimized to Detect Multiple HIV Subtypes. **PLOS ONE**, v. 10, n. 2, p. e0117852, 12 fev. 2015.

ODARI, E. O. et al. Establishment and evaluation of a loop-mediated isothermal amplification (LAMP) assay for the semi-quantitative detection of HIV-1 group M virus. **Journal of Virological Methods**, v. 212, p. 30–38, fev. 2015.

PAN, Y. et al. Potential False-Negative Nucleic Acid Testing Results for Severe Acute Respiratory Syndrome Coronavirus 2 from Thermal Inactivation of Samples with Low Viral Loads. **Clinical Chemistry**, v. 66, n. 6, p. 794–801, 1 jun. 2020.

PARIDA, M. et al. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. **Reviews in Medical Virology**, v. 18, n. 6, p. 407, nov. 2008.

PASOMSUB, E. et al. Saliva sample as a non-invasive specimen for the diagnosis of coronavirus disease 2019: a cross-sectional study. **Clinical Microbiology and Infection**, v. 27, n. 2, p. 285.e1-285.e4, fev. 2021.

PENG, D. et al. One-step reverse transcription loop-mediated isothermal amplificationassay for detection of Apple chlorotic leaf spot virus. **Journal of Virological Methods**, v. 248, p. 154–158, out. 2017.

PHAM, T. et al. The Rotavirus NSP4 Viroporin Domain is a Calcium-conducting Ion Channel. **Scientific Reports**, v. 7, 3 mar. 2017.

PHUA, J. et al. Intensive care management of coronavirus disease 2019 (COVID-19): challenges and recommendations. **The Lancet Respiratory Medicine**, v. 8, n. 5, p. 506–517, maio 2020.

POURCEL, C.; SALVIGNOL, G.; VERGNAUD, G. CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. **Microbiology**, v. 151, n. 3, p. 653–663, 1 mar. 2005.

QIN, R.; STEEL, A.; FAZEL, N. Oral mucosa biology and salivary biomarkers. **Clinics in Dermatology**, v. 35, n. 5, p. 477–483, 1 set. 2017.

RUAN, Q. et al. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. **Intensive Care Medicine**, v. 46, n. 5, p. 1, 1 maio 2020.

SAHAJPAL, N. S. et al. Clinical Validation of a Sensitive Test for Saliva Collected in Healthcare and Community Settings with Pooling Utility for Severe Acute Respiratory Syndrome Coronavirus 2 Mass Surveillance. **The Journal of Molecular Diagnostics : JMD**, v. 23, n. 7, p. 788, jul. 2021.

SALIAN, V. S. et al. COVID-19 Transmission, Current Treatment, and Future Therapeutic Strategies. **Molecular Pharmaceutics**, v. 18, n. 3, p. 754–771, 1 mar. 2021.

SANTOS, C. N. et al. Saliva: an important alternative for screening and monitoring of COVID-19 in children. **Brazilian Oral Research**, v. 34, 2020.

SCHOEMAN, D.; FIELDING, B. C. Coronavirus envelope protein: current knowledge. **Virology Journal**, v. 16, n. 1, 27 maio 2019.

SCOLA, B. LA et al. Viral RNA load as determined by cell culture as a management tool for discharge of SARS-CoV-2 patients from infectious disease wards. **European Journal of Clinical Microbiology & Infectious Diseases**, v. 39, n. 6, p. 1059, 1 jun. 2020.

SILVA, S. J. R. DA; PARDEE, K.; PENA, L. Loop-Mediated Isothermal Amplification (LAMP) for the Diagnosis of Zika Virus: A Review. **Viruses**, v. 12, n. 1, 23 dez. 2020.

SINGH, H. et al. Cutaneous Manifestations of COVID-19: a systematic review. **Adv Wound Care (New Rochelle)**, v. 10, n. 2, p. 51–80, 23 dez. 2020.

SULE, W. F.; OLUWAYELU, D. O. Real-time RT-PCR for COVID-19 diagnosis: challenges and prospects. **The Pan African Medical Journal**, v. 35, n. Suppl 2, p. 121, 2020.

SUNGNAK, W. et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelialcells together with innate immune genes. **Nature Medicine 2020 26:5**, v. 26, n. 5, p. 681–687, 23 abr. 2020.

SWIFT, A.; HEALE, R.; TWYCROSS, A. What are sensitivity and specificity? **Evidence Based Nursing**, v. 23, n. 1, p. 2–4, jan. 2020.

TABATABAEIZADEH, S.-A. Airborne transmission of COVID-19 and the role of face mask to prevent it: a systematic review and meta-analysis. **European Journal of Medical Research**, v. 26, n. 1, p. 1, 1 dez. 2021.

TEO, A. K. J. et al. Saliva is more sensitive than nasopharyngeal or nasal swabs for diagnosis of asymptomatic and mild COVID-19 infection. **Scientific Reports 2021 11:1**, v. 11, n. 1, p. 1–8, 4 fev. 2021.

TERNS, M. P. CRISPR-based Technologies: Impact of RNA-targeting Systems. **Molecular cell**, v. 72, n. 3, p. 404, 1 nov. 2018.

TIAN, B. et al. Attomolar Zika virus oligonucleotide detection based on loop-mediated isothermal amplification and AC susceptometry. **Biosensors and Bioelectronics**, v. 86, p. 420–425, dez. 2016.

TIAN, X. et al. CRISPR/Cas9 – An evolving biological tool kit for cancer biology and oncology. **npj Precision Oncology**, v. 3, n. 1, p. 8, 18 dez. 2019.

TO, K. K.-W. et al. Consistent Detection of 2019 Novel Coronavirus in Saliva. **Clinical Infectious Diseases**, v. 71, n. 15, p. 841–843, 28 jul. 2020.

TO, K. K.-W. et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. **The Lancet. Infectious Diseases**, v. 20, n. 5, p. 565, 1 maio 2020.

VANDENBERG, O. et al. Considerations for diagnostic COVID-19 tests. **Nature Reviews Microbiology**, v. 19, n. 3, p. 171–183, 14 mar. 2021.

VOGELS, C. B. F. et al. Analytical sensitivity and efficiency comparisons of SARS-CoV- 2 RT–qPCR primer–probe sets. **Nature Microbiology 2020 5:10**, v. 5, n. 10, p. 1299–1305, 10 jul. 2020.

WANG, W.-K. et al. Detection of SARS-associated Coronavirus in Throat Wash andSaliva in Early Diagnosis. **Emerging Infectious Diseases**, v. 10, n. 7, p. 1213, 2004.

WHO. Syndrome coronavirus (MERS-CoV). 7 April 2022. Acesso em 20 de Abril de 2022. Disponivel em: https://www.who.int/emergencies/disease-outbreak-news/item/middle- east-respiratory-syndrome-coronavirus-(mers-cov)-saudi-arabia-2022.

WILLIAMS, E. et al. Detection of SARS-CoV-2 in saliva: implications for specimen

transport and storage. **Journal of Medical Microbiology**, v. 70, n. 2, 1 fev. 2021.

WIT, E. DE et al. SARS and MERS: recent insights into emerging coronaviruses. **Nature Reviews. Microbiology**, v. 14, n. 8, p. 523, 1 ago. 2016.

WÖLFEL, R. et al. Virological assessment of hospitalized patients with COVID-2019. **Nature 2020 581:7809**, v. 581, n. 7809, p. 465–469, 1 abr. 2020.

WONG, S. C. Y. et al. Posterior Oropharyngeal Saliva for the Detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). **Clinical Infectious Diseases**, v. 71, n. 11, p. 2939–2946, 31 dez. 2020.

WOO, P. C. Y. et al. Discovery of Seven Novel Mammalian and Avian Coronaviruses in the Genus Deltacoronavirus Supports Bat Coronaviruses as the Gene Source of Alphacoronavirus and Betacoronavirus and Avian Coronaviruses as the Gene Source of Gammacoronavirus and Deltacoronavirus. **Journal of Virology**, v. 86, n. 7, p. 3995, 1 abr. 2012.

WRAPP, D. et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. **Science (New York, N.y.)**, v. 367, n. 6483, p. 1260, 13 mar. 2020.

WYLLIE, A. L. et al. Saliva or Nasopharyngeal Swab Specimens for Detection of SARS-CoV-2. **New England Journal of Medicine**, v. 383, n. 13, p. 1283–1286, 24 set. 2020.

XU, J. et al. Salivary Glands: Potential Reservoirs for COVID-19 Asymptomatic Infection. **Journal of Dental Research**, v. 99, n. 8, p. 989, 1 jul. 2020.

Y, Z. et al. Network-based drug repurposing for novel coronavirus 2019-nCoV/SARS- CoV-2. **Cell discovery**, v. 6, n. 1, 1 dez. 2020.

ZAKI, A. M. et al. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. **N Engl J Med**, v. 367, n. 19, p. 1814–1820, 7 nov. 2012.

ZETSCHE, B. et al. Cpf1 is a single RNA-guided endonuclease of a Class 2 CRISPR- Cas system. **Cell**, v. 163, n. 3, p. 759, 22 out. 2015.

ZHANG, R. et al. The ORF4a protein of human coronavirus 229E functions as a viroporin that regulates viral production. **Biochimica et Biophysica Acta. Biomembranes**, v.1838, n. 4, p. 1088, 2014.

ZHANG, X.-H. et al. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. **Molecular Therapy. Nucleic Acids**, v. 4, n. 11, p. e264, 1 nov. 2015.

ZHOU, P. et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. **Nature**, v. 579, n. 7798, p. 270, 12 mar. 2020.

