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IMPACTO DE MUTAÇÕES DE RESISTÊNCIA NO VÍRUS DA HEPATITE C NA RESPOSTA AO TRATAMENTO COM OS NOVOS ANTIVIRAIS DE AÇÃO DIRETA

VANESSA DUARTE DA COSTA

Rio de Janeiro
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Ministério da Saúde

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Impacto de mutações de resistência no vírus da hepatite C na resposta ao tratamento com os novos antivirais de ação direta

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

Orientador: Prof. Dr. Francisco Campello do Amaral Mello

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Rio de Janeiro, 25 de Setembro de 2020

DEDICATÓRIA

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“No meio da confusão, encontre a simplicidade.

A partir da discórdia, encontre a harmonia.

No meio da dificuldade reside a oportunidade”

Albert Einstein

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RESUMO

TESE DE DOUTORADO EM BIOLOGIA CELULAR E MOLECULAR

Vanessa Duarte da Costa

O tratamento da hepatite C tem evoluído de forma considerável ao longo dos anos a partir do desenvolvimento de moléculas denominadas antivirais de ação direta (DAAs) que inibem proteínas não estruturais do vírus da hepatite C (HCV), como por exemplo, simeprevir (SMV), daclatasvir (DCV) e sofosbuvir (SOF), inibidores das proteínas NS3, NS5A e NS5B do HCV, respectivamente. O licenciamento de esquemas terapêuticos com DAAs permitiu um aumento das taxas de resposta virológica sustentada (RVS), porém a seleção de estirpes virais com substituições associadas à resistência (RASs) aos antivirais pode ser considerada um dos fatores determinantes para a ocorrência de falha terapêutica. A presente tese teve como objetivo avaliar a frequência de resistência basal através da identificação de RASs nas proteínas NS3, NS5A e NS5B do HCV e analisar seu possível impacto clínico no tratamento antiviral com DAAs para diferentes grupos de indivíduos com hepatite C crônica: monoinfectados pelos genótipos 1 e 3 do HCV e coinfectados HCV/HIV, tendo em ambos os grupos pacientes experimentados por terapias prévias com DAAs e virgens de tratamento. Como principais resultados obtidos nos três manuscritos produzidos, destacamos (i) a prevalência de RASs na proteína NS3 do HCV em pacientes previamente experimentados que falharam a terapia com os IPs telaprevir/boceprevir foi maior em comparação com a dos pacientes não experimentados, provavelmente devido à pressão seletiva imposta pela droga; (ii) as RASs V36M e R155K podem reduzir a susceptibilidade a IPs aprovados no Brasil, tais como o SMV, paritaprevir e grazoprevir, sendo que a mutação no resíduo 36 foi a mais prevalente nas sequências de aminoácidos da proteína NS3 para o genótipo 1a do HCV; (iii) considerando a baixa prevalência da RAS Q80K nas estirpes do HCV circulantes no Brasil, não há necessidade de testes de resistência no pré-tratamento com SMV; (iv) as altas taxas de RVS de portadores crônicos brasileiros na prática de rotina clínica foram semelhantes a dos ensaios clínicos controlados para a terapia combinada com o uso de SOF e DCV (acima de 95%); (v) a ocorrência e persistência das RASs específicas nas posições 30 e 93 da proteína NS5A do HCV pode ter influenciado na não resposta de dois pacientes com hepatite C crônica submetidos ao tratamento com SOF e DCV. Em conclusão, nossos achados sugeriram que a ocorrência de mutações basais nas proteínas NS3, NS5A e NS5B do HCV não seria um fator limitante para o sucesso terapêutico com os DAAs aprovados em protocolos clínicos brasileiros. Entretanto, a combinação de fatores de prognóstico negativo para o alcance da RVS em alguns casos clínicos particulares justifica a realização de testes moleculares de resistência no sentido de definir uma melhor terapia de resgate.

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IMPACT OF HEPATITIS C VIRUS RESISTANCE MUTATIONS IN RESPONSE TO DIRECT ACTING ANTIVIRALS TREATMENT

ABSTRACT

PHD THESIS IN CELLULAR AND MOLECULAR BIOLOGY

Vanessa Duarte da Costa

Hepatitis C treatment has evolved considerably over the years from the development of molecules called direct-acting antivirals (DAAs) that inhibit hepatitis C virus (HCV) non-structural proteins, such as simeprevir (SMV), daclatasvir (DCV) and sofosbuvir (SOF), inhibitors of HCV NS3, NS5A and NS5B proteins, respectively. The licensing of therapeutic regimens with DAAs has enabled an increase in sustained virological response (SVR) rates, but the selection of viral strains with resistance-associated substitutions (RASs) at specific amino acid positions can be considered one of the limiting factors for the occurrence of therapeutic failures. This thesis aimed to evaluate the frequency of baseline resistance through RASs identification in HCV NS3, NS5A and NS5B proteins and to analyze their possible clinical impact in DAAs antiviral treatment for different groups of individuals with chronic hepatitis C: monoinfected by HCV genotypes 1 and 3 and HCV/HIV-coinfected patients, having in both groups experimented patients by previous therapies with DAAs and treatment-naive. As the main results obtained in the three manuscripts produced, we highlighted (i) the prevalence of HCV NS3 RASs for previously experienced patients who failed therapy with PIs telaprevir/boceprevir was higher compared to non-experienced patients, probably due the selective pressure imposed by the drug; (ii) NS3 RASs V36M and R155K can reduce PIs susceptibility approved in Brazil, such as SMV, paritaprevir and grazoprevir, with the mutation at residue 36 being the most prevalent in NS3 protein aminoacid sequences for HCV genotype 1a; (iii) considering RAS Q80K low prevalence in HCV strains circulating in Brazil, there is no need for resistance tests in pretreatment with SMV; (iv) High SVR rates of chronic brazilian patients in routine clinical practice was similar to randomized clinical trials for combined therapy with the use of SOF and DCV (above 95%); (v) the occurrence and persistence of specific RASs at positions 30 and 93 of HCV NS5A protein may have influenced the non-response of two patients with chronic hepatitis C undergoing treatment with SOF and DCV. In conclusion, our findings suggested that the occurrence of baseline mutations in HCV NS3, NS5A and NS5B proteins would not be a limiting factor for therapeutic success with approved DAAs in Brazilian clinical protocols. However, the combination of negative prognostic factors for achieving SVR in some particular clinical cases justifies conducting molecular resistance tests in order to define a better rescue therapy.

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G	Glicina
A	Alanina
L	Leucina
V	Valina
I	Isoleucina
P	Prolina
F	Fenilalanina
S	Serina
T	Treonina
C	Cisteína
Y	Tirosina
N	Asparagina
Q	Glutamina
D	Aspartato ou ácido aspártico
E	Glutamato ou ácido glutâmico
R	Arginina
K	Lisina
H	Histidina
W	Triptofano
M	Metionina
Anti-HCV	Anticorpo contra o vírus da hepatite C
ALT	Alanina aminotransferase
AST	Aspartato aminotransferase
CD81	*Cluster of Differentiation 81*
CDC	*Centers for Disease Control and Prevention*
cDNA	DNA complementar
CHC	Carcinoma Hepatocelular
DAA	*Direct-acting antiviral*
DCV	Daclatasvir
DNA	Ácido desoxirribonucléico
E1	Glicoproteína do envelope 1
E2	Glicoproteína do envelope 2
ELISA	*Enzyme-linked immunosorbent assay*

GBV-B	*GB virus B*
GBV-C	*GB virus C*
GGT	Gama glutamil transferase
HAV	Vírus da hepatite A
HBV	Vírus da hepatite B
HBsAg	Antígeno de superfície do vírus da Hepatite B
HCV	Vírus da hepatite C
HGV	Vírus da hepatite G
HIV	Vírus da Imunodeficiência Humana
HRV1	Região hipervariável 1
HSC	*Hepatic stellate cells*
ICTV	*International Committee on Taxonomy of Viruses*
IFN	Interferon
IP	Inibidor de protease
IRES	Sítio interno de entrada ribossomal
LDL	Lipoproteína de baixa densidade
MHC	Complexo principal de histocompatibilidade
miR-122	MicroRNA-122
NADPH	Fosfato de dinucleotídeo de adenina e nicotinamida
NANB	Hepatite não-A não-B
NC	Não codificante
NI	*Nucleoside inhibitor*
NIH	*National Institutes of Health*
NK	*Natural killer*
NNI	*Non-nucleoside inhibitor*
NPHV	*Non-primate hepacivirus*
mg	Miligrama
NS2	Proteína não estrutural 2
NS3	Proteína não estrutural 3
NS4A	Proteína não estrutural 4A
NS4B	Proteína não estrutural 4B
NS5A	Proteína não estrutural 5A
NS5B	Proteína não estrutural 5B
ORF	*Open reading frame*
PCDT	Protocolo Clínico e Diretrizes Terapêuticas para Hepatite C e

	Coinfecções
PCR	Reação em cadeia da polimerase
PD-1	*Programmed death-1*
Peg-IFN	Interferon peguilado
RAS	*Resistance-associated substituition*
RBV	Ribavirina
RFLP	*Restriction Fragment Length Polymorphism*
RNA	Ácido ribonucléico
RVS	Resposta Virológica Sustentada
RVS12	Resposta Virológica Sustentada 12 semanas pós-tratamento
SMV	Simeprevir
Sinan	Sistema de Informação de Agravos de Notificação
SIM	Sistema de Informação sobre Mortalidade
SOF	Sofosbuvir
SRB1	*Scavenger receptor class B type I*
SREBP-1c	*Sterol regulatory element-binding protein-1*
TCD4+	Linfócito T auxiliar
TCD8+	Linfócito T citotóxico
TMA	Amplificação mediada por transcrição
TR	Teste rápido
Treg	Células T reguladoras

1 INTRODUÇÃO

1.1 Histórico

Em 1975, estudo de Feinstone *et al.* (1975) [1] destacou a ocorrência de vinte e dois casos de pacientes com hepatite pós-transfusional após serem submetidos a cirurgias cardíacas no *National Institutes of Health* (NIH) nos Estados Unidos. Até a década de 1980, cerca de 10% de todos os pacientes que recebiam transfusão de sangue e derivados nos Estados Unidos apresentavam hepatite pós-transfusional. Testes sorológicos visando à detecção de anticorpos para o vírus da hepatite A (HAV) foram realizados, e concluiu-se que nenhum dos pacientes soronegativos (9/22) ou soropositivos (13/22) antes da transfusão apresentou soroconversão ou aumento nos níveis de anticorpos para HAV, respectivamente. Ainda se referindo à pesquisa de Feinstone *et al.* (1975) [1], amostras de soro de vinte e um pacientes foram coletadas e testadas através de radioimunoensaio para a detecção do antígeno de superfície do vírus da Hepatite B (HBV), HBsAg, então denominado antígeno Austrália [2], e o resultado foi negativo. Um paciente havia sido testado previamente através da técnica de *countereletrophoresis*, sendo o resultado também negativo. Além disso, todos os vinte e dois pacientes apresentavam anticorpo preexistente ao vírus Epstein-Barr antes da transfusão. Concluiu-se, portanto, que outro agente infeccioso ainda não identificado poderia ser transmitido via transfusão sanguínea e causar hepatite pós-transfusional. Por essa razão, foi introduzido o termo hepatite não-A não-B (NANB) para a designação desses casos. A pesquisa de um novo agente etiológico nesses casos tornou-se de extrema importância já que, através de metodologias diagnósticas bem definidas, outros estudos realizados nas décadas de 70 e 80 também evidenciaram que casos de hepatite provenientes de transfusão sanguínea não foram causados por HAV ou HBV, enfatizando a hipótese de existência de outros agentes infecciosos responsáveis pela infecção [3-5].

Em 1989, grupo composto por Choo *et al.* [6] elucidou a natureza do vírus responsável pela hepatite NANB pós-transfusional. A partir do soro de chimpanzés infectados com sangue de pacientes com hepatite NANB, os autores isolaram o ácido nucléico e, a partir desse *pool*, obtiveram uma biblioteca de fragmentos de

DNA complementar usando transcriptase reversa com iniciadores randômicos. Esse processo deu origem a cerca de seis milhões de sequências complementares aos ácidos nucléicos encontrados no soro infeccioso. As sequências foram inseridas em fagos vetores e expressos em *Escherichia coli*. Cada polipeptídio resultante foi testado contra soros de pacientes com hepatite NANB crônica para detectar reatividade com anticorpos séricos. Após cerca de um milhão de testes terem sido realizados foi identificada um polipeptídeo que reagia com anticorpos dos soros infectados, mas não reagia com os soros-controle (não infectados). O fragmento de DNA complementar que foi usado para dar origem a esse polipeptídeo foi então, usado como sonda de hibridização para extrair o ácido nucléico original a partir do qual o fragmento havia sido gerado. Dessa maneira, o genoma completo do agente suspeito foi identificado. Foi encontrado RNA de fita simples com cerca de 10.000 nucleotídeos. Esse agente foi denominado de vírus da Hepatite C (HCV). Outros estudos indicaram que o novo vírus tem menos de 80 nm de diâmetro e, devido à sua sensibilidade comprovada a solventes orgânicos, possui um envelope composto de lipídios essenciais [2, 7]. Além disso, foi evidenciado que a infecção pelo HCV está associada à hepatite aguda e crônica [8] e câncer de fígado [9, 10].

1.2 Classificação taxonômica do HCV

O HCV pertence à família *Flaviviridae*, gênero *Hepacivirus* e espécie *Hepacivirus C* [11]. Até 2011, o único outro membro classificado do gênero *Hepacivirus* era o *GB virus B* (GBV-B), isolado em micos de laboratório [12-14]. Desde então, o conhecimento sobre a diversidade de hepacivírus foi ampliado pela identificação de vírus homólogos ao HCV em várias espécies de mamíferos, incluindo cavalos, primatas do Velho Mundo, roedores e morcegos [15-20]. A identificação de hepacivírus não primatas (NPHV, do inglês *non-primate hepacivirus*) inicialmente em cães e subsequentemente em cavalos foi a primeira indicação de uma distribuição mais ampla de hepacivírus em mamíferos [15, 17]. A família *Flaviviridae* também inclui outros três gêneros: (i) *Pestivirus*, tendo como membros o vírus da diarréia viral bovina e o vírus da peste suína clássica que causam doenças em bovinos e suínos, respectivamente, (ii) *Flavivirus*, que inclui membros como os vírus da Febre Amarela, Zika, Nilo Ocidental e Dengue os quais representam agentes etiológicos causadores de doenças virais transmitidas por artrópodes em

humanos e (iii) *Pegivirus*, incluindo o vírus humano *GB virus C* (GBV-C), também conhecido como vírus da hepatite G (HGV) [14, 21].

1.3 Características estruturais, moleculares e replicativas do HCV

1.3.1 Organização do genoma do HCV

O HCV é um vírus envelopado e apresenta um genoma de RNA de fita simples de polaridade positiva que consiste em aproximadamente 9.600 nucleotídeos [6] **(figura 1.1)**. O genoma viral possui duas regiões não codificantes (5' e 3' NC), sendo uma em cada extremidade, e uma fase de leitura aberta (ORF, do inglês *open reading frame*) que codifica uma poliproteína com cerca de 3000 aminoácidos que, pela ação de proteases virais e celulares é clivada em proteínas estruturais (*core*, E1 e E2) e não estruturais (p7, NS2, NS3, NS4A, NS4B, NS5A e NS5B) [22], com suas funções descritas abaixo.

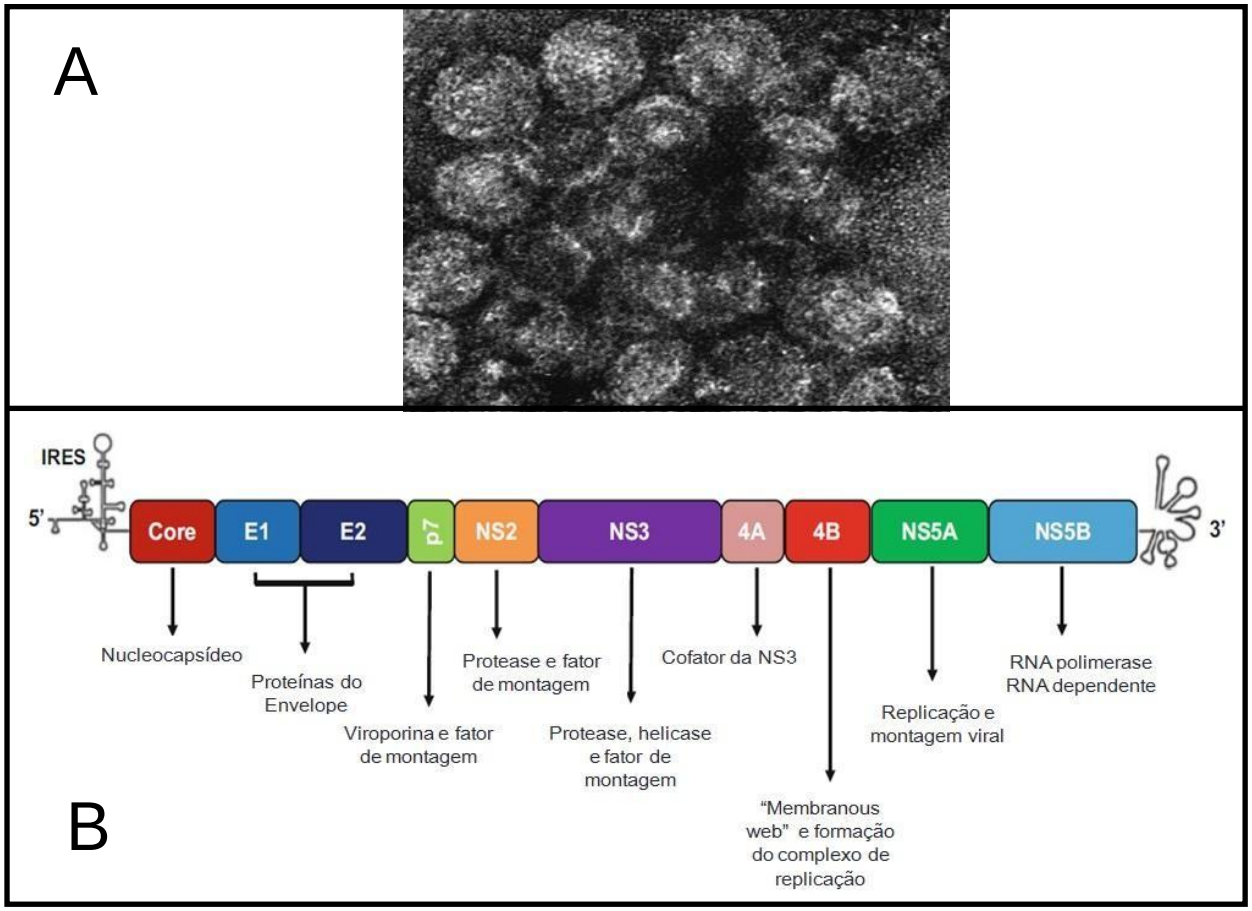

Figura 1.1. A) Microscopia eletrônica do HCV. Fonte: Yu X *et al.*, 2007 [23]; **B)** Organização genômica do HCV. Fonte: Adaptado de Li DK *et al.* (2019) [24]. IRES: Sítio interno para entrada ribossomal; E: Envelope; NS: Não estruturais.

Em relação às regiões 5’ e 3’ NC, ambas formam estruturas secundárias e terciárias e interagem com proteínas virais e celulares e entre si [25, 26]. Parte da região 5’ NC e do gene do *core* são ocupados pelo sítio interno para entrada ribossomal (IRES), essencial para a tradução *cap*-independente do RNA viral [27]. O IRES abrange uma região de aproximadamente 341 nucleotídeos e se dobra em quatro domínios estruturalmente distintos (I-IV) [28]. Considerando que o domínio I é dispensável para a atividade do IRES, mesmo substituições de um único nucleotídeo ou *indels* nos domínios II-IV podem ter um impacto severo na eficácia do IRES no início da tradução [29]. Já em relação à região 3’ NC, acredita-se que esta seja importante na iniciação da replicação do genoma viral, além de ser composta por uma pequena região variável, uma cauda poli-U de 80 nucleotídeos e uma região altamente conservada composta de 98 nucleotídeos, denominada cauda X [30, 31].

As proteínas estruturais do genoma do HCV consistem no *core* ou capsídeo e nas glicoproteínas do envelope E1 e E2. A proteína do capsídeo é produzida no retículo endoplasmático e consiste nos primeiros 191 aminoácidos da poliproteína traduzida do RNA de fita simples de polaridade positiva [32]. Ela é removida da poliproteína por peptidases do hospedeiro que realizam uma clivagem na região C-terminal promovendo a liberação da forma imatura desta proteína [33]. Posteriormente, esta forma imatura é processada também por peptidases do hospedeiro resultando na proteína madura. O *core* maduro consiste de um domínio hidrofílico na porção N-terminal e um domínio hidrofóbico menor na porção C-terminal [34]. Já as proteínas de envelope E1 e E2 são glicoproteínas altamente glicosiladas e apresentam pesos moleculares de aproximadamente 35 kDa e 70 kDa, respectivamente. Essas proteínas são essenciais para a entrada do vírus na célula e para a montagem das partículas infecciosas [35, 36]. A glicoproteína E2 apresenta em sua extremidade N-terminal uma região denominada região hipervariável 1 (HVR1, do inglês *Hypervariable region 1*), o qual é considerada o principal alvo da resposta imune adaptativa, contendo alvos de epítopos de células B e T [37].

Considerando as proteínas não estruturais do genoma do HCV, englobam-se as proteínas p7, NS2, NS3, NS4A, NS4B, NS5A e NS5B. A viroporina p7, uma proteína que possui dois domínios transmembranares e um domínio transmembranar alfa-helicoidal anfipático, forma um canal iônico de permeabilidade

entre membranas, os quais estudos sugerem que a mesma atue de maneira multifuncional no ciclo replicativo do HCV. Tedbury *et al.* (2011) [38] sugeriram que a p7 possa atuar de forma conjunta com a proteína NS2 na montagem de vírions, o que não está relacionado à sua atividade como canal iônico. Além disso, Lee *et al.* (2016) [39] sugeriram que a p7 estaria associada com o processo de adesão da membrana no processo final de formação do envelope do vírion e o seu respectivo brotamento.

Outra proteína não estrutural é a NS2 que apresenta 217 aminoácidos e domínio N-terminal composto por três segmentos transmembranares cuja localização é direcionada para o interior do retículo endoplasmático. Com o domínio N-terminal da NS3, forma a autoprotease NS2-NS3 que catalisa a clivagem na localização NS2/NS3 [40, 41]. A atividade de protease da NS2 é estimulada pelo domínio serino-protease da NS3, definindo este domínio como um cofator estimulatório para a NS2 [42]. Já a NS3 é uma proteína hidrofílica que apresenta peso molecular de 70 kDa e 217 aminoácidos. Seu domínio N-terminal atua como serino-protease e é responsável pela proteólise de toda a região não estrutural da poliproteína viral [43]. A NS3 forma um complexo não covalente com a proteína NS4A que é um polipeptídeo ancorado na membrana. A NS4A age como um cofator da NS3 e também a estabiliza. Para que a clivagem da poliproteína seja eficiente, é necessária a presença deste cofator, especialmente no sítio NS4B/NS5A, sugerindo que NS3 e NS4A formam um complexo estável [44]. Em adição ao seu papel na ativação e estabilização da protease NS3, a proteína NS4A, de aproximadamente 8 kDa e composta por 54 aminoácidos, também apresenta a função de ancoragem da NS3 na membrana do retículo endoplasmático via o seu domínio hidrofóbico N-terminal [45]. A proteína NS4B está envolvida na formação de complexos de replicação e na montagem de partículas virais [46].