ZHU, H.; WEI, L.; NIU, P. The novel coronavirus outbreak in Wuhan, China. **Global Health Research and Policy**, v. 5, n. 1, 1 dez. 2020.

ZHU, N. et al. Brief Report: A Novel Coronavirus from Patients with Pneumonia in China, 2019. **The New England Journal of Medicine**, v. 382, n. 8, p. 727, 20 fev. 2020.

Apêndices

Genomic Evidence of SARS-CoV-2 Reinfection Involving E484K Spike Mutation, Brazil

Carolina Kymie Vasques Nonaka, Marília Miranda Franco, Tiago Gräf, Camila Araújo de Lorenzo Barcia, Renata Naves de Ávila Mendonça, Karoline Almeida Felix de Sousa, Leila Machado Costa Neiva, Vagner Fosenca, Ana Verena Almeida Mendes, Renato Santana de Aguiar, Marta Giovanetti, Bruno Solano de Freitas Souza

Author affiliations: D'Or Institute for Research and Education (IDOR), São Rafael Hospital Center for Biotechnology and Cell Therapy, Salvador, Brazil (C.K. Vasques Nonaka, B. Solano de Freitas Souza); São Rafael Hospital Department of Infectology, Salvador (M.M. Franco, C. Araújo de Lorenzo Barcia, R. Naves de Ávila Mendonça, K. Almeida Felix de Sousa, A. Verena Almeida Mendes); Fundação Oswaldo Cruz, Gonçalo Moniz Institute, Salvador (T. Gräf); Coordenação Geral dos Laboratórios de Saúde Pública/Ministério da Saúde, Brasília, Brazil (V. Fosenca); University of KwaZulu-Natal College of Health Sciences, Durban, South Africa (V. Fosenca); Universidade Federal de Minas Gerais Instituto de Ciências Biológicas, Belo Horizonte, Brazil (V. Fosenca, R.S. de Aguiar, M. Giovanetti); Fundação Oswaldo Cruz Instituto Oswaldo Cruz, Rio de Janeiro (M. Giovanetti); University Campus Bio-Medico of Rome Unit of Medical Statistics and Molecular Epidemiology, Rome, Italy (M. Giovanetti)

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Uncertainty remains about how long the protective immune responses against severe acute respiratory syndrome coronavirus 2 persists, and suspected reinfection in recovered patients has been reported. We describe a case of reinfection from distinct virus lineages in Brazil harboring the E484K mutation, a variant associated with escape from neutralizing antibodies.

Viral evolution might favor reinfections (*1*), and the recently described spike mutations, particularly in the receptor binding domain in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) lineages circulating in the United Kingdom, South Africa, and most recently in Brazil (A. Rambaut et al., unpub. data, https://virological.org/t/preliminary-genomic-characterisation-of-an-emergent-sars-cov-2-lineage-in-the-uk-defined-by-a-novel-set-of-spike-mutations/563; H. Tegally et al., unpub. data, https://doi.org/10.1101/2020.12.21.20248640 ; C.M. Voloch et al., unpub. data, https://doi.org/10.1101/2020.12.23.20248598), have raised concern on their potential impact in infectivity, immune escape, and reinfection. We report a case of reinfection from distinct SARS-CoV-2 lineages in Brazil harboring the E484K mutation, a variant associated with escape from neutralizing antibodies (*2*; A.J. Greaney, unpub. data, https://doi.org/10.1101/2020.12.31.425021; Z. Liu, unpub. data, https://doi.org/10.1101/2020.11.06.372037).

A 45-year-old woman residing in Salvador (Bahia State, northeast Brazil) with no underlying conditions had symptoms of viral infection on 2 occasions (May 26, 2020, and October 26, 2020). In the first episode, the patient had diarrhea, myalgia, asthenia, and odynophagia for ≈7 days. She took 40 mg prednisone for 5 days and returned to normal activities 21 days later, after resolution of symptoms without sequelae or complaints. In the second episode, which was symptomatically more severe in terms of intensity and duration, the patient had headache, malaise, diarrhea, cough, and sore throat that evolved to myalgia and ageusia, muscle fatigue, insomnia, mild dyspnea on exertion, and shortness of breath. In both episodes, however, disease was classified as mild, and she was treated at home, not requiring hospitalization.

The patient was a healthcare executive. Identified workplace exposure included frequent meetings with coronavirus disease (COVID-19) frontline physicians and healthcare teams. Also, before the second episode, she attended a meeting with a group of physicians, one of whom had COVID-19 diagnosed in the days following.

On both occasions, viral RNA was extracted from nasopharyngeal swab specimens and tested for SARS-CoV-2 by multiplex real-time reverse transcription PCR (rRT-PCR) Allplex SARS-CoV-2 assay (Seegene, https://www.seegene.com). Both times, results of rRT-PCR tests targeting 3 genes (N, E, and RdRp) were positive for SARS-CoV-2 (Figure, panel A). Cycle threshold values of N, E, and RdRp targets were 25, 26, and 27 in the first episode and 21, 12, and 17 in the second episode, respectively. In the second episode, the patient had a high viral load (presumed because of low cycle threshold values detected). Four weeks after the patient tested positive by rRT-PCR in the second episode, an IgG test against S1 protein by chemiluminescence (VITROS, Ortho Clinical Diagnostics, https://www.orthoclinicaldiagnostics.com) yielded a positive result. We then sequenced swab specimens by using PGM Ion Torrent (ThermoFisher, https://www.thermofisher.com), according to the manufacturer's instructions. A total of 1,405,009 mapped reads for sample A (from the

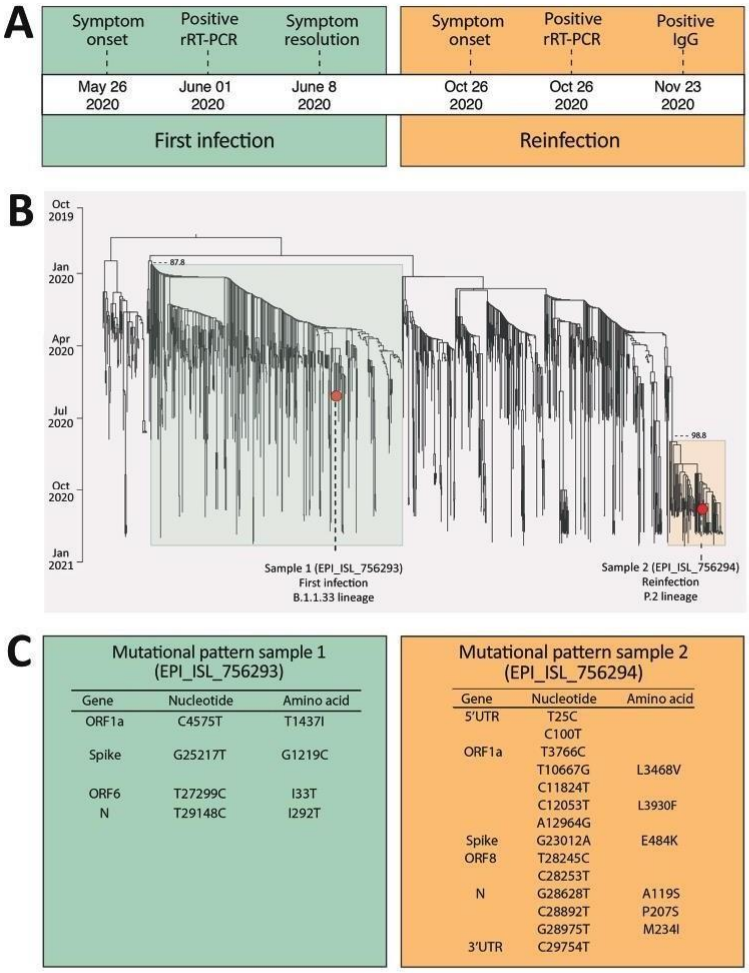

Figure. Molecular characterization of a severe acute respiratory syndrome coronavirus 2 reinfection case in Salvador, Bahia State, northeast Brazil. A) Timeline of symptom onset and molecular and serologic diagnosis. B) Time-scaled maximum-likelihood tree, including the new genomes (GISAID accession nos. EPI_ISL_756293 and EPI_ISL_756294; https://www.gisaid.org) recovered from a 45-year-old woman residing in Salvador and full-length viral genomes from Brazil available through GISAID as of January 14, 2021 (Appendix Table, https://wwwnc.cdc.gov/EID/article/27/5/21-0191-App1.xlsx). New genomes are highlighted with red circles. Branch support (SH-aLTR >0.8) is shown at key nodes. C) Mutational pattern of the 2 isolates obtained from the same patient within a 147-day interval. Only unique mutations and lineage defining mutations for B.1.1.33 and P.2 are shown. ORF, open reading frame; rRT-PCR, real-time reverse transcription PCR; UTR, untranslated region.

first episode) and 2,570,182 reads for sample B (from the second episode) were obtained, resulting in a sequencing mean depth >1,000× for both samples and a coverage of >99%.

We further assessed the distinct viral origin of the 2 infections by phylogenetic inference, comparing the 2 new isolates (GISAID accession nos. EPI_ISL_756293 and EPI_ISL_756294; https://www.gisaid.org) with all SARS-CoV-2 genomes from Brazil available through GISAID as of January 14, 2021. Only genomes >29,000 bp and <1% of ambiguities were retrieved (n = 1,164). Sequences were aligned by using MAFFT (*3*) and submitted to IQ-TREE for maximum-likelihood phylogenetic analysis (*4*). We inferred time-scaled trees by using TreeTime (*5*).