Através da ação conjunta entre NS3 e NS4A, duas proteínas codificadas a partir da região NS5 são liberadas, são elas, NS5A e NS5B. A proteína NS5A é uma fosfoproteína associada à membrana que pode ser encontrada na forma fosforilada basal de 56 kDa e na forma hiperfosforilada de 58 kDa. A proteína é fosforilada em resíduos de serina e treonina, sendo hiperfosforilada na presença de NS4A [47]. A utilização do clone JFH-1 revelou que a NS5A apresenta funções diretas e independentes na replicação e montagem do genoma do vírus. Dentre os três

domínios da NS5A, os domínios I e II são necessários para a replicação do genoma, enquanto que o domínio III tem um papel na montagem [48]. A proteína NS5B apresenta 591 aminoácidos e peso molecular de 86 kDa [37]. Ela foi identificada como a RNA polimerase RNA dependente do HCV e é responsável pelas etapas de síntese de RNA de cadeia negativa complementar, usando o genoma como molde e pela síntese subsequente da cadeia positiva de RNA a partir desse molde de RNA de cadeia negativa [49]. Os sítios para a atividade da proteína NS5B possuem afinidade de ligação com segmentos de poli U, como aquele presente na extremidade da região 3' NC do HCV [50]. A existência de um elemento de replicação *cis* no seu domínio C-terminal, em conjunto com a região 3' NC, garante a iniciação da replicação do genoma completo a partir desta região [50].

1.3.2 Replicação viral

O processo replicativo se inicia com a entrada da partícula viral nos hepatócitos, sendo um processo altamente coordenado que envolve diversas moléculas de superfície celular em passos sequenciais **(figura 1.2)**. A adsorção à superfície da célula hospedeira envolve as glicoproteínas E1 e E2, apolipoproteínas presentes na superfície de lipoviropartículas e outras moléculas da superfície celular [51]. Glicosaminoglicanos e o receptor de lipoproteínas de baixa densidade (LDL) parecem estar envolvidos na ligação inicial de baixa afinidade e, posteriormente, as glicoproteínas E1 e E2 interagem com os receptores *Cluster of Differentiation 81* (CD81) da família das tetraspaninas e *scavenger receptor class B member 1* (SRB1) [51]. A entrada do HCV na célula se dá por endocitoce, sendo mediada por vesículas recobertas por clatrina [52] compartimentalizada em vesículas de endossoma ácido [53]. As proteínas de junção claudina 1 e ocludina também são necessárias para a entrada na célula [54]. Os receptores do HCV estão aparentemente associados a junções celulares, o que permite a transmissão direta célula-célula[55].

Após a entrada do HCV nas células, a liberação do nucleocapsídeo para o citosol ocorre após a fusão do envelope do HCV à membrana do endossoma. Acredita-se que este processo é desencadeado de uma forma independente do receptor, mas dependente do pH, e está estreitamente relacionado com a composição lipídica [56]. Além do pH ácido, o colesterol das membranas também apresenta um forte efeito na fusão entre vírus e célula hospedeira [57]. A partícula

viral é então submetida a um processo de desnudamento, com posterior liberação do RNA genômico no citosol que serve de RNA mensageiro para a síntese da poliproteína [51]. A tradução da proteína se inicia com a formação de um complexo estável entre o IRES na região 5' NC com a subunidade 40S ribossomal, fatores de iniciação da célula hospedeira e proteínas virais [58]. Posteriormente, ocorre o processamento da poliproteína viral com a clivagem das proteínas não-estruturais por proteases virais e celulares [26]. São necessárias pelo menos duas peptidases celulares hospedeiras (signalase e peptidade do peptídeo sinal) para o processamento das proteínas estruturais do HCV, enquanto duas peptidases virais (NS2 e NS3/4A) estão envolvidas no processamento das proteínas não estruturais do HCV [51].

Após o processamento da poliproteína, forma-se o complexo de replicação denominado “rede membranosa”, incluindo proteínas virais não-estruturais e proteínas celulares [59]. A replicação é catalisada pela proteína NS5B. A proteína NS5A e o domínio helicase-NTPase da proteína NS3 desempenham um papel regulador importante na replicação do vírus [60]. Enquanto que a proteína NS5A atua como um dímero facilitando a ligação do RNA à rede membranosa, a função de helicase da proteína NS3 apresenta um papel importante na separação de filamentos de RNA molde e recém-sintetizados [61]. Na replicação, o RNA do HCV de polaridade positiva é utilizado como molde para a síntese de RNA de polaridade negativa (replicativo intermediário), o qual por sua vez serve de molde para a síntese do RNA genômico. A seguir, o RNA sintetizado atua como RNA mensageiro para a síntese de proteínas virais ou interage com cópias da proteína do *core*, formando o nucleocapsídeo [60]. Vários fatores do hospedeiro também demonstram apresentar funções importantes no ciclo replicativo do HCV. A ciclofilina A, também conhecida como peptidilprolil isomerase A, se liga às proteínas NS5A e NS5B, induzindo alterações conformacionais necessárias para a replicação [51]. O microRNA-122 (miR-122), um miRNA abundante específico do fígado, se liga a dois locais conservados no local de entrada interno do ribossomo, o que é necessário para a estabilização do RNA. Para a formação de novos vírions, é realizada a montagem de proteínas virais, glicoproteínas e o RNA genômico a partir de um processo que envolve múltiplas etapas, incluindo componentes virais e da célula hospedeira. Após

a montagem, ocorre a maturação do vírion no interior da vesícula de transporte e sua posterior liberação da célula hospedeira [62].

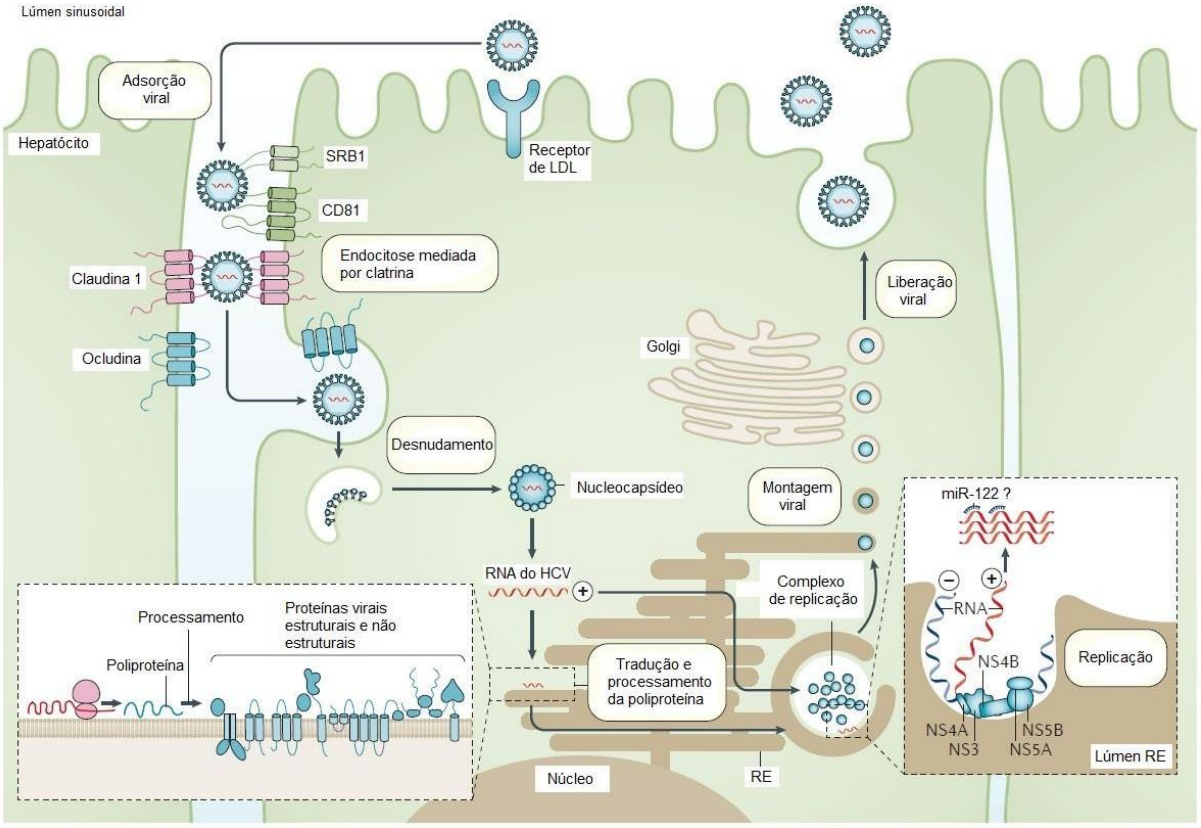

Figura 1.2. Representação esquemática do ciclo replicativo do HCV envolvendo ligação viral, endocitose mediada por clatrina, tradução, processamento de poliproteínas, replicação do RNA e, finalmente, montagem e liberação viral. Fonte: Adaptado de Manns *et al.* (2017) [51].

1.4 Epidemiologia da infecção pelo HCV

Globalmente, estima-se que 71 milhões de pessoas apresentem infecção crônica pelo HCV [63]. Nos países onde as práticas de controle de infecção são ou foram historicamente insuficientes, tais como, divulgação limitada sobre a existência de métodos de diagnóstico e de fatores de risco para a transmissão do HCV no que tange ao compartilhamento de objetos de higiene pessoal e de instrumentos para uso de drogas injetáveis além da ausência de triagem nos bancos de sangue. Embora a qualidade dos dados epidemiológicos e as estimativas de prevalência

variem entre países e regiões, as estimativas globais mais recentes indicam que a prevalência virêmica da infecção pelo HCV (prevalência do RNA do HCV) é <1,0% na maioria dos países desenvolvidos **(figura 1.3)**. A prevalência é consideravelmente mais alta em alguns países da Europa Oriental (3,3% na Rússia e 2,2% na Letônia), África (6,3% no Egito e 7,0% no Gabão), Oriente Médio (3,0% na Síria), Sul do Cáucaso e Ásia Central (4,2% na Geórgia e 4,3% no Uzbequistão) e sul e leste da Ásia (3,8% no Paquistão, 6,4% na Mongólia e 2,1% em Taiwan) [63].

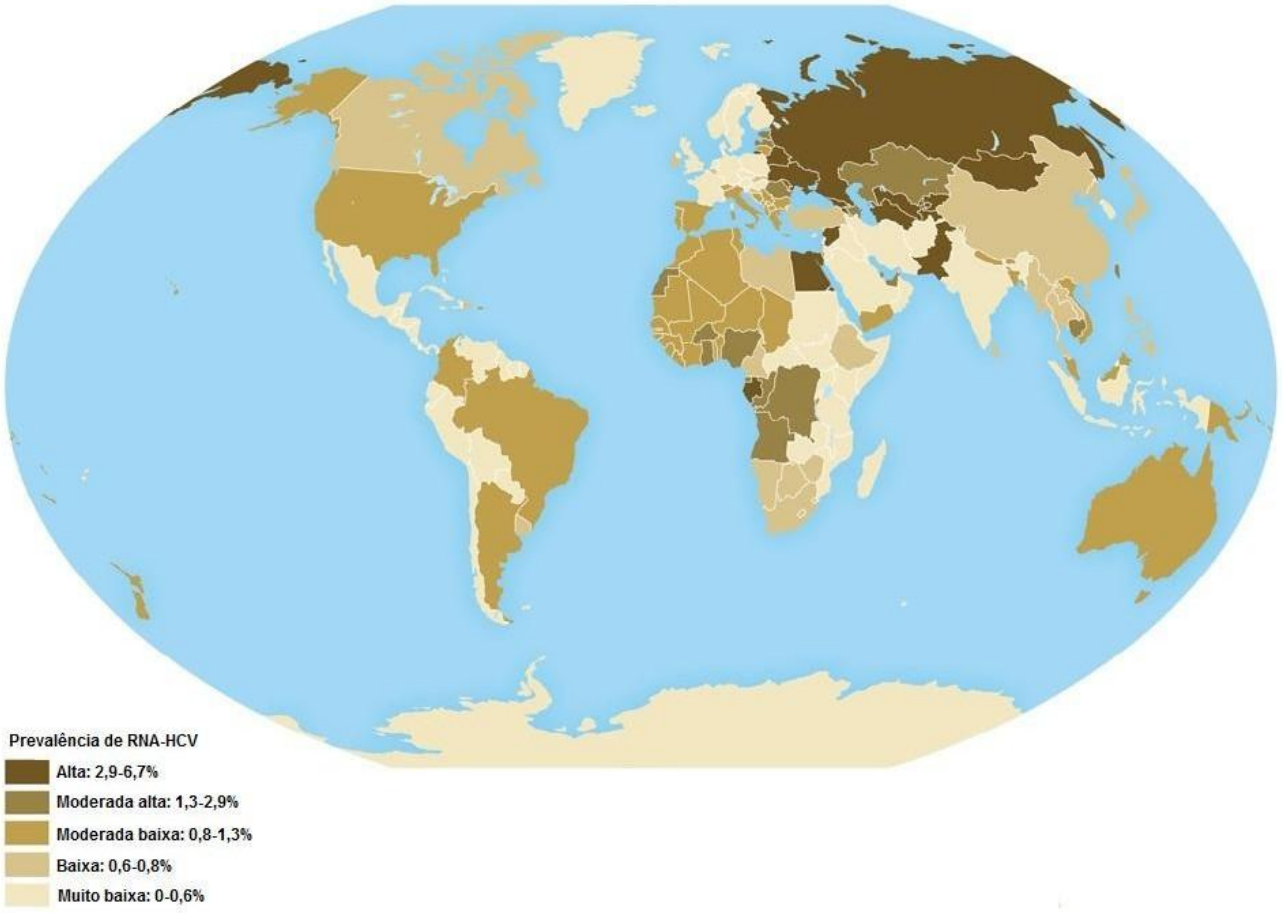

Figura 1.3. Prevalência global de HCV-RNA. Fonte: Adaptado de *Centers for Disease Control and Prevention* (CDC) (2020) [63].