Comparison of the phylogenetic profiles of the 2 new sequences with contemporaneous sequences from Brazil (Appendix Table, https://wwwnc.cdc.gov/EID/article/27/5/21-0191-App1.xlsx) clearly demonstrated that the 2 COVID-19 episodes, separated by a 147-day interval, were indeed caused by different SARS-CoV-2 lineages, confirming reinfection (Figure, panel B). In the first episode, the lineage B.1.1.33 was detected, whereas lineage P.2 (an alias for B.1.1.28.2) was detected in the second infection (Figure, panel B), according to the Pangolin lineage

classification (https://github.com/hCoV-2019/pangolin [accessed 2021 Jan 11]). Further, we identified several mutations distinguishing the 2 genomes (Figure, panel C), 2 of which were in the SARS-CoV-2 spike glycoprotein. In the first infection, the retrieved genome had the S:G1219C mutation, whereas the mutation S:E484K was observed in the second infection.

This reinfection case aligns with another reinfection recently described in Brazil in which a first infection with the B.1.1.33 lineage was followed by a second one with the P.2 lineage (P.C. Resende et al., unpub. data, https://virological.org/t/spike-e484k-mutation-in-the-first-sars-cov-2-reinfection-case-confirmed-in-brazil-2020/584). The E484K mutation, located in the viral receptor binding domain, has been emerging independently in several SARS-CoV-2 variants, and its monitoring is of pivotal importance in the current stage of the pandemic. At least 3 main lineages harbor E484K: B.1.351, first identified in South Africa and widespread worldwide (H. Tegally et al.); P.1, recently described in Manaus, Brazil, which harbors a constellation of new mutations (including N501Y) (N.R. Faria et al., unpub. data, https://virological.org/t/genomic-characterisation-of-an-emergent-sars-cov-2-lineage-in-manaus-preliminary-findings/586); and P.2, also described in Brazil (C.M. Voloch et al.) and already detected in the United Kingdom, United States, Canada, and Argentina (https://cov-lineages.org/lineages.html). Our report of SARS-CoV-2 reinfection with a E484K variant corroborates in vitro and in silico studies that estimated the potential of lineages carrying this mutation to escape from neutralizing antibodies (3; Z. Liu et al.) and highlights the importance of genomic surveillance to detect and monitor the emergence of new viral linages with possible implications for public health policies and immunization strategies.

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This research was approved by the São Rafael Hospital Ethics Review Committee (approval no. 41528620.1.0000.0048).

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Author contributions include conception and design: C.K.V.N., A.V.A.M., R.S.A., B.S.F.S.; investigations: C.K.V.N., M.M.F., T.G., C.A.L.B., R.N.A.M., K.A.F.S., A.V.A.M., R.S.A., M.G., and BSFS; data curation: C.K.V.N., T.G., M.G., and B.S.F.S.; formal analysis: C.K.V.N., T.G., M.G., V.F., and B.S.F.S.; manuscript writing: C.K.V.N., T.G., M.G., and B.S.F.S.; manuscript revision: C.K.V.N., M.M.F., T.G., A.V.A.M., R.S.A., M.G., and B.S.F.S.; and resources: B.S.F.S. and R.S.A.

About the Author

Dr. Nonaka is a researcher at the Biotechnology and Cell Therapy Centre of São Rafael Hospital, Salvador, Brazil. Her research focuses on the implementation of next-generation sequencing techniques to track the spread of emerging and reemerging viral pathogens and to identify novel disease biomarkers.

References

1. Tillett RL, Sevinsky JR, Hartley PD, Kerwin H, Crawford N, Gorzalski A, et al. Genomic evidence for reinfection with SARS-CoV-2: a case study. Lancet Infect Dis. 2021;21:52–8. https://doi.org/10.1016/S1473-3099(20)30764-7
2. Weisblum Y, Schmidt F, Zhang F, DaSilva J, Poston D, Lorenzi JC, et al. Escape from neutralizing antibodies by SARS-CoV-2 spike protein variants. eLife. 2020;9:3–10. https://doi.org/10.7554/eLife.61312
3. Nakamura T, Yamada KD, Tomii K, Katoh K. Parallelization of MAFFT for large-scale multiple sequence alignments. Bioinformatics. 2018;34:2490–2. https://doi.org/10.1093/bioinformatics/bty121
4. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015;32:268–74. https://doi.org/10.1093/molbev/msu300
5. Sagulenko P, Puller V, Neher RA. TreeTime: Maximum-likelihood phylodynamic analysis. Virus Evol. 2018;4:vex042. https://doi.org/10.1093/ve/vex042

Address for correspondence: Bruno S.F. Souza, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Candeal, Salvador, Bahia, CEP 40296-710, Brazil; email: bruno.solano@fiocruz.br; or Marta Giovanetti, Reference Laboratory of Flavivirus, Oswaldo Cruz Institute (Fundação Oswaldo Cruz–RJ), Av. Brasil, 4365, Manguinhos, Rio de Janeiro, RJ, CEP 21040-360, Brazil; email: giovanetti.marta@gmail.com

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Case Report

Early detection of P.1 variant of SARS-CoV-2 in a cluster of cases in Salvador, Brazil

Isadora Cristina de Siqueira[a,*], Aquiles Assunção Camelier[b,c,d], Elves A.P. Maciel[b], Carolina Kymie Vasques Nonaka[e,f], Margarida Celia L.C. Neves[b,g], Yasmin Santos Freitas Macêdo[a], Karoline Almeida Félix de Sousa[a], Victor Costa Araujo[d], Aurea Angelica Paste[b,g], Bruno Solano de Freitas Souza[a,e,f], Tiago Gräf[a]

[a] Instituto Gonçalo Moniz, Fiocruz, Salvador, Brazil
[b] Hospital Aliança D'Or, Salvador, Brazil
[c] Programa de Pós-graduação em Medicina e Saúde Pública — Escola Bahiana de Medicina e Saúde Pública, Salvador, Brazil
[d] Universidade do Estado da Bahia (Uneb), Salvador, Brazil
[e] Centro de Biotecnologia e Terapia Celular, Hospital São Rafael, Salvador, Brazil
[f] Instituto D'Or de Pesquisa e Ensino (IDOR), Salvador, Brazil
[g] Faculdade de Medicina da Bahia — Universidade Federal da Bahia, Salvador, Brazil

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ABSTRACT

We report 3 cases of severe COVID-19 due to the SARS-CoV-2 P.1 lineage in a familial cluster detected in Salvador, Bahia–Brazil. All cases were linked to travel by family members from the state of Amazonas to Bahia in late December 2020. This report indicates the cryptic transmission of the SARS-CoV-2 P.1 lineage across Brazil and highlights the importance of genomic surveillance to track the emergence of new SARS-CoV-2 variants of concern.

Introduction

The SARS-CoV-2 virus, which causes COVID-19, reportedly emerged in December 2019 in Wuhan, China (Zhu et al., 2020). SARS-CoV-2 rapidly spread around the world, and on March 11, 2020, the World Health Organization (WHO) declared the outbreak as a pandemic (WHO/Europe, 2020). More than one year since its emergence, with more than 153 954 491 reported cases and 3 221 052 deaths (WHO, 2021), the COVID-19 pandemic continues to impact countries worldwide. In addition, in late 2020, new SARS-CoV-2 Variants of Concern (VOC) were identified, including the 501Y.V1 (B.1.1.7 lineage) reported in the United Kingdom (Public Health England, 2020) and 501Y.V2 (B.1.351 lineage) first detected in South Africa (Tegally et al., 2021).

In early January 2021, another SARS-CoV-2 VOC, termed P.1 (or 20J/501Y.V3), was first identified (Fujino et al., 2021). This variant is believed to have emerged in early December in Manaus in northern Brazil and has been associated with increased transmissibility and high viral load (Faria et al., 2021; Naveca et al., 2021a).

Herein, we report and characterize 3 cases of severe COVID-19 linked to the SARS-CoV-2 P.1 lineage detected in Salvador, Bahia, northeast Brazil, part of a familial cluster of infections resulting from travel between the states of Amazonas and Bahia in late December 2020.

Case series

Three members of the same family, residents of Manaus (Amazonas, Brazil), traveled to Salvador (Bahia, Brazil) on December 19, 2020, to visit 2 relatives for the holidays. Table 1 lists the clinical characteristics and laboratory results from all 5 cases of infection.

Case 1

On December 23, 2020, a 40-year-old female with no comorbidities began to experience myalgia and nasal obstruction lasting for 3 days and then promptly recovered.

* Corresponding author at: Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, R. Waldemar Falcão, 121, Candeal, Salvador, BA CEP 40296-710, Brazil.
E-mail address: isadora.siqueira@fiocruz.br (I.C. de Siqueira).

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Table 1
Demographic and clinical characteristics of 5 cases of COVID-19 linked to the P.1 variant of SARS-CoV-2.

	Case 1	Case 2	Case 3	Case 4	Case 5
Age (years)	40	19	69	41	71
Sex	Female	Male	Male	Male	Female
Place of residence	Manaus	Manaus	Salvador	Manaus	Salvador
RT-PCR for SARS-CoV-2	Detectable	Detectable	Detectable	Detectable	Detectable
SARS-CoV-2 sequencing	ND	ND	P.1 lineage	P.1 lineage	P.1 lineage
Onset of symptoms	23/12/2020	27/12/2020	25/12/2020	27/12/2020	25/12/2020
Comorbidities	None	None	SAH	None	COPD
Hospital admission	No	No	Yes	Yes	Yes
SpO_2 at admission	ND	ND	92%	90%	80%
% lung involvement on Thorax HRCT	ND	ND	50%–75%	50%	50%–75%
D-dimer at admission (ng/mL)	ND	ND	1054	1038	755
C reactive protein at admission (mg/L)	ND	ND	17.8	5.3	14.7
ICU admission	No	No	Yes	Yes	Yes
Respiratory support	No	No	HFNC	HFNC	Mechanical ventilation
Days in ICU	0	0	12	11	65
Days in hospital	0	0	19	21	65
Outcome	Recovery	Recovery	Recovery	Recovery	Death

ND = not done; SpO_2 = oxygen saturation (breathing room air); HRCT = high resolution computed tomography; SAH = systemic arterial hypertension; COPD = chronic obstructive pulmonary disease; HFNC = high-flow nasal cannula; RT-PCR = real-time polymerase chain reaction; ICU = intensive care unit.