Em estudo de relevância global de Gower *et al.* (2014) [64], a prevalência global de anti-HCV foi estimada em 1,6% correspondendo a 115 milhões de infecções. A maioria dessas infecções, aproximadamente 104 milhões, está relacionada a adultos (definidos como maiores de 15 anos) com uma taxa de anti-HCV de 2,0% [64]. Além disso, destacou-se que a prevalência virêmica seja de 1,4% para adultos, correspondendo a 75 milhões de infecções. Entretanto, o estudo

indicou que, considerando os artigos incluídos para análise de prevalência global, havia relativamente poucas pesquisas relacionadas a crianças, o que dificultou uma análise mais precisa a respeito da prevalência de anti-HCV e RNA do HCV em populações mais jovens.

Considerando dados epidemiológicos do Brasil, Gower *et al.* (2014) [64] definiram uma prevalência virêmica de 0,9%, o qual corroborou com estimativas definidas pelo CDC [63] que incluiu o Brasil na faixa de prevalência moderada baixa variando entre 0,8 a 1,3%. De acordo com o Boletim Epidemiológico de Hepatites Virais publicado em 2019 pelo Ministério da Saúde do Brasil [65], 632.814 casos confirmados de hepatites virais entre 1999 e 2018 foram notificados no Sistema de Informação de Agravos de Notificação (Sinan), sendo que destes, 359.673 (57%) referem-se aos casos de hepatite C ao considerar apenas um dos marcadores reagente (anti-HCV ou RNA-HCV reagente). Além disso, de 2000 a 2017, 70.671 óbitos associadas às hepatites virais dos tipos A, B, C e D foram identificados pelo Sistema de Informação de Mortalidade (SIM) sendo 76% (53.709) associadas à hepatite C. No Brasil, as taxas de hepatite B apresentaram discreta tendência de queda nos últimos cinco anos, enquanto que a hepatite C mostrou tendência de aumento. Tal fato pode ter sido influenciado pela mudança de definição dos casos para fins de vigilância epidemiológica a partir de 2015, ou seja, os casos, que previamente eram notificados com dois marcadores reagentes, passaram, então, a ser notificados com apenas um deles (anti-HCV ou RNA-HCV reagente). De acordo com o Sinan, ao avaliar por região do Brasil, somente para a região Sudeste, foram 205.740 (57,2%) casos notificados entre 1999 e 2018. No mesmo período, referindo-se à abragência de dados estaduais, o Rio de Janeiro correspondeu a 7,4% (26.658) do total de casos do Brasil que foram notificados com um dos marcadores reagentes.

1.5 Transmissão do HCV

O HCV é transmitido principalmente através da exposição percutânea ao sangue, devido a procedimentos médicos ou compartilhamento de instrumentos contaminados para uso de drogas injetáveis [51]. De modo geral, a transmissão sexual é pouco comum e ocorre em relações sem uso de preservativo.

Há também a possibilidade de transmissão vertical, em menor proporção dos casos [66]. As causas de infecções iatrogênicas incluem transfusão de sangue ou administração de fatores de coagulação (com sangue contaminado antes do início da triagem de sangue nos anos 90), hemodiálise em longo prazo, compartilhamento da mesma seringa, realização de tatuagens e falha de esterilização de equipamentos de manicure em ambientes não regulamentados [51]. No passado, a exposição aos cuidados de saúde, com a reutilização de seringas de vidro para injeções médicas, era uma importante fonte de transmissão.

1.6 Manifestações clínicas da infecção pelo HCV

Após a exposição ao HCV, o período de incubação da infecção pode variar entre 2 e 25 semanas (média de 6 a 7 semanas). A infecção pelo HCV dificilmente é diagnosticada na fase inicial que é marcada por uma evolução silenciosa cujos sintomas são inespecíficos e autolimitados [67]. Na maioria dos casos, a infecção aguda ocorre sem sintomas e sem doença clinicamente evidente podendo durar até seis meses. De modo geral, a hepatite C apresenta evolução subclínica: cerca de 80% dos casos têm apresentação assintomática e anictérica, o que dificulta o diagnóstico. Os sinais e sintomas são comuns às demais doenças parenquimatosas crônicas do fígado e costumam manifestar-se apenas em fases mais avançadas da doença [68]. Posteriormente podem aparecer outras manifestações clínicas como dor abdominal, icterícia, prurido, colúria, acolia e artralgias [68].

Um aspecto clínico importante da hepatite C é o alto índice de progressão a cronicidade, sendo que após a exposição ao vírus, 75-85% evoluem para a infecção crônica e, em média, 20% podem evoluir para cirrose ao longo de um período de 20 a 30 anos e 1% a 5% dos pacientes desenvolve carcinoma hepatocelular (CHC) [51]. Antes de desenvolver sintomas, os pacientes podem experimentar a ocorrência de fadiga, perda de peso, dores musculares e articulares ou desconforto abdominal superior direito, dor ou coceira.

A progressão não é necessariamente um processo linear e pode ser acelerado por vários fatores, incluindo idade do paciente, sexo masculino, consumo de álcool e coinfecção com outros vírus, como o HBV e o vírus da Imunodeficiência Humana (HIV) [51]. Muitos pacientes com infecção crônica pelo HCV permanecem

assintomáticos ao longo dos anos e só tomam conhecimento desta doença com risco de vida uma vez que já tenham desenvolvido cirrose. O curso natural da infecção e o resultado clínico da infecção aguda e crônica pelo HCV são como em todas as doenças infecciosas: o resultado da interação entre o agente infeccioso e o hospedeiro. Os níveis séricos de ALT apresentam elevações intermitentes em 60% a 70% daqueles que têm infecção crônica [68]. Nos casos mais graves, ocorre progressão para cirrose e descompensação hepática. A ocorrência de casos fatais da doença está associada a complicações, tais como a hepatopatia crônica e o desenvolvimento de CHC, reforçando a necessidade de identificar a doença precocemente.

1.7 Patogênese da infecção pelo HCV

O HCV é um vírus não citopático que entra na célula hepática e, a partir do processo de replicação, causa necrose celular por vários mecanismos, incluindo citólise mediada por mecanismos imunológicos (respostas imune inata e adaptativa) ou metabólicos como esteatose hepática, estresse oxidativo e resistência à insulina [69]. Após a entrada e replicação do vírus nas células hepáticas, as moléculas virais são transportadas para o retículo endoplasmático e se associam às moléculas do complexo principal de histocompatibilidade (MHC), que são transportadas para a superfície celular. Essas moléculas na superfície celular são reconhecidas pelas células T por sua ação imunológica. A maioria dos linfócitos T citotóxicos é TCD8+ e reconhece antígenos presentes nas moléculas da classe MHC [69]. Um infiltrado linfomononuclear representado principalmente por células TCD8+ desempenha um papel importante na contenção viral, embora outros subconjuntos, como células TCD4+, *Natural Killer* (NK) e células T reguladoras (Treg) também exerçam função similar [70]. No entanto, o HCV desenvolveu mecanismos para evitar o reconhecimento pelos linfócitos T citotóxicos. O vírus reduz a expressão de moléculas de MHC ou impede a apresentação do peptídeo viral na superfície celular [69]. Outro mecanismo de persistência do HCV é a presença de células TCD8+ específicas para o HCV que expressam o gene do receptor *Programmed death-1* (PD-1), um marcador inibitório evidenciado no momento da fase aguda da infecção pelo HCV [70]. A aquisição de um fenótipo de memória e a recuperação de uma função eficiente das células TCD8+ diminuem a expressão de PD-1 nas infecções

por HCV, enquanto que, quando o HCV persiste e as células TCD8+ permanecem disfuncionais, altos níveis de PD-1 são mantidos. Ainda, a ligação de PD-1 provavelmente fornece um sinal inibitório geral para as células Tregs [70].

A fibrose hepática é causada por células inflamatórias do infiltrado intra-hepático secretando citocinas e quimiocinas para ativar células estreladas hepáticas (HSC, do inglês *hepatic stellate cells*) a fim de secretar colágeno [70]. As HSCs podem existir com vários fenótipos, funções e características moleculares diferentes o que contribui significativamente para a homeostase hepática. As células estreladas quiescentes são críticas para o funcionamento metabólico normal do fígado. A lesão hepática provoca a diferenciação do estrelato quiescente ao seu fenótipo ativado, e consequentemente, aumenta a autofagia e a amplificação da lesão parenquimatosa. Através dessas mudanças, as células estreladas ativadas conduzem a resposta fibrótica à lesão e ao desenvolvimento de cirrose. À medida que a lesão hepática diminui, as células estreladas ativadas podem ser eliminadas por uma de três vias: apoptose, senescência ou reversão para um fenótipo inativado [70].

Condições metabólicas, como por exemplo, esteatose hepática, estresse oxidativo e resistência à insulina, podem estar associadas com a patogênese da infecção pelo HCV. A resistência à insulina aumenta a lipogênese, isto é, a síntese de ácidos graxos via superexpressão e maturação da proteína 1 de ligação ao elemento regulador de esterol (SREBP-1c, do inglês *Sterol regulatory element-binding protein-1*). Isso, por sua vez, aumenta as atividades de enzimas lipogênicas, incluindo acetil CoA carboxilase e ácido graxo sintase. Concomitantemente, intermediários da biossíntese de triglicerídeos também ativam inibidores da sinalização de insulina [69]. O estresse oxidativo é uma parte importante do dano hepático induzido pelo HCV através de componentes moleculares do vírus. A proteína do *core* do HCV presente na membrana externa das mitocôndrias induz a oxidação da glutationa e, após esse processo, há um aumento na produção de espécies reativas de oxigênio pelo complexo de transporte de elétrons mitocondriais. Já a proteína não estrutural NS5A do HCV promove a produção de espécies reativas de oxigênio na membrana do retículo endoplasmático, e consequentemente, ativa a liberação de Ca^{2+} induzindo um estresse oxidativo. A proteína não estrutural NS3 induz produção de espécies reativas de oxigênio por ativação da NADPH oxidase [69]. Em relação à esteatose hepática, o HCV pode estimular a ocorrência dessa

condição metabólica de três maneiras diferentes: (i) secreção prejudicada de lipídios dos hepatócitos; (ii) aumento da síntese de ácidos graxos livres; e (iii) degradação prejudicada dos ácidos graxos [69].

1.8 Abordagem diagnóstica da hepatite C

O diagnóstico da infecção pelo HCV baseia-se na detecção de anticorpos anti-HCV (testes indiretos), na detecção do RNA viral e do antígeno do *core* (testes diretos) e técnicas de genotipagem [51]. O RNA do HCV é o marcador molecular que pode ser detectado no soro ou plasma a partir da 1ª semana após a exposição ao HCV, constituindo-se a melhor ferramenta para diagnóstico precoce da infecção aguda [71]. A janela sorológica entre a infecção e soroconversão, quando o anti-HCV se torna detectável, é variável entre duas e oito semanas, portanto, testar apenas o anti-HCV pode significar perda da infecção precoce [51].

De acordo com o Protocolo Clínico e Diretrizes Terapêuticas para Hepatite C e Coinfecções (PCDT) publicado em 2019 pelo Ministério da Saúde do Brasil [72], é importante destacar as definições de hepatite C aguda e crônica com base no diagnóstico laboratorial, apresentadas na tabela a seguir **(Tabela 1.1)**.

Tabela 1.1 Definição de diagnóstico laboratorial para as fases aguda e crônica da hepatite C.

HEPATITE C AGUDA
Soroconversão recente (há menos de seis meses) e com documentação de anti-HCV não reagente no início dos sintomas ou no momento da exposição, e anti-HCV reagente na segunda dosagem, realizada com intervalo de 90 dias;
OU
Anti-HCV não reagente e detecção do HCV-RNA em até 90 dias após o início dos sintomas ou a partir da data de exposição, quando esta for conhecida.
HEPATITE C CRÔNICA
Anti-HCV reagente por mais de seis meses;
E
Confirmação diagnóstica com HCV-RNA detectável por mais de seis meses.

1.8.1 Testes sorológicos

Atualmente, o ensaio imunoenzimático (ELISA, do inglês *enzyme-linked immunosorbent assay*) de terceira geração é utilizado para a detecção do anti-HCV no soro ou plasma [51]. Esse ensaio imunoenzimático é comumente utilizado no diagnóstico laboratorial com sensibilidade estimada em 99% para pacientes na fase crônica da doença [70]. Na ausência de exposição recente ou de supressão imunológica, um resultado negativo indica a ausência de infecção [51]. Os testes de diagnóstico rápido (TR) são utilizados para a detecção qualitativa do anti-HCV, por método imunocromatográfico, na triagem de indivíduos infectados pelo HCV. Uma vantagem adicional é que eles podem ser usados não apenas com soro ou plasma, mas também com sangue total coletado por punção digital. Apenas TRs com desempenho analítico validado (alta sensibilidade e especificidade) devem ser utilizados [51].

1.8.2 Testes moleculares

Uma vez que o RNA do HCV é detectável dentro de alguns dias após a infecção, os testes moleculares baseados na detecção de ácidos nucléicos são eficientes no diagnóstico precoce da hepatite C aguda e devem ser considerados obrigatórios. A detecção e quantificação do RNA do HCV são úteis para diagnosticar a infecção ativa (caracterizada pela replicação do vírus), identificar pacientes com indicação para terapia e avaliar a resposta à terapia antiviral [51]. O teste molecular também representa uma alternativa eficiente para a confirmação dos resultados anti-HCV reagentes por teste sorológico [70].

A detecção e quantificação do RNA do HCV são baseadas em métodos de PCR em tempo real ou de Amplificação Mediada por Transcrição (TMA), que são sensíveis e específicos [51]. A PCR em tempo real é uma metodologia quantitativa que envolve a técnica de PCR com sistema de detecção de sinais fluorescentes cuja fluorescência emitida pela clivagem da sonda específica é detectada por um sensor anexado ao termociclador. A quantidade de fluorescência é proporcional a quantidade de DNA (ou cDNA) presente na amostra. Esses ensaios quantitativos podem detectar e quantificar o RNA do HCV em uma faixa muito ampla, de aproximadamente 10 UI/mL a 10 milhões UI/mL [70]. O sistema de TMA amplifica grandes quantidades de RNA em ensaios isotérmicos que utilizam coordenamente

as enzimas transcriptase reversa (efetua uma cópia de cDNA de fita simples a partir do RNA), RNase H (destrói o RNA do híbrido RNA-cDNA) e RNA polimerase (produz inúmeras cópias do RNA de fita simples). A reação também depende de dois iniciadores, porém, diferentemente da PCR, amplifica o RNA ao invés do DNA [70].

1.8.3 Detecção do antígeno do core

O antígeno do *core* do HCV, através de epítopos que são expressos no nucleocapsídeo do HCV, é um marcador da replicação do HCV e pode ser detectado e quantificado no sangue dos pacientes. Dessa forma, pode ser utilizado como alternativa aos ensaios de RNA do HCV no diagnóstico da infecção e no monitoramento do tratamento antiviral. A detecção e quantificação do antígeno do *core* são baseadas em um ensaio imunoenzimático automatizado. Seu limite inferior de detecção é equivalente a 500-3.000 UI/mL. No entanto, este teste não é amplamente utilizado na prática clínica [51].

1.8.4 Genotipagem do HCV

A determinação de diferentes genótipos do HCV é importante para prever a probabilidade de resposta ao final do tratamento da hepatite C, bem como determinar o esquema terapêutico e a duração da terapia antiviral estabelecida em protocolos clínicos disponibilizados pelo Ministério da Saúde. O método de referência para a determinação dos genótipos e subtipos do HCV é o sequenciamento nucleotídico de regiões específicas do genoma do HCV com posterior análise filogenética. Os ensaios iniciais foram projetados para analisar exclusivamente a região 5' NC, porém altas taxas de classificação incorreta, especialmente a nível do subtipo, justificaram o aprimoramento dos ensaios com análise de regiões codificadoras, em particular as proteínas do *core* e NS5B [70]. Entretanto, outras técnicas também podem ser realizadas, tais como: a técnica de Polimorfismo de Comprimento dos Fragmentos de Restrição (RFLP, do inglês *Restriction Fragment Length Polymorphism*), no qual o produto é submetido à digestão por enzimas de restrição, gerando fragmentos de tamanhos característicos para cada um dos genótipos virais [73], hibridização reversa [70] ou PCR em tempo real, onde sondas marcadas com fluoróforos são específicas para cada genótipo/subtipo do HCV sendo a técnica mais utilizada na rotina laboratorial por minimizar a contaminação a partir da manipulação de produtos amplificados [74].

1.9 Variabilidade genética

1.9.1 Genótipos, subtipos e quasispécies

Uma característica intrínseca do HCV é sua extensa heterogeneidade genética o que corresponde à emergência de diferentes subpopulações virais durante a evolução da infecção no indivíduo [75]. Em 1993, a análise filogenética de sequências da região NS5 do HCV recuperadas de isolados de pacientes de todo o mundo demonstrou que o vírus pode ser classificado em seis genótipos [76]. Em 2015, Murphy *et al.* [77] identificou um novo genótipo infectante do HCV em pacientes originários da República Democrática do Congo. Com os avanços para obtenção de sequências nucleotídicas de genomas completos ou parciais, houve um aumento no número de isolados de HCV sequenciados e, a partir da análise filogenética realizada por Smith *et al.* (2014) [78], foi possível confirmar a existência de sete genótipos e 67 subtipos do HCV. Entretanto, estudo prévio de Borgia *et al.* (2018) [79] confirmou a circulação de um novo genótipo do HCV em quatro pacientes originários do distrito de Punjab na Índia através de sequenciamento do genoma completo do HCV e análise filogenética **(figura 1.4)**. Diante do achado, em Maio de 2019, o Comitê Internacional de Taxonomia Viral (ICTV, do inglês *International Committee on Taxonomy of Viruses*) atualizou a classificação do HCV para oito genótipos e 90 subtipos **(figura 1.5)** [80].

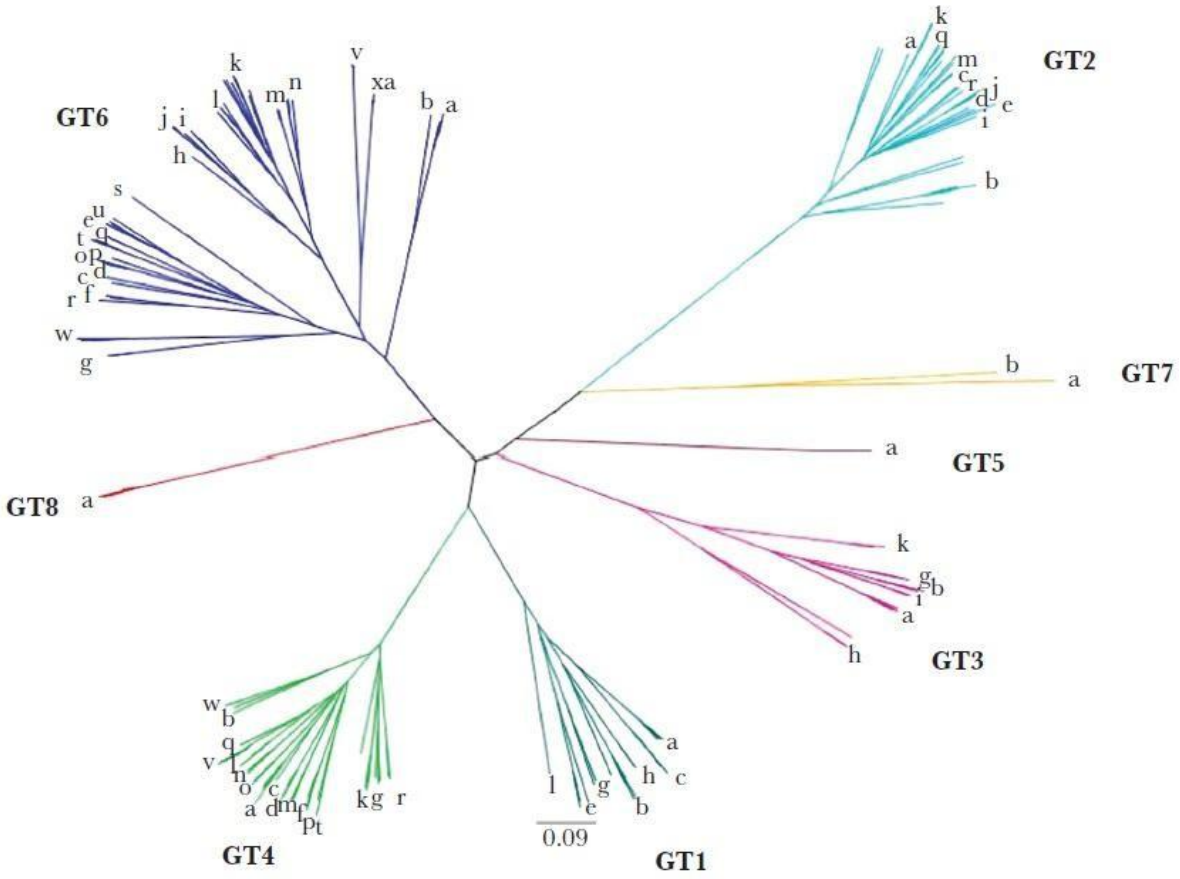

Figura 1.4. Árvore filogenética comparativa composta por sequências dos genótipos e subtipos do HCV. GT = Genótipo. Fonte: Borgia *et al.*(2018) [79].