Case 2

On December 27, 2020, a previously healthy 19-year-old male reported fever and myalgia and recovered after 3 days of mild symptoms.

Case 3

On December 25, 2020, a 69-year-old male with systemic arterial hypertension and dyslipidemia began to experience fever, chills, headache, and myalgia. After 10 days of symptoms and the onset of dyspnea, he was admitted to the emergency service of a local hospital. His pulse oxygen saturation (SpO_2) level was 92% when breathing room air, and high-resolution computed tomography (HRCT) of the thorax revealed 50%–75% of bilateral ground-glass pulmonary opacities. The day after admission, the patient was transferred to the hospital's intensive care unit (ICU) for oxygen support by high-flow nasal cannula (HFNC). He remained in the ICU for 12 days and was discharged after 19 days. Methylprednisolone was administered during his hospital stay.

Case 4

On December 27, 2020, a 41-year-old male reported myalgia and fever. After 9 days of persistent fever and the onset of dyspnea, he was admitted to the same hospital for oxygen support. His SpO_2 was 90% when breathing room air, with an arterial oxygen pressure (pO_2) of 60.5 mmHg. Thorax HRCT revealed 50% of bilateral ground-glass pulmonary opacities. After 3 days, he was transferred to the ICU due to worsening respiratory symptoms. During 11 days in the ICU, the patient required oxygen delivery by HFNC. Methylprednisolone was administered, and the patient was discharged after 21 days.

Case 5

On December 25, 2020, a 71-year-old female with chronic obstructive pulmonary disease and dyslipidemia experienced fever, odynophagia and diarrhea. After 7 days, worsening dyspnea prompted her to seek medical attention. Upon arrival at the same hospital, she was admitted to the ICU due to severe respiratory distress. Her SpO_2 was 80% when breathing room air, and thorax HRCT revealed 50%–75% of bilateral ground-glass pulmonary opacities. The patient initially received non-invasive oxygen support with HFNC, but due to worsening respiratory distress, endotracheal intubation and invasive mechanical ventilation support for moderate acute respiratory distress syndrome were required. Methylprednisolone was administered upon admission. Her course became complicated by nosocomial infection, persistent respiratory failure, and multiple organ failure, culminating in death after 65 days in the ICU.

SARS-CoV-2 diagnosis, sequencing, and lineage designation

All 5 cases reported herein tested positive for SARS-CoV-2 by real-time polymerase chain reaction (RT-PCR). Nasopharyngeal swab samples were tested by multiplex RT-PCR using the Allplex SARS-CoV-2 assay (Seegene Inc., Seoul, Korea) on an ABI7500 Fast real-time PCR instrument (Thermo Fisher Scientific). SARS-CoV-2 viral genome sequencing was run on a PGM Ion System; reads were analyzed using Torrent Suite Software v.5.12.1 (Thermo Fisher Scientific).

High-quality SARS-CoV-2 genome sequences were generated for Cases 3–5 and submitted to the online pangolin tool for lineage classification (Rambaut et al., 2020). All 3 sequences were classified as the P.1 lineage and are available at the EpiCov database, maintained by the GISAID initiative, with accession codes EPI_ISL_1443197, EPI_ISL_1443196 and EPI_ISL_1443198. A phylogenetic tree was constructed considering the available P.1 diversity. All high-quality Brazilian P.1 sequences (>29,000 bp and <1% Ns) were retrieved from GISAID on March 25, 2021. Alignment was performed using the MAFFT sequence alignment program, and the phylogenetic tree was inferred using the IQ-TREE software package with Maximum Likelihood (ML) analysis, as previously described (Naveca et al., 2021a). This approach resulted in a tree trunk with very low branch support, reflecting the rapid spread of P.1 and low genetic genome diversity in this early phase of dissemination (Figure 1). The majority of the obtained sequences branching out from the tree trunk had been isolated in the Amazonas region or were cases of individuals with a history of travel to the region. Cases 3–5 clustered within the obtained sequences, indicating that these originated from the early diversity of P.1 prior to the further diversification that can be observed in more recently sampled genomes.

Discussion

The emergence of SARS-CoV-2 VOCs in different parts of the world presents new challenges to COVID-19 pandemic control.

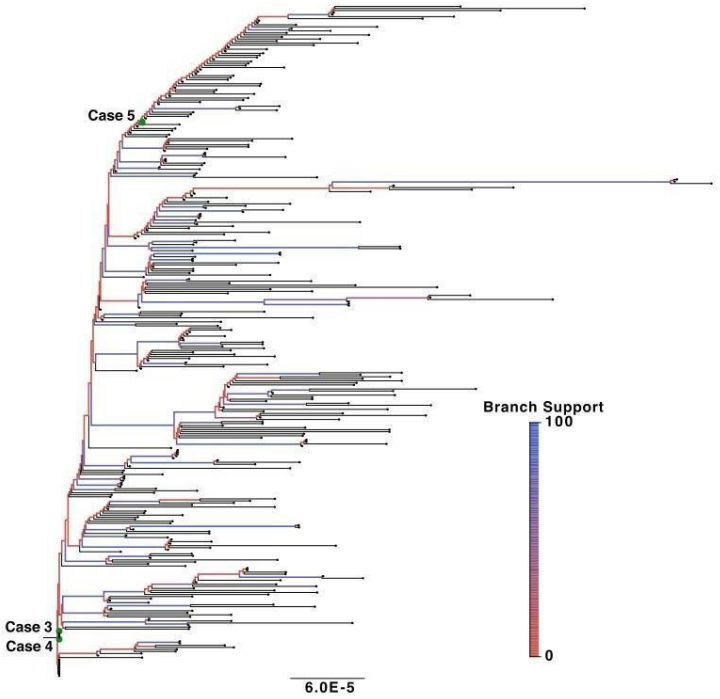

Figure 1. Maximum likelihood phylogenetic tree detailing the P.1 lineage in Brazil.

Increased transmissibility (Tegally et al., 2021) and mortality (Davies et al., 2021) have been associated with VOCs, and cases of reinfection have also been reported (Naveca et al., 2021b). The capacity to decrease the efficacy of currently available vaccines (Madhi et al., 2021) represents an additional threat.

With COVID-19 incidence of 7104 cases/100 000 individuals, corresponding to a mortality rate of 197 cases/100 000 individuals, Brazil is currently facing a critical phase of the pandemic, with alarming increases in cases and deaths notified across all regions of the country (https://covid.saude.gov.br/, accessed on May 5, 2021). One underlying cause behind the surge in case numbers is the emergence of SARS-CoV-2 lineage P.1, which has already been detected in all regions of Brazil (http://www.genomahcov.fiocruz.br, accessed on April 21, 2021).

The 3 cases of P.1 lineage identified herein exemplify the cryptic transmission of this VOC throughout Brazil before it had been identified. P.1 could have been introduced into other Brazilian states due to holiday-associated travel at the end of 2020 and early 2021. The cases reported here predate 11 other cases identified in the state of Bahia in mid-January, all of which were associated with travel from/to Manaus (Tosta et al., 2021). For instance, in the Southern Brazilian region, community transmission of P.1 was detected in January 2021 (Martins et al., 2021), and the first local P.1 case was identified in a popular Brazilian tourist destination (Salvato et al., 2021).

In conclusion, the present report details a familial cluster of COVID-19 linked to the SARS-CoV-2 P.1 lineage. Notably, 3/5 reported cases evolved as severe COVID-19, entailing long ICU stays with 1 associated death. One patient, a 41-year-old male, had no prior risk factors that would predispose him to severe disease. On the basis of the alarming surge in COVID-19 deaths recently reported in Brazil, it has been assumed that the P.1 lineage could imply an increased risk of severe infection or higher mortality. This association is speculative; further studies are urgently needed to assess this assumption more comprehensively.

The present report further supports the cryptic transmission of the SARS-CoV-2 P.1 lineage across Brazil, highlighting the importance of genomic surveillance to track the emergence of new viral variants. Coordinated implementation of non-pharmaceutical interventions, including social distance, airport screening and quarantine for travelers, are pivotal tools to slow down viral transmission and diminish the burden on the national public health system.

Three newly obtained genomic sequences were analyzed together with 327 P.1 background genomes retrieved from GISAID. Internal branches are colored according to support (SH-like aLRT), and sequences from the reported cases are highlighted with green circles. The tree was rooted in the branch connecting to the earliest sampled genome.

Ethical approval

This study was approved by National Commission for Ethics and Research (3.980.128/2020). All participants provided written informed consent.

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Conflict of interest

A.A.C. works as a part-time Internal Expert Consultant at GSK-Brazil, and has no conflicts of interest with regard to the present publication.

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The authors are grateful to the physicians and nurses involved in the patients' clinical care, the GISAID team, and all researchers who submitted sequences to the EpiCoV database. The GISAID table containing detailed data on the sequences used in this study and the associated researchers is included as Appendix Table 1. The authors also thank Andris K. Walter for English language revision and manuscript copy editing assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2021.05.010.