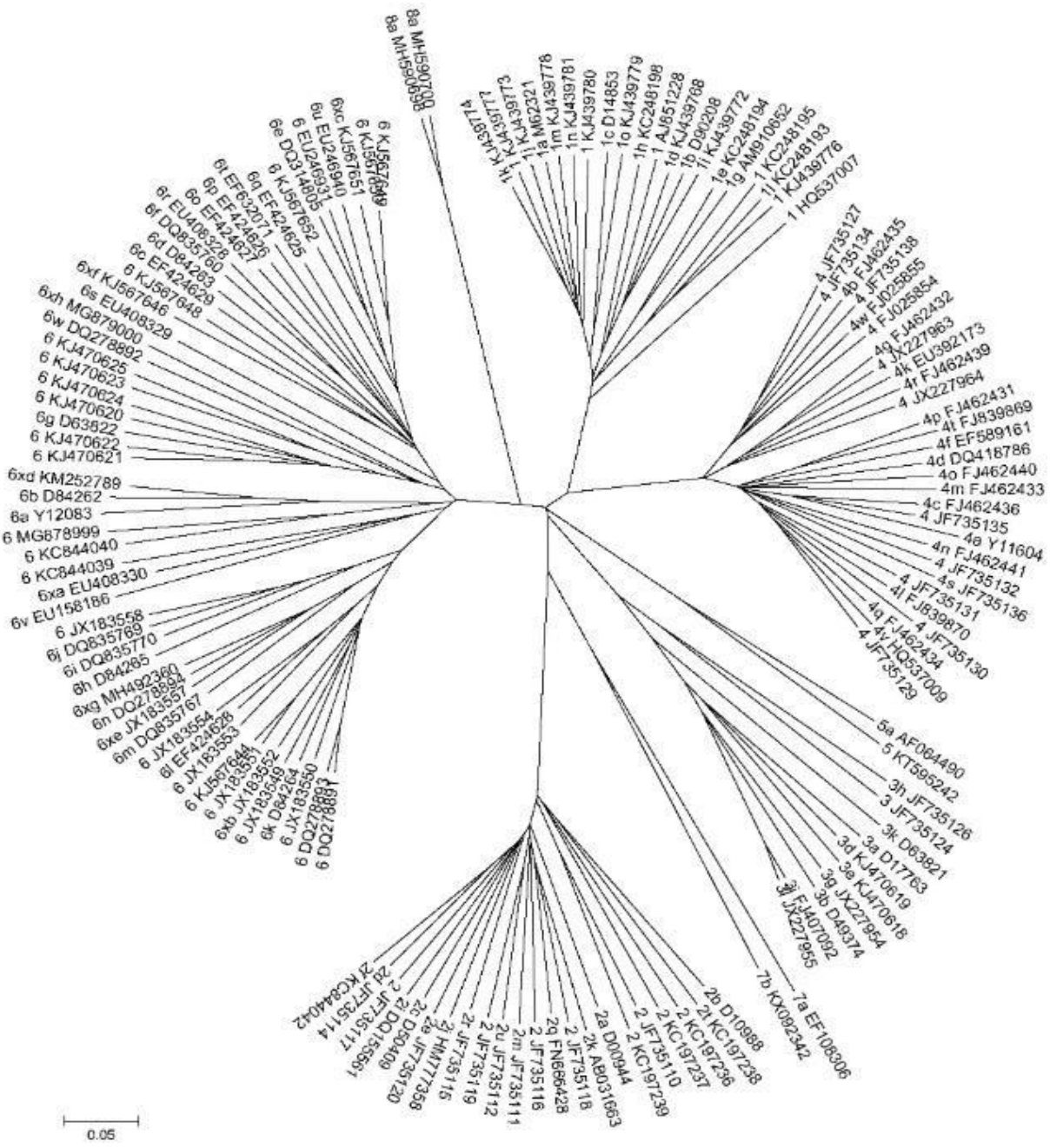

Figura 1.5. Árvore filogenética comparativa composta por sequências dos 8 genótipos e 90 subtipos do HCV. Fonte: Comitê Internacional de Taxonomia Viral [80].

Os genomas codificantes dos isolados do HCV pertencentes a diferentes genótipos diferem em média de 30% a 35% em relação aos nucleotídeos enquanto que os subtipos diferem de 15% a 25% [81]. A elevada taxa de replicação viral e a ausência de atividade de revisão da RNA polimerase RNA dependente do HCV acarretam numa classificação heterogênea e na detecção de subpopulações geneticamente distintas denominadas quasispécies [82]. O conceito de quasispécies foi definido por Eigen *et al.* (1981) [83] como um grupo de moléculas autoreplicantes

que eram diferentes, mas intimamente relacionadas, evoluindo como uma única unidade ao confrontar-se com mudanças no ambiente. Consequentemente, devido à impossibilidade de definir um genoma viral como uma sequência única do vírus infectante em um indivíduo, é considerada a “média ponderada de sequências individuais” [84]. Após uma mudança ambiental, a ampla gama de variantes preexistentes na população original e a geração contínua de novas variantes durante a replicação levarão a uma rápida seleção dos mutantes mais favoráveis para novos ambientes [37]. O termo "seleção positiva" indica situações em que um genótipo se torna dominante em uma população em evolução. O uso do termo quasispécies é justificado *in vivo* porque transmite o conceito de um conjunto de sequências genômicas geradas por um processo dinâmico de emergência de mutações [37]. Mais especificamente, a taxa de mutação do HCV foi estimada entre $1{,}5\times10^{-3}$ a $2{,}0\times10^{-3}$ substituições de nucleotídeo por sítio de nucleotídeo por ano [85]. A evolução natural das infecções virais é definida pela dinâmica das quasispécies, que por sua vez influencia a progressão da doença através de vários parâmetros, como taxa de replicação, carga viral e heterogeneidade genética [86].

1.9.2 Distribuição geográfica dos genótipos do HCV

Embora os genótipos do HCV sejam identificados de maneira cosmopolita, existem diferenças a respeito de sua distribuição geográfica **(figura 1.6)**. Um estudo global evidenciou que os genótipos 1 e 3 são os mais prevalentes, representando 46,2% (83,4 milhões de casos) e 30,1% (54,3 milhões) de todas as infecções, respectivamente. Os genótipos 2, 4 e 6 são responsáveis por um total de 22,8% de todos os casos enquanto que o genótipo 5 compreende menos de 1% do total [87]. Embora os genótipos 1 e 3 sejam mais prevalentes na maioria dos países, independentemente do *status* econômico, as maiores prevalências dos genótipos 4 e 5 estão nos países de baixa renda [87].

Na Europa, cerca de 90% de todas as infecções por HCV correspondem aos genótipos 1, 2 e 3 [87]. Entre os genótipos 1 e 2, os subtipos 1a e 2b são os mais prevalentes no norte da Europa, enquanto que os subtipos 1b e 2c são os mais prevalentes no sul da Europa [75]. As infecções pelo genótipo 3 são representadas quase exclusivamente pelo subtipo 3a. Na maioria dos países das Américas, a maioria das infecções está associada ao genótipo 1 (subtipos 1a e 1b); as infecções

restantes correspondem aos genótipos 2 (em particular 2a) e 3a [87]. O genótipo 3 é encontrado com pouca frequência na África, sendo os genótipos 1, 2 e 4 mais prevalentes na África do Norte e Central e os genótipos 1 e 5 na África do Sul [75]. No Japão, a maioria das infecções são referentes à infecção pelos genótipos 1b e 2a. O genótipo 6 é encontrado em 10 a 20% da população em muitas áreas no leste e sudeste da Ásia. A alta prevalência relativa do subtipo 6a, originalmente identificada em um paciente de Hong Kong [88], é o resultado da disseminação de usuários de drogas intravenosas no Vietnã e Hong Kong [75].

Considerando o Brasil, estudo de Amorim *et al.* (2004) [89] caracterizou a prevalência dos genótipos do HCV em doadores de sangue do Distrito Federal. Dentre 41 pacientes com anti-HCV reagente e HCV-RNA detectável, 60,9% corresponderam ao genótipo 1 e 39,1% ao genótipo 3. Maior prevalência do genótipo 1 (66,1%) seguida do genótipo 3 (33,9%) foi verificada na Bahia [90]. Análise de Campiotto *et al.* (2005) [91] demonstrou uma maior prevalência do genótipo 1 do HCV (64,9%; 1095/1688) seguido dos genótipos 3 (30,2%; 510/1688), 2 (4,6%; 78/1688), 4 (0,2%; 3/1688) e 5 (0,1%; 2/1688). O mesmo estudo identificou que em todas as regiões o genótipo 1 foi o mais frequente (51,7 a 74,1%), atingindo o maior ocorrência na região Norte enquanto que o genótipo 2 foi mais frequente na região Centro-Oeste (11,4%), principalmente no Estado de Mato Grosso (25,8%), e o genótipo 3 foi mais comum na região Sul (43,2%). Em pesquisa de Lampe E *et al.* (2013) [92], um total de 698 sequências parciais do gene NS5B do HCV referentes à sete Estados brasileiros foram analisadas e o genótipo 1 correspondeu à 491 sequências (subtipo 1a: 321; subtipo 1b: 170; genótipo 2: 13; subtipo 3a: 185; genótipo 4: 6; subtipo 5a: 3). Além disso, análise filogenética dos isolados do subtipo 1a de diferentes regiões brasileiras formaram dois clusters monofiléticos, e que a maioria das sequências (98%) se agrupou no clado 1 dentre os dois clados previamente descritos por Pickett BE *et al* (2011) [93]. A pesquisa conduzida por Gower *et al.* (2014) [64] foi utilizada como referência na estimativa global de distribuição de genótipos do HCV conduzida por um grupo de epidemiologistas da "CDA Foundation" a partir de 6754 estudos publicados nas bases de dados bibliográficos PudMed e Embase [94]. Em referência ao Brasil, a revisão sistemática relatou uma maior prevalência do genótipo 1 do HCV (33,4% para o subtipo 1b e 31% para o subtipo 1a) seguidos dos genótipos 3 (30,2%), 2 (4,6%), 4 (0,2%) e 5 (0,1%).

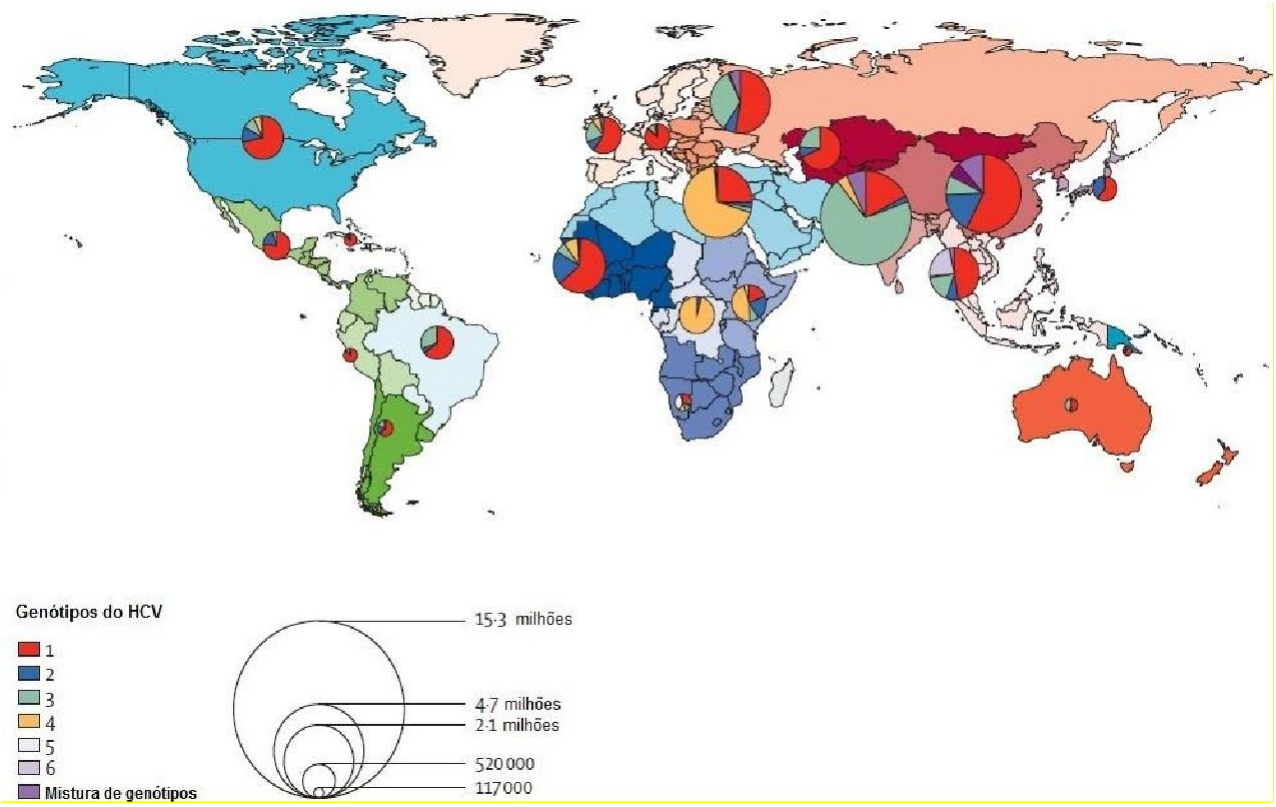

Figura 1.6. Distribuição geográfica dos genótipos do HCV. Fonte: Adaptado de The Polaris Observatory HCV Collaborators (2017) [94].

1.10 Abordagem terapêutica do HCV

1.10.1 Evolução do tratamento da hepatite C no Brasil

A partir de 1996, as hepatites virais foram incluídas na lista de doenças de notificação compulsória do Brasil e, desde então, a coleta de dados sobre essas patologias passou a compor as práticas de vigilância epidemiológica do Ministério da Saúde. O comitê técnico assessor em Hepatites Virais, criado em 2008, apresentava um caráter consultivo a respeito de aspectos técnico-científicos no manejo das hepatites virais [95]. A implantação de diretrizes terapêuticas tornou-se necessária, principalmente para o manejo terapêutico dos casos de hepatite C sendo, então, publicado pelo Ministério da Saúde em 2011, um protocolo clínico com recomendações para a abordagem clínica e terapêutica dos portadores da hepatite C [95]. O guia terapêutico incluiu orientações quanto à prevenção, aconselhamento, acolhimento, abordagem clínica e laboratorial e orientações sobre adesão ao

tratamento e manejo de eventos adversos, buscando assim, potencializar a qualidade de vida dos pacientes e os resultados da terapia antiviral.

O PCDT de 2011 indicou que a detecção precoce da infecção aguda pelo HCV, sintomática ou não, é considerada uma importante medida de controle e que o início tardio da terapia está associado com menores taxas de resposta virológica sustentada (RVS), definida neste protocolo como HCV-RNA indetectável na 24ª semana após o término do tratamento [95]. Logo, recomendava-se iniciar o tratamento imediatamente após o diagnóstico para indivíduos assintomáticos, em média quatro semanas após a exposição, principalmente para pacientes de alto risco de infecção (indivíduos expostos a acidentes com instrumentos perfurocortantes, pacientes de hemodiálise e usuários de drogas endovenosas). Os esquemas terapêuticos para pacientes portadores de hepatite C aguda, que incluíam os agentes indiretos Interferon (IFN) e/ou Ribavirina (RBV), independetemente do genótipo está exposto na **tabela 1.2**. O IFN é uma citocina que compõe a resposta inata do hospedeiro. A adição de uma molécula de polietilenoglicol à molécula do IFN prolonga a ação, eleva a velocidade de absorção, aumenta a meia-vida e reduz o *clearance* do IFN. A RBV é um antiviral análogo de nucleosídeo da guanosina utilizado por via oral.

Tabela 1.2. Esquema terapêutico para hepatite C aguda de acordo com o exposto em protocolo clínico publicado pelo Ministério da Saúde em 2011.

ESQUEMAS TERAPÊUTICOS - HEPATITE C AGUDA
Monoterapia com IFN convencional em dose diária
Primeiras 4 semanas: IFN alfa-2a 6MUI ou IFN alfa-2b 5MUI
20 semanas restantes: IFN alfa-2a 3MUI ou IFN alfa-2b 3MUI
Via de administração subcutânea
OU
IFN alfa-2a 3MUI ou IFN alfa-2b 3MUI **+** RBV 15mg/kg/dia (3x/semana)
IFN: Via de administração subcutânea
RBV: Via de administração oral
Duração do tratamento: 24 semanas

Considerando a infecção crônica pelo HCV, a terapia antiviral tem como objetivo controlar a progressão da doença hepática por meio da inibição da

replicação viral. De forma geral, a redução da atividade inflamatória impede a evolução para cirrose e CHC. Considerando a terapia antiviral com IFN e/ou RBV incluída no PCDT de 2011, alguns fatores virais e do hospedeiro estão associados com uma melhor resposta ao tratamento, são eles: genótipo do HCV não-1, carga viral baixa (<600.000 UI/mL), ausência de fibrose, atividade inflamatória inexistente ou mínima e ausência de obesidade [96]. O protocolo clínico de 2011 destacou um sistema de classificação de alteração arquitetural (estágio de fibrose hepática) denominado escala METAVIR com classificações F0 (fígado sem fibrose), F1 (fibrose leve), F2 (fibrose moderada), F3 (fibrose avançada) e F4 (fibrose severa ou cirrose) [97]. Além disso, a cirrose descompensada distingue-se da compensada por meio do escore de Child-Turcotte-Pugh (Child-Pugh), utilizado para avaliar o grau de deterioração da função hepática. As classes de Child-Pugh são A (escore de 5 a 6), B (7 a 9) ou C (acima de 10). Em geral, a descompensação indica cirrose com um escore de Child-Pugh >7 (classe B de Child-Pugh) [98, 99]. Conforme exposto na **tabela 1.3**, os esquemas terapêuticos para a hepatite C crônica do PCDT 2011 são organizados de acordo com o genótipo infectante do HCV.

Tabela 1.3. Esquema terapêutico para hepatite C crônica de acordo com o exposto em protocolo clínico publicado pelo Ministério da Saúde em 2011.

ESQUEMAS TERAPÊUTICOS - HEPATITE C CRÔNICA	
Genótipo 1	1x/semana: IFN peguilado (Peg-IFN) alfa-2a **ou** Peg-IFN alfa-2b **+** RBV 15 mg/kg/dia (12/12h) Via de administração subcutânea (IFN) e oral (RBV) Duração do tratamento: 48 a 72 semanas
Genótipos 2 e 3	**Na inexistência de fatores preditores de baixa RVS*:** 3x/semana: IFN alfa-2a 3MUI ou IFN alfa-2b 3MUI **+** RBV 15 mg/kg/dia (12/12h) Via de administração subcutânea (IFN) e oral (RBV) Duração do tratamento: 24 semanas **Na existência de fatores preditores de baixa RVS*:** 1x/semana: IFN peguilado (Peg-IFN) alfa-2a 180 mcg **ou** Peg-IFN alfa-2b 1,5 mcg/kg **+** RBV 15 mg/kg/dia (12/12h) Via de administração subcutânea (IFN) e oral (RBV) Duração do tratamento: 24 a 48 semanas

*Escore METAVIR≥ F3 e/ou manifestações clínicas de cirrose hepática e/ou carga viral superior a 600.000UI/mL.

A principal restrição ao uso desses imunomodulares está relacionada à ocorrência de graves efeitos colaterais, como por exemplo, alopecia, distúrbios autoimunes (tireoidite e fibrose pulmonar), depressão, retinopatia, difusão da tireóide, trombocitopenia e neutropenia para o IFN e anemia hemolítica, gota, tosse e dispneia para a RBV [95]. Além disso, baixas taxas de RVS (40-50% para o genótipo 1 e 70-80% para os genótipos 2 e 3), o tempo de terapia prolongado e a administração de alta quantidade de medicamentos por semana também representavam fatores limitantes para uma melhor tolerabilidade e eficácia desses imunomodulares justificando assim a necessidade de desenvolvimento de novos medicamentos [51].

Nos últimos anos, avanços tecnológicos em metodologias moleculares e de cultura de células possibilitaram o melhor conhecimento das etapas do ciclo replicativo do HCV, permitindo assim, a descoberta de diversas moléculas denominadas antivirais de ação direta (DAAs, do inglês *direct-acting antivirals*) que bloqueiam especificamente três proteínas do genoma do HCV: a protease NS3, a polimerase NS5B e a proteína NS5A (figura 1.7) [51, 100, 101]. Os inibidores da NS3 (nomenclatura com sufixo “previr”) se ligam ao sítio ativo da protease e, em geral, apresentam moderada barreira genética para resistência antiviral [102], ou seja, no início do tratamento um rápido declínio de carga viral é evidenciado. Entretanto, variantes resistentes são rapidamente selecionadas e a capacidade replicativa dessas subpopulações virais é mantida ao longo da exposição à droga, o que configura um *breakthrough* viral com o HCV-RNA detectável novamente. Os inibidores da NS5A (nomenclatura com o sufixo “asvir”) inibem a formação da chamada “rede membranosa” durante o ciclo replicativo do HCV já que a NS5A consiste em uma fosfoproteína de ligação do RNA com três domínios, e subsituições de aminoácidos são identificadas no dominio I [103]. Os DAAs voltados para a proteína NS5A apresentam baixa barreira genética para resistência com uma rápida seleção de variantes resistentes com alto *fitness* replicativo ao iniciar a terapia antiviral e persistência das RASs na NS5A por longo período após término do tratamento [102]. Os inibidores da polimerase NS5B (nomenclatura com sufixo “buvir”) são classificados em nucleosídeos (NIs, do inglês *nucleoside inhibitors*), cuja ligação ocorre em sítios ativos da polimerase, e não nucleosídeos (NNIs, do inglês *non-nucleoside inhibitors*) que se ligam a sítios alostéricos da polimerase. Os inibidores nucleosídeos apresentam alta barreira genética para resistência, o que

permite baixas taxas de *breakthrough* viral e baixo *fitness* replicativo de variantes resistentes ao longo da exposição à droga [102].

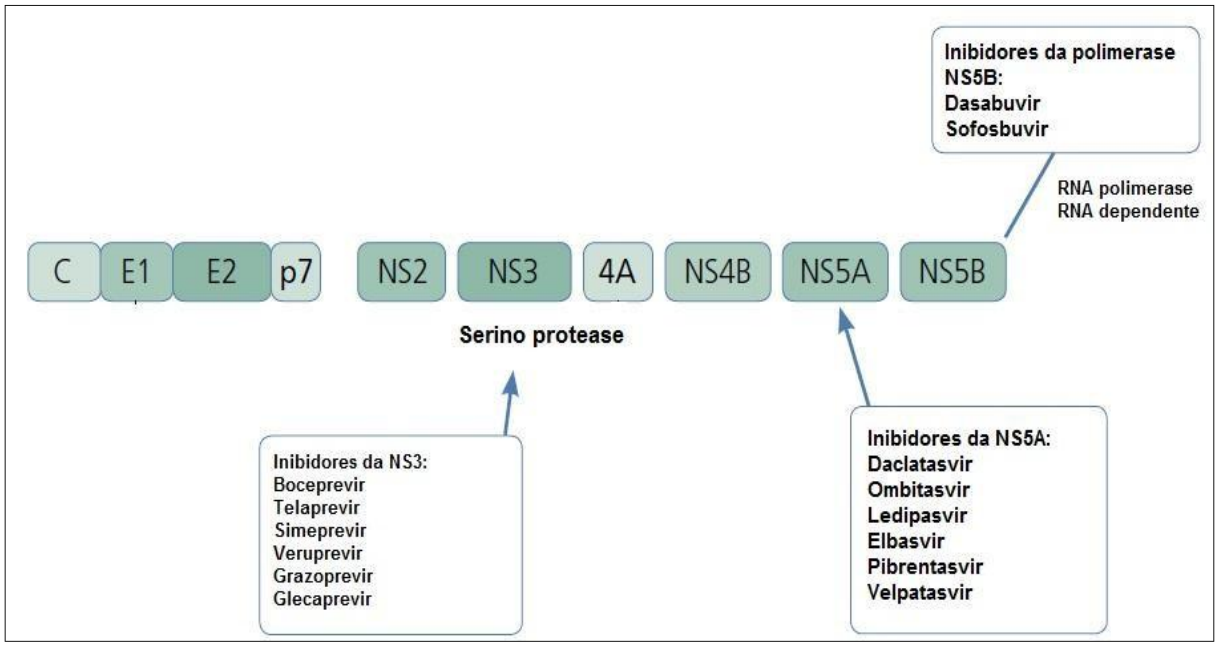

Figura 1.7. Inibidores das proteínas NS3, NS5A e NS5B do HCV. Fonte: Adaptado de Naggie S. *et al*. (2019) [104].

Em 2013, o Ministério da Saúde publicou um novo PCDT com as recomendações de uso clínico para os primeiros DAAs aprovados no Brasil [105]. Os inibidores de protease (IP) de primeira geração ("primeira-onda") telaprevir e boceprevir foram incorporados ao protocolo clínico com o objetivo de tratar pacientes cronicamente infectados pelo genótipo 1 do HCV. O telaprevir estaria recomendado para pacientes com cirrose hepática compensada (METAVIR F4 ou evidências menos invasivas de cirrose) e para pacientes METAVIR F3 nulos de resposta a tratamento prévio com Peg-IFN e RBV **(figura 1.8)**. Já o boceprevir poderia ser utilizado para pacientes com fibrose avançada e cirrose (METAVIR F3 e F4). O tratamento com boceprevir deve obrigatoriamente ser precedido por 4 semanas de uso da terapia dupla com Peg-IFN e RBV. Esse período é denominado "*lead-in*" e é obrigatório no esquema de tratamento com esse DAA **(figura 1.8)**. Importante ressaltar que a terapia tripla envolvendo os IP de primeira geração telaprevir e boceprevir em combinação com Peg-IFN e RBV não é indicada para o tratamento dos pacientes infectados pelos genótipos 2 e 3 do HCV [105].

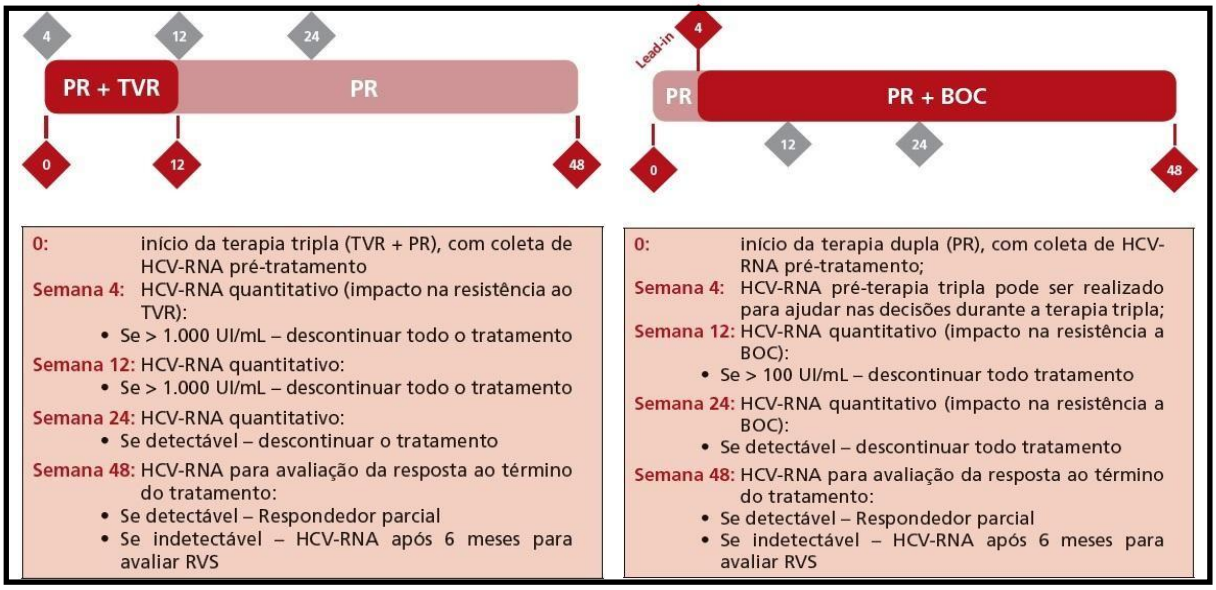

Figura 1.8. Esquemas terapêuticos baseados na terapia tripla entre Peg-IFN, RBV e telaprevir ou boceprevir. Fonte: PCDT 2013 [105].

De acordo com estudos clínicos randomizados, o uso da terapia tripla resultou em maiores taxas de RVS no caso de pacientes virgens de tratamento (61-75%) quando comparada à terapia dupla (38-49%) [106, 107]. Contudo, apesar dos avanços terapêuticos com estes tratamentos, os resultados obtidos ainda não eram considerados satisfatórios por diversos fatores: longo tempo de terapia (24 a 48 semanas), necessidade de ingestão de grande número de comprimidos (10 a 16 comprimidos/dia) associado ao uso de medicamento injetável semanalmente e dificuldade no tratamento do paciente portador de coinfecção HCV/HIV. Além desses fatores, os IP de primeira geração estão associados com efeitos colaterais importantes. No caso do telaprevir, o paciente pode ter náuseas, diarréia, *rash* e anemia. Recentemente, descobriu-se que o telaprevir está relacionado a uma redução da função renal (medida a partir da taxa de filtração glomerular), o que provoca a diminuição da eliminação renal de RBV, e consequentemente, um grau maior de anemia hemolítica. Já o boceprevir, está associado à anemia, dor de cabeça e náuseas [105].

Diante dos fatores apresentados e da necessidade de acesso a novos tratamentos, em 2015, o Ministério da Saúde publicou um novo PCDT [108] com a inclusão de três novos DAAs disponíveis no mercado brasileiro com a aprovação da Agência Nacional de Vigilância Sanitária (Anvisa): sofosbuvir (SOF), um análogo nucleotídeo que inibe a polimerase do HCV (NS5B), simeprevir (SMV), um IP ainda

de primeira geração porém de “segunda onda” e daclatasvir (DCV), um inibidor da proteína NS5A. Esse novo guia terapêutico considera RVS como o resultado de HCV-RNA indetectável na 12ª (em esquemas sem Peg-IFN) ou 24ª semana de seguimento pós-tratamento (em esquemas com Peg-IFN), conforme estipulado pela Associação Europeia para Estudos do Fígado em 2015 [109]. A respeito das indicações de tratamento, a terapia é indicada aos pacientes não tratados anteriormente com DCV, SMV ou SOF, que apresentem resultados de exame indicando fibrose hepática avançada ou cirrose (METAVIR F3 ou F4) ou que apresentem as seguintes condições: coinfecção HCV/HIV, manifestações extra-hepáticas, sinais clínicos ou evidências ecográficas sugestivas de cirrose hepática, insuficiência hepática, insuficiência renal crônica, pós-transplante de fígado, linfoma, gamopatia monoclonal, mieloma múltiplo e outras doenças hematológicas malign as, biópsia hepática com resultado METAVIR F2 presente há mais de três anos e púrpura trombocitopênica idiopática [108].

De acordo com o PCDT de 2015, o tratamento da hepatite C aguda continua semelhante ao apresentado no protocolo clínico de 2011, ou seja, terapia dupla com IFN convencional associado ou não a RBV durante 24 semanas. Já a terapia antiviral para pacientes com hepatite C crônica engloba novos regimes terapêuticos que apresentam vantagens como redução da duração do tratamento e menos efeitos adversos. Os novos DAAs também permitem que o tratamento de pacientes coinfectados HCV/HIV seja realizado de forma análoga ao de monoinfectados pelo HCV [108]. A **tabela 1.4** destaca os esquemas terapêuticos, sua duração e o perfil da infecção pelo HCV correspondentes a cada genótipo infectante.

Tabela 1.4. Esquema terapêutico para hepatite C crônica de acordo com o exposto em protocolo clínico publicado pelo Ministério da Saúde em 2015.

Genótipo	Perfil da infecção	Regime terapêutico	Tempo
Genótipo 1	Monoinfecção HCV	sofosbuvir + simeprevir	12 semanas
	Monoinfecção HCV	sofosbuvir + daclatasvir	12 semanas
	Cirrose Child-Pugh B e C, paciente experimentado com BOC/TEL ou coinfecção HCV/HIV	sofosbuvir + daclatasvir	24 semanas
Genótipo 2	Não há distinção entre perfis	sofosbuvir + ribavirina	12 semanas
Genótipo 3	PR autorizado	sofosbuvir + PR	12 semanas
	PR contraindicado	sofosbuvir + daclatasvir	12 semanas
Genótipo 4	PR autorizado	daclatasvir + Peg-IFN + ribavirina	24 semanas
	PR contraindicado	Sofosbuvir + daclatasvir	12 semanas

Legenda: BOC: boceprevir; TEL: telaprevir; PR: Peg-IFN + RBV

Estudo clínico randomizado COSMOS avaliou o tratamento combinado entre SMV (150 mg) e SOF (400 mg). Foram incluídos pacientes monoinfectados pelo genótipo 1 do HCV previamente tratados com Peg-IFN/RBV ou sem tratamento prévio. O regime terapêutico foi administrado por 24 semanas com ou sem RBV ou por 12 semanas com ou sem RBV. As taxas de RVS foram de 96% (n=27) e 93% (n=14) para esquemas de tratamento de 12 semanas e de 79% (n=24) e 93% (n=15) para tratamentos de 24 semanas com e sem RBV, respectivamente. Em população monoinfectada pelo genótipo 1 do HCV (80% subtipo 1a), portadora de fibrose avançada (METAVIR F3-F4), tanto com falha em tratamento prévio com Peg-IFN/RBV quanto sem tratamento prévio, as taxas de RVS para o mesmo tratamento foram de 93% para grupos tratados por 12 semanas com (n=27) e sem (n=14) RBV e de 93% (n=30) e 100% (n=16) para tratados por 24 semanas com ou sem a adição de RBV, respectivamente [110].

Considerando o esquema terapêutico entre SOF (400mg/1x/dia) e DCV (60mg/1x/dia) com ou sem RBV por 24 semanas, estudo clínico de Sulkowsky *et al.* (2014) [111] incluiu análise de 44 pacientes não tratados anteriormente com infecção pelo genótipo 1 do HCV e 44 pacientes infectados pelos genótipos 2 ou 3 do HCV. O estudo foi expandido para incluir 123 pacientes adicionais com infecção pelo genótipo 1 do HCV (82 pacientes não tratados anteriormente e 41 pacientes que

tiveram falha terapêutica prévia com telaprevir ou boceprevir e Peg-IFN/RBV). No geral, 211 pacientes receberam tratamento. Entre os pacientes com infecção pelo genótipo 1 do HCV, 98% dos 126 pacientes não tratados anteriormente e 98% dos 41 pacientes que falharam previamente aos IPs do HCV obtiveram RVS na semana 12 após o término da terapia. Para os pacientes infectados pelo genótipo 3 do HCV, 89% dos 18 pacientes obtiveram RVS na semana 12 pós-tratamento. Foram observadas altas taxas de RVS entre pacientes com subtipos 1a e 1b do HCV (98% e 100%, respectivamente) e, bem como entre os pacientes que receberam RBV e os que não receberam (94% e 98%, respectivamente). Os eventos adversos mais comuns foram fadiga, dor de cabeça e náusea.

Recentemente, seguindo a ação do Ministério da Saúde do Brasil de incorporar alternativas terapêuticas com DAAs aos portadores crônicos da infecção pelo HCV, um novo PCDT foi disponibilizado no final do ano de 2018 (versão definitiva em 2019) [72] e resumiu os esquemas terapêuticos aprovados pela Anvisa e utilizados na rotina clínica **(tabela 1.5)** com exceção à terapia 3D que não foi prescrita para o tratamento de pacientes crônicos infectados por nenhum genótipo do HCV.

Tabela 1.5. Esquemas terapêuticos para hepatite C crônica disponibilizados pelo Ministério da Saúde de acordo com o ano de inclusão no PCDT.

Data de inclusão no PCDT	Esquemas terapêuticos	Disponibilidade para genótipos/subtipos do HCV
2017	**Terapia 3D** Ombitasvir (12,5 mg) + veruprevir (75 mg) + ritonavir (50 mg) - 2 cp 1x/dia **e** dasabuvir (250 mg) – 1cp 2x/dia - com RBV	Subtipo 1a com monoinfecção HCV sem cirrose; Subtipo 1b com monoinfecção HCV com ou sem cirrose Child-Pugh A.
Junho/2018	SOF (400 mg) + ledipasvir (90 mg) com ou sem RBV 1cp/1x/dia	Pacientes não tratados e experimentados Subtipos 1a e 1b: Não cirróticos (12 semanas) e cirróticos por 12 (Child-Pugh A) ou 24 semanas (Child-Pugh B ou C).
	Elbasvir (50 mg) + grazoprevir (100 mg) 1cp/1x/dia	Pacientes não tratados Subtipo 1a e genótipo 4: Cirróticos (Child-Pugh A) e não cirróticos por 16 semanas (com RBV) ou 12 semanas (sem RBV); Subtipo 1b: Cirróticos (Child-Pugh A) e não cirróticos 12 semanas (sem RBV).
Dez/2018 (versão diagramada) e Março de 2019 (versão definitiva)	Glecaprevir (100 mg) + pibrentasvir (40 mg) 3cp/1x/dia	Pacientes não tratados Subtipos 1a e 1b, genótipos 2, 3, 4, 5 e 6: Cirróticos (Child-Pugh A) por 12 semanas e não cirróticos por 8 semanas; Pacientes experimentados Subtipos 1a, 1b e genótipos 2 e 3: Cirróticos (Child-Pugh A) e não cirróticos por 12 ou 16 semanas (genótipo 3 apenas).
	SOF (400 mg) + velpatasvir (100 mg) 1cp/1x/dia	Pacientes não tratados Subtipos