References

Davies NG, Jarvis CI, CMMID COVID-19 Working Group, Edmunds WJ, Jewell NP, Diaz-Ordaz K, et al. Increased mortality in community-tested cases of SARS-CoV-2 lineage B.1.1.7. Nature 2021;593(May (7858))270–4, doi:http://dx.doi.org/10.1038/s41586-021-03426-1 Epub 2021 March 15. PMID: 33723411.

Faria NR, Mellan TA, Whittaker C, Claro IM, Candido DDS, Mishra S, et al. Genomics and epidemiology of a novel SARS-CoV-2 lineage in Manaus, Brazil. medRxiv 2021;, doi:http://dx.doi.org/10.1101/2021.02.26.21252554 2021.02.26.21252554.

Fujino T, Nomoto H, Kutsuna S, Ujiie M, Suzuki T, Sato R, et al. Novel SARS-CoV-2 variant identified in travelers from Brazil to Japan. Emerg Infect Dis 2021;27(April (4))1243–5, doi:http://dx.doi.org/10.3201/eid2704.210138 Epub 2021 February 10. PMID: 33567247; PMCID: PMC8007308.

Madhi SA, Baillie V, Cutland CL, Voysey M, Koen AL, Fairlie L, et al. Efficacy of the ChAdOx1 nCoV-19 Covid-19 vaccine against the B.1.351 variant. N Engl J Med 2021;384(May (20))1885–98, doi:http://dx.doi.org/10.1056/NEJMoa2102214 Epub 2021 March 16. PMID: 33725432; PMCID: PMC7993410.

Martins AF, Zavascki AP, Wink PL, Volpato FCZ, Monteiro FL, Rosset C, et al. Detection of SARS-CoV-2 lineage P.1 in patients from a region with exponentially increasing hospitalisation rate, February 2021, Rio Grande do Sul, Southern Brazil. Euro Surveill 2021;26(March (12))2100276, doi:http://dx.doi.org/10.2807/1560-7917.ES.2021.26.12.2100276 PMID: 33769251; PMCID: PMC7995561.

Naveca FG, Nascimento V, de Souza VC, Corado AL, Nascimento F, Silva G, et al. COVID-19 in Amazonas, Brazil, was driven by the persistence of endemic lineages and P .1 emergence. Nat Med 2021a;(May (25)), doi:http://dx.doi.org/10.1038/s41591-021-01378-7 Epub ahead of print. PMID: 34035535.

Naveca F, da Costa C, Nascimento V, Souza V, Corado A, Nascimento F, et al. Three SARS-CoV-2 reinfection cases by the new Variant of Concern (VOC) P.1/501Y.V3. 2021 15 March 2021. PREPRINT (Version 1) available at Research Square [https://doi.org/10.21203/rs.3.rs-318392/v1].

Public Health England. Investigation of novel SARS-COV-2 variant: variant of concern 202012/01. 2020. www.gov.uk/government/publications/investigation-of-novel-sars-cov-2-variant-variant-of-concern-20201201.

Rambaut A, Holmes EC, O'Toole Á, Hill V, McCrone JT, Ruis C, et al. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. Nat Microbiol 2020;5(November (11))1403–7, doi:http://dx.doi.org/10.1038/s41564-020-0770-5 Epub 2020 July 15. PMID: 32669681; PMCID: PMC7610519.

Salvato RS, Gregianini TS, Campos AAS, Crescente LV, Vallandro MJ, Ranieri TMS, et al. Epidemiological investigation reveals local transmission of SARS-CoV-2 lineage P.1 in Southern Brazil. 2021 March 2 [cited 2021 Apr 21]; Available from: https://doi.org/10.21203/rs.3.rs-280297/v1.

Tegally H, Wilkinson E, Giovanetti M, Iranzadeh A, Fonseca V, Giandhari J, et al. Detection of a SARS-CoV-2 variant of concern in South Africa. Nature 2021;592(April (7854))438–43, doi:http://dx.doi.org/10.1038/s41586-021-03402-9 Epub 2021 March 9. PMID: 33690265.

Tosta S, Giovanetti M, Nardy VB, da Silva LRO, Gómez MKA, Lima JG, et al. Early genomic detection of SARS-CoV-2 P.1 variant in Northeast Brazil. medRxiv 2021;, doi:http://dx.doi.org/10.1101/2021.02.25.21252490 2021.02.25.21252490.

WHO/Europe. Coronavirus disease (COVID-19) outbreak — WHO announces COVID-19 outbreak a pandemic. 2020. . [13 March 2021] https://www.euro.who.int/en/health-topics/health-emergencies/coronavirus-covid-19/news/news/2020/3/who-announces-covid-19-outbreak-a-pandemic.

World Health Organization. COVID-19 dashboard. [Accessed 5 May 2021]. https://covid19.who.int.

Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 2020;382(February (8))727–33, doi:http://dx.doi.org/10.1056/NEJMoa2001017 Epub 2020 January 24. PMID: 31978945; PMCID: PMC7092803.

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SARS-CoV-2 variant of concern P.1 (Gamma) infection in young and middle-aged patients admitted to the intensive care units of a single hospital in Salvador, Northeast Brazil, February 2021

Carolina Kymie Vasques Nonaka[a,b], Tiago Gräf[c], Camila Araújo de Lorenzo Barcia[d], Vanessa Ferreira Costa, Department of Quality Management[a], Janderson Lopes de Oliveira, Information technology department[a], Rogério da Hora Passos[e], Iasmin Nogueira Bastos[c], Maria Clara Brito de Santana[c], Ian Marinho Santos[a,c], Karoline Almeida Felix de Sousa[c], Thamires Gomes Lopes Weber[f], Isadora Cristina de Siqueira[c], Clarissa Araújo Gurgel Rocha[a,b,c], Ana Verena Almeida Mendes[d], Bruno Solano de Freitas Souza[a,b,c,*]

[a] Center for Biotechnology and Cell Therapy, São Rafael Hospital, Salvador, Brazil
[b] D'Or Institute for Research and Education (IDOR), Salvador, Brazil
[c] Gonçalo Moniz Institute, FIOCRUZ, Salvador, Brazil
[d] Department of Infectology, São Rafael Hospital, Salvador, Brazil
[e] Intensive Care Unit, São Rafael Hospital, Salvador, Brazil
[f] Clinical Laboratory, São Rafael Hospital, Salvador, Brazil

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ABSTRACT

Objectives: To evaluate changes in the characteristics of patients with coronavirus disease 2019 (COVID-19) after the emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variant of concern (VOC) P.1 (Gamma), by comparing the clinical, demographic, and laboratory profiles of patients hospitalized during the first (May to July 2020) and second (December 2020 to February 2021) pandemic waves.

Methods: Data were collected from the records of COVID-19 patients ($n = 4164$) admitted to a single hospital in Salvador, Northeast Brazil. SARS-CoV-2 genome sequencing was performed on nasopharyngeal swab samples from 12 patients aged <60 years admitted to the intensive care unit (ICU) in February 2021.

Results: Between June 2020 and February 2021, the median age of patients admitted to the ICU decreased from 66 to 58 years ($P < 0.05$). This was accompanied by an increased proportion of patients without comorbidities (15.32% vs 32.20%, $P < 0.0001$). A significant reduction in the cycle threshold values of SARS-CoV-2 RT-PCR tests was observed in the second wave ($P < 0.0001$). Sequencing analysis detected lineage Gamma in all 12 ICU patients sampled in February 2021.

Conclusions: The results of this study demonstrated an increased proportion of younger adults without comorbidities with severe disease during the second COVID-19 wave, shortly after the confirmation of local Gamma circulation.

1. Introduction

There is concern regarding the increased infectivity and possible immune escape of new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants (Chakraborty et al., 2021). Ac-

* Corresponding author: Bruno S. F. Souza, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Salvador, CEP: 40296-710, Brazil. Tel: +55 7131762260. Fax: +55 7131762272.
E-mail address: bruno.solano@fiocruz.br (B.S.d.F. Souza).

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cording to the World Health Organization (WHO), there are currently four variants of concern (VOC), all with key mutations in the receptor binding domain (RBD) of the spike protein (Korber et al., 2020). These are the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617) lineages.

In Brazil, two SARS-CoV-2 lineages – B.1.1.28 and B.1.1.33 – were predominant during the first surge of infections in the first 6 months of 2020 (Resende et al., 2021, Candido et al., 2020). The emerging Brazilian Gamma variant, derived from the B.1.1.28 lineage, began spreading rapidly later in 2020. Initially detected in the state of Amazonas, Gamma was responsible for a public health calamity in that state (Faria et al., 2021; Naveca et al., 2021; Sabino et al., 2021). The Gamma variant started to be detected at increasing rates from January 2021 onwards throughout Brazil, and became the predominant lineage associated with the second wave of infections (http://www.genomahcov.fiocruz.br). Following an acceleration of the transmission rates in 2021, Brazil became the epicenter of the coronavirus disease 2019 (COVID-19) pandemic, with over 13 million confirmed cases and 350 000 deaths (https://www.covid.saude.gov.br).

Gamma has 21 lineage-defining mutations, including 10 in the spike protein, three of them in the RBD (K417T, E484K and N501Y), showing a surprising convergence with the B.1.351 RBD. These three mutations in the RBD combined have been shown to increase the receptor binding affinity (Nelson et al., 2021). The mutations found in Gamma have been associated with increased transmissibility (Naveca et al., 2021), a higher viral load (Faria et al., 2021) and propensity for immune evasion (Dejnirattisai et al., 2021), and SARS-CoV-2 reinfection (Naveca et al., 2021).

Along with the dissemination of the Gamma variant, there have also been reports of an increased percentage of young patients developing severe disease (Dejnirattisai et al., 2021). However, many other questions remain unanswered, including a possible increased fatality rate, increased disease severity in people without known SARS-CoV-2 risk comorbidities, reduced time from symptom onsent to hospitalization.

The public health authorities in the state of Bahia (Northeast Brazil) confirmed the circulation of Gamma in the state in February, 2021. The first reports of Gamma detection in Salvador date from late December 2020 and early January 2021, and these were linked to individuals with a history of travel to Manaus (Tosta et al., 2021). This was followed by a rampant increase in the number of hospitalizations and deaths due to COVID-19 in Bahia in February and March 2021 (https://bi.saude.ba.gov.br/transparencia/). However, the clinical and demographic features of this epidemic second wave, as well as the association with SARS-CoV-2 lineages, still need to be characterized. Herein, we report the changes in the profile of patients admitted to the intensive care unit (ICU) of a private hospital in Salvador, Bahia capital city, in February 2021, due to COVID-19 with possible involvement of the locally circulating Gamma VOC.

2. Methods

2.1. Study design and procedures

A cross-sectional study was performed at São Rafael Hospital, a private general reference hospital in Salvador, Bahia, Northeast Brazil. Two time periods with increased numbers of hospital admissions were selected for data analysis, corresponding to the first wave (May to July 2020) and second wave (December 2020 to February 2021) of COVID-19 hospital admissions.

Data regarding the number of confirmed COVID-19 patients admitted to the hospital, along with their clinical and demographic characteristics, were obtained from the hospital health information system and electronic medical records (n = 4164). Data on confirmed COVID-19 cases in the city of Salvador and the state of Bahia were obtained from https://bi.saude.ba.gov.br.

The nasopharyngeal swabs were obtained and tested by multiplex real-time PCR Allplex SARS-CoV-2 assay (Seegene Inc., Seoul, Korea) in the laboratory of São Rafael Hospital, as part of routine diagnostic procedures. Cycle threshold (Ct) values were evaluated in positive samples from the peak months of each wave, June 2020 and February 2021 (n = 2360). Nucleic acid sequencing was performed of viral RNA extracted from 12 nasal swab samples selected from patients aged less than 60 years, who were admitted to the São Rafael Hospital ICU in February 2021. Written informed consent was obtained. The SARS-CoV-2 viral genome sequencing was performed in accordance with the manufacturer's instructions, using the Ion Torrent PGM system (Life Technologies, USA). cDNA was synthesized with the SuperScript VILO reverse transcriptase kit (Invitrogen, USA). The libraries were prepared using the Ion AmpliSeq SARS-CoV-2 assay panel, the Ion AmpliSeq Library Kit, and Ion Torrent PGM (Thermo Fisher Scientific, USA). Genomes were submitted to the Nextclade tool (https://clades.nextstrain.org) for quality control assessment and the Pangolin tool (https://pangolin.cog-uk.io/) for SARS-CoV-2 lineage assignment. Maximum likelihood (ML) phylogenetic analysis was performed in IQ-TREE for all detected Gamma variant genomes, and tree branch support was calculated with the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT), as described previously (Minh et al., 2020). Background high quality (>29 000 bp and <1% N) Brazilian Gamma variant sequences were obtained from the EpiCoV database in GISAID, as available on March 25, 2021 (**Supplementary Material** Table S1) and aligned with the genomes generated here in MAFFT (Katoh and Standley, 2013). The ML tree was visualized using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.2. Statistical analysis

Categorical variables were compared using the Fisher test. Continuous data were presented as the median and 95% confidence interval (CI). The Mann–Whitney test and Kruskal–Wallis test were used for comparisons of non-parametric data (Ct values and median age). P-values <0.05 were considered significant. Data were analyzed with Prism software v.9.1 (GraphPad).

3. Results

Accompanying the evolution of the pandemic in the city of Salvador, the data analysis demonstrated two waves of admission to the hospital due to COVID-19, the first during May to July 2020 and the second during December 2020 to February 2021. A similar pattern of cumulative confirmed COVID-19 cases reported by Bahia State, Salvador, and São Rafael Hospital in Salvador in the northeast of Brazil was observed (Figure 1A). There was a rapid increase in patients requiring hospitalization and ICU treatment in February 2021 (Figure 1B).

From May 2020 to February 2021, 2087 patients were admitted to the ICU of São Rafael Hospital; 672 were admitted during the first wave (May to July 2020) and 943 in the second wave (December 2020 to February 2021). The median age of the patients hospitalized and admitted to the ICU was lower in February 2021 (58 years) when compared to May and June 2020 (66 years), and this decrease in median age was found to be statistically significant (Figure 2A). A progressive increase in the percentage of patients aged <60 years being admitted to the ICU was observed in the second wave, differing from the demographic pattern observed in the first wave (Figure 2B).

Along with the increase in percentage of young and middle-aged patients, there was also an increase in the percentage of patients entering the ICU without known comorbidities (cardiovascu-

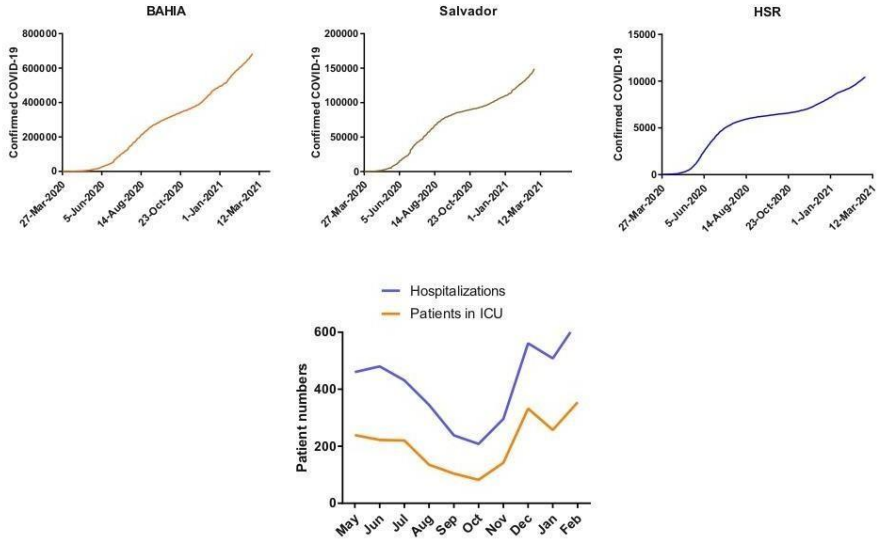

Figure 1. Confirmed COVID-19 hospitalizations. (A) Cumulative confirmed COVID-19 cases in Bahia State, Salvador, and patients admitted to the São Rafael Hospital (HSR) since May 2020. (B) The total number of patients hospitalized ($n = 4164$, blue line) and admitted to the ICU ($n = 2087$, orange line) from May 2020 to February 2021.

lar diseases, hypertension, diabetes, obesity, liver diseases, asthma, kidney diseases, and immunosuppression). Patients without comorbidities represented 32.20% of hospitalized COVID-19 patients admitted to the ICU due to COVID-19 in February 2021, compared to 15.32% in June 2020 ($P < 0.0001$). Table 1 shows the frequency of patients with different comorbidities stratified by age group.

In addition to the changes in clinical and demographic profile found, possible alterations in the pattern of the results obtained in the RT-qPCR analysis were also evaluated. A significant reduction in the median Ct values from nasopharyngeal swab samples analyzed by RT-qPCR was observed when comparing June 2020 to February 2021 ($P < 0.0001$) (Figure 3). During the same periods, similar average values for the time between symptom onset and sample collection were observed: 4.4 days (95% CI 4.3–4.9 days) and 5.1 days (95% CI 4.8–5.4 days) for June 2020 and February 2021, respectively.

To investigate the viral diversity associated with the second COVID-19 wave in Salvador, 12 swab samples from patients admitted to the ICU in February 2021, who were within the age range of 18–59 years, were sequenced. The characteristics of these patients are shown in Table 2. Five patients had no comorbidities. Half of the patients required invasive mechanical ventilation, while the other half were treated with non-invasive ventilation. Sequencing analysis identified the SARS-CoV-2 Gamma variant in all of the samples evaluated. Although all sequences were classified by the Pangolin tool as Gamma with high probability, one of the genomes presented low coverage and was not submitted to phylogenetic analysis. All of the genomes generated herein are available in the EpiCoV database, maintained by the GISAID initiative, with accession codes **EPI_ISL_160861** to **EPI_ISL_1608171**. The ML phylogenetic tree revealed that nine of the 11 genomes clustered together with high support (SH-aLRT = 87), meaning that the Gamma lineage has already established local community transmission in Salvador (Figure 4). Two other highly supported clusters of Gamma genomes isolated in Bahia State were also observed in the tree, being composed of travelers returning from Amazonas (Tosta et al., 2021). These three clusters provide evidence of multiple introductions of the Gamma lineage into Bahia State.

4. Discussion

Here we report the identification of the P.1 or Gamma variant in all sequenced clinical samples from patients admitted to the ICU in February 2021, in Salvador, Brazil. These results suggest that the Gamma variant is responsible for a large share of the COVID-19 cases in Salvador, in line with different reports finding a high proportion of the Gamma variant across Brazil (Slavov et al., 2021).

Recently, researchers have reported a significant increase in case fatality rates among young and middle-aged adults in Parana, Brazil, which may be associated with the Gamma strain (de Oliveira et al., 2021). The data presented herein also give support to a role of the Gamma variant in the acceleration of the pandemic in Salvador and Bahia State seen in early 2021.

The observed increase in the percentage of patients being admitted to the ICU with no risk factors for severe COVID-19, such as increased age, hypertension, and diabetes, is notable (de Oliveira et al., 2021). However, these are preliminary data and more studies are urgently required to clarify whether changes in the virulence may be attributable to the Gamma variant.

It is also necessary to investigate the influence of the vaccine rollout, which started in mid-January, first recruiting healthcare workers and then progressing to elderly individuals older than 80 years of age by the end of February, utilizing either CoronaVac (a two-dose, 28-day interval vaccine scheme) or AstraZeneca ChAdOx nCoV-19 (a two-dose, 3-month interval vaccine scheme). Therefore, during the period evaluated in this study, a very low percentage of elderly patients had received two doses of the vaccine.

Table 1
Comorbidity assessment in patients entering the ICU during the first wave (June 2020) and second wave (February 2021), stratified by age group.

Comorbidities	18–29 years			30–39 years			40–49 years			50–59 years			≥60 years		
	Admission period		*P*-value[a]	Admission period		*P*-value[a]	Admission period		*P*-value[a]	Admission period		*P*-value[a]	Admission period		*P*-value[a]
	Jun 2020 ($n = 3$)	Feb 2021 ($n = 10$)		Jun 2020 ($n = 19$)	Feb 2021 ($n = 40$)		Jun 2020 ($n = 17$)	Feb 2021 ($n = 66$)		Jun 2020 ($n = 29$)	Feb 2021 ($n = 41$)		Jun 2020 ($n = 118$)	Feb 2021 ($n = 123$)	
Obesity	-	20.0%	0.999	31.6%	22.5%	0.528	41.2%	10.6%	0.007*	17.2%	19.5%	0.999	16.9%	9.8%	0.1
Cardiovascular diseases	-	-		26.3%	12.5%	0.266	47.0%	24.2%	0.078	48.3%	51.2%	0.999	85.6%	69.1%	0.002*
Hematological	-	-		-	2.5%	0.999	-	-		3.4%	-	0.414	3.4%	1.6%	0.439
Liver disease	-	-		5.3%	-	0.322	-	4.5%	0.999	-	-		2.5%	-	0.116
Asthma	-	-		5.3%	-	0.322	11.8%	1.5%	0.105	-	-		3.4%	3.3%	0.999
Diabetes	-	-		-	2.5%	0.999	17.6%	15.2%	0.724	27.6%	20.0%	0.461	44.9%	40.5%	0.49
Neurological	-	10.0%	0.999	10.5%	-	0.999	-	-		-	2.4%	0.999	13.5%	9.8%	0.357
Lung disease	-	-		-	-		-	-		-	-		6.8%	4.1%	0.351
Immunosuppression	-	-		-	-		5.9%	-	0.205	6.9%	2.4%	0.566	3.4%	1.6%	0.439
Kidney disease	-	-		-	-		17.6%	3.0%	0.056	13.8%	4.9%	0.224	17.8%	5.7%	0.003*

ICU, intensive care unit.
[a] *P*-value of the Chi-square test or Fisher's exact test; *P-value <0.05, significant.

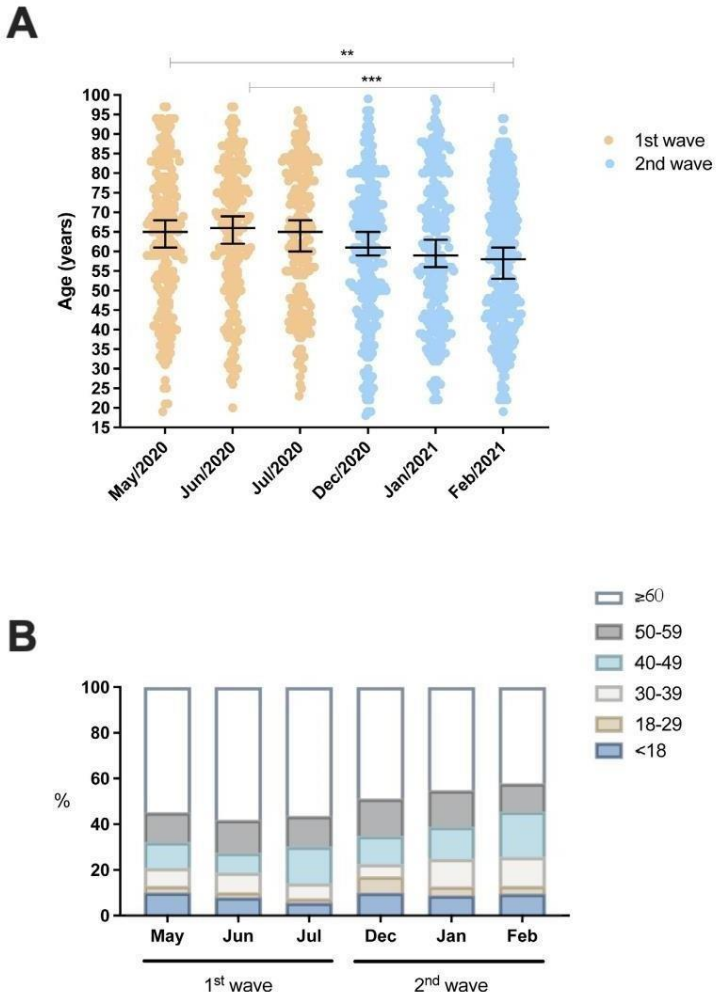

Figure 2. Temporal changes in the demographic profile of the patients admitted to the ICU due to COVID-19. (A) Individual age values (≥18 years old) are represented, along with median and 95% CI. The first wave includes the months of May, June, and July 2020; the second wave includes the months of December 2020, January 2021, and February 2021. (B) Total numbers of patients admitted to the ICU stratified by age group. $^{**}P < 0.001$, $^{***}P < 0.0001$.

Previous studies have suggested an increased Gamma viral load in nasopharyngeal swab samples compared to previous SARS-CoV-2 lineages (Resende et al., 2021). The present study demonstrated that positive swab samples in February 2021 presented lower Ct values than samples evaluated in June 2020. Although Ct values are subject to intrinsic sample collection variability and do not utilize reference measurements, the comparison of the results found in the peaks of the first wave and the second wave suggest that patients presented increased viral loads in February 2021, which was also reported when Gamma samples were compared to non-Gamma samples (Nelson et al., 2021, Faria et al., 2021).

Confirming that Gamma is a highly transmissible variant, associated with increased viral replication and disease severity, would require restrictive control measures to be revisited in populations with confirmed Gamma variant circulation, to adequately prevent viral spread and pressure on the healthcare system, as has been observed in Brazil. The data presented herein do not give support to an increased mortality rate among hospitalized COVID-19 patients after the emergence of the Gamma variant, since mortality rates and invasive mechanical ventilation rates decreased during the period evaluated. Since these data may be influenced by other confounders, such as improved disease management and evolving therapeutic protocols, future properly designed studies will be re-

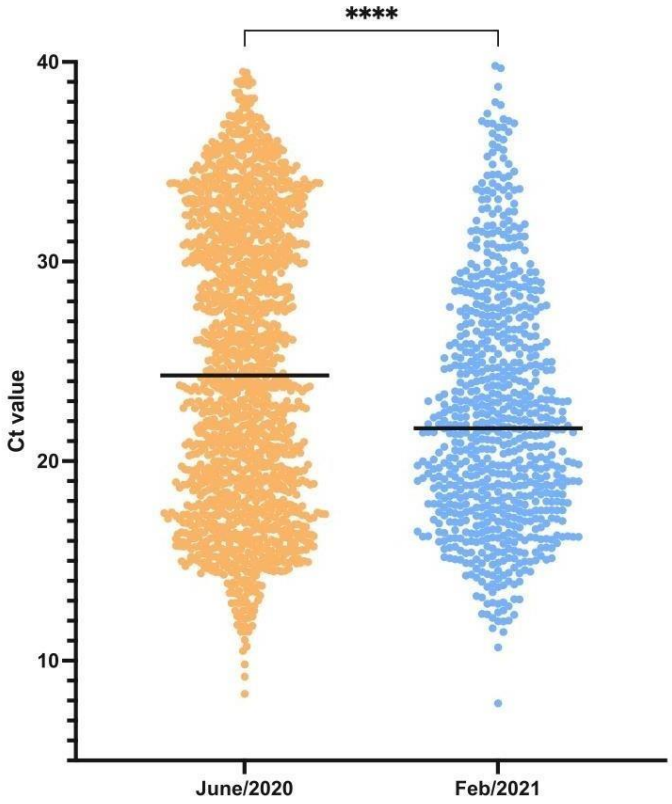

Figure 3. Temporal change in the SARS-CoV-2 RT-PCR cycle threshold (Ct) values. N gene Ct values were evaluated and compared between samples from COVID-19 patients referred to the hospital in June 2020 ($n = 1589$) and in February 2021 ($n = 771$). Single Ct values and the median are plotted. $^{****}P < 0.0001$.

Table 2
Clinical and demographic characteristics of patients admitted to the ICU in February 2021 selected for sequencing.

Sex	Age (years)	Symptom onset (days before ICU admission)	Ventilatory support	Comorbidities	Extent of ground glass opacitites (Thorax CT)
Male	35	7	Non-invasive ventilation	None	25%
Male	31	7	Non-invasive ventilation	Obesity, hypertension, diabetes	25–50%
Male	41	9	Non-invasive ventilation	None	25–50%
Male	59	11	Non-invasive ventilation	Obesity, hypertension	40%
Male	37	10	Invasive mechanical ventilation	Obesity	25–50%
Male	46	9	Invasive mechanical ventilation	Obesity	>75%
Male	44	5	Invasive mechanical ventilation	None	50%
Male	41	10	Non-invasive ventilation	None	75%
Male	56	8	Invasive mechanical ventilation	Hypertension	>75%
Female	36	5	Non-invasive ventilation	Obesity	50–75%
Male	24	8	Invasive mechanical ventilation	Obesity	25–50%
Male	35	7	Invasive mechanical ventilation	None	50%

CT, computed tomography; ICU, intensive care unit.

quired to determine whether the Gamma variant may be associated with any changes in disease severity.

In summary, the findings of this study contribute to describe the characteristics of the patients with severe COVID-19 during the second pandemic wave, which appears to be associated with an increased proportion of young and middle-aged adults. Additionally, the results suggest that the Gamma variant may have spread rapidly in Salvador, Brazil, leading to increased numbers of cases, hospitalizations, and ICU admissions shortly after its first detection in Bahia State. These preliminary findings reinforce the immediate need to adopt measures to reduce its spread and accelerate the vaccine rollout.

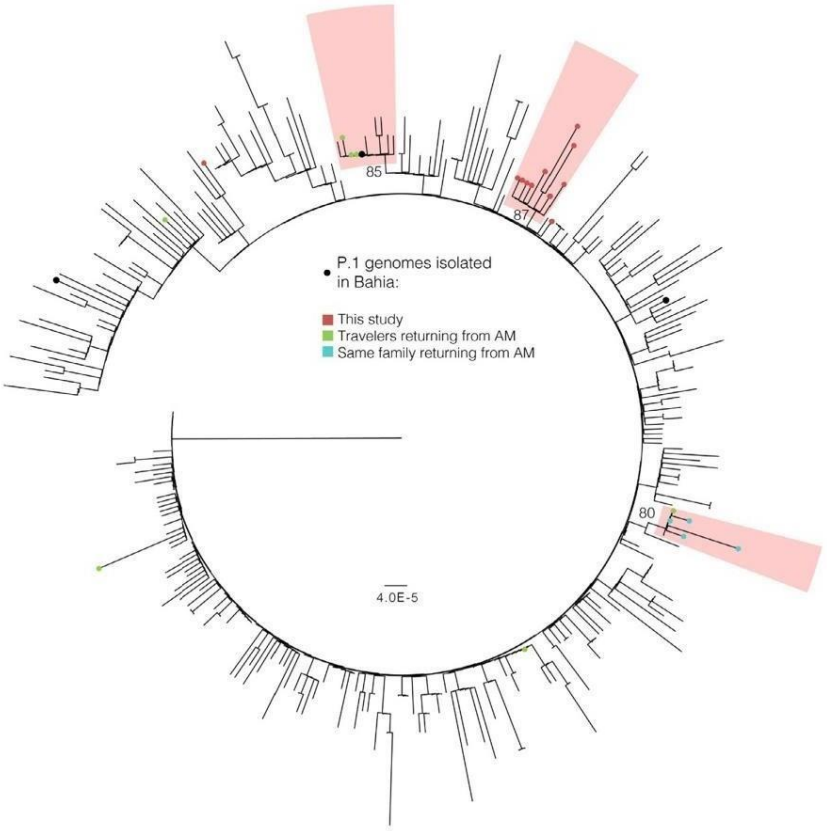

Figure 4. Circular maximum likelihood phylogenetic tree of the P.1 (Gamma) lineage diversity in Brazil. Samples isolated in Bahia are shown with circles, and the colors represent the origin of the sample, when available. Travel history data are those reported by Tosta et al., 2021 (Tosta et al., 2021). Three clusters with samples isolated in Bahia are highlighted and SH-aLRT support is shown. The tree was rooted in the oldest P.1 sampled genome.

CRediT authorship contribution statement

Carolina Kymie Vasques Nonaka: Writing – original draft, Writing – review & editing, Investigation, Formal analysis, Methodology. **Tiago Gräf:** Writing – original draft, Writing – review & editing, Investigation, Formal analysis, Methodology. **Camila Araújo de Lorenzo Barcia:** Investigation, Methodology. **Vanessa Ferreira Costa:** Investigation, Methodology. **Janderson Lopes de Oliveira:** Investigation, Methodology. **Rogério da Hora Passos:** Investigation, Methodology. **Iasmin Nogueira Bastos:** Investigation, Methodology. **Maria Clara Brito de Santana:** Investigation, Methodology. **Ian Marinho Santos:** Investigation, Methodology. **Karoline Almeida Felix de Sousa:** Investigation, Methodology. **Thamires Gomes Lopes Weber:** Methodology. **Isadora Cristina de Siqueira:** Writing – review & editing. **Clarissa Araújo Gurgel Rocha:** Writing – original draft, Writing – review & editing, Investigation, Methodology. **Ana Verena Almeida Mendes:** Writing – review & editing, Conceptualization. **Bruno Solano de Freitas Souza:** Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis, Funding acquisition.

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Ethics statement

This study was reviewed by local IRBs and received ethical approval by the National Committee for Ethics on Research (CONEP; CAAE: 29496920.8.0000.5262, 46821621.5.0000.0048 and

3.980.128 /2020). All sampled patients have provided written informed consent.

Conflict of interest

The authors claim no conflict of interest.

Access to data

C.K.V.N. and A.V.A.M. have full access to the data and are the guarantor for the data. Data are available upon reasonable request.

References

Candido DS, Claro IM, de Jesus JG, Souza WM, Moreira FR, Dellicour S, et al. Evolution and epidemic spread of SARS-CoV-2 in Brazil. Science 2020;369(6508):1255–60. doi:10.1126/science.abd2161.

Dejnirattisai, W., Zhou, D., Supasa, P., Liu, C, Mentzer, A.J., Ginn, H. M. et al. Antibody evasion by the Brazilian P.1 strain of SARS-CoV-2. bioRxiv preprint. 2021. 10.1101/2021.03.12.435194

Chakraborty D, Agrawal A, Maiti S. Rapid identification and tracking of SARS-CoV-2 variants of concern. The Lancet 2021;397(10282):1346–7. doi:10.1016/S0140-6736(21)00470-0.

Faria NR, Mellan TA, Whittaker C, Claro IM, Candido DDS, Mishra S, et al. Genomics and epidemiology of a novel SARS-CoV-2 lineage in Manaus, Brazil. Science 2021;372(6544):815–21 http://www.ncbi.nlm.nih.gov/pubmed/33688664.

Faria NR, Mellan TA, Whittaker C, Claro IM, Candido DDS, Mishra S, et al. Genomics and epidemiology of a novel SARS-CoV-2 lineage in Manaus, Brazil. medRxiv. 2021. doi:10.1101/2021.02.26.21252554.

Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usa-bility. Mol Biol Evol 2013;30:772–80. doi:10.1093/molbev/mst010.

Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, et al. Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell 2020;182(4):812–27. doi:10.1016/j.cell.2020.06.043.

Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Von Haeseler A, et al. IQ-TREE 2: New models and efficient methods for phylogenetic inference in the Genomic Era. Molecular Biology and Evolution 2020;37(5):1530–4. doi:10.1093/molbev/msaa015.

Naveca, F., Nascimento, V., Souza, Corado, A., Nascimento, F., Silva, G. et al. Phylogenetic relationship of SARS-CoV-2 sequences from Amazonas with emerging Brazilian variants harboring mutations E484K and N501Y in the Spike protein. Virological.org 2021;

Naveca F, da Costa C, Nascimento V, Souza V, Corado A, Nascimento F, et al. Three SARS-CoV-2 reinfection cases by the new Variant of Concern (VOC) P.1/501Y.V3. Res Sq 2021. doi:10.21203/rs.3.rs-318392/v1.

Nelson G, Buzko O, Spilman PR, Niazi K, Rabizadeh S, Soon-Shiong PR. Molecular dynamic simulation reveals E484K mutation enhances spike RBD-ACE2 affinity and the 1 combination of E484K, K417N and N501Y mutations (501Y.V2 variant) induces conformational 2 change greater than N501Y mutant alone, potentially resulting in an escape mutant. bioRxiv 2021. doi:10.1101/2021.01.13.426558.

de Oliveira MHS, Lippi G, Henry BM. Sudden rise in COVID-19 case fatality among young and middle-aged adults in the south of Brazil after identification of the novel B. 1.1. 28.1 (P. 1) SARS-CoV-2 strain: analysis of data from the state of Parana. medRxiv 2021. doi:10.1101/2021.03.24.21254046.

Resende PC, Delatorre E, Gräf T, Mir D, Motta FC, Appolinario LR, et al. Evolutionary dynamics and dissemination pattern of the SARS-CoV-2 lineage B.1.1.33 during the early pandemic phase in Brazil. Front Microbiol 2021;11:1–14. doi:10.3389/fmicb.2020.615280.

Sabino EC, Buss LF, Carvalho MP, Prete CA, Crispim MA, Fraiji NA, et al. Resurgence of COVID-19 in Manaus, Brazil, despite high seroprevalence. The Lancet 2021;397(10273):452–5. doi:10.1016/S0140-6736(21)00183-5.

Slavov SN, Patane J, Bezerra RS, Giovanetti M, Fonseca V, Martins AJ, et al. Genomic monitoring unveil the early detection of the SARS-CoV-2 B.1.351 lineage (20H/501Y.V2) in Brazil. medRxiv 2021. doi:10.1101/2021.03.30.21254591.

Tosta S, Giovanetti M, Nardy VB, da Silva LRDO, Gomez MKA, Lima JG, et al. Early genomic detection of SARS-CoV-2 P. 1 variant in Northeast Brazil. medRxiv 2021. doi:10.1101/2021.02.25.21252490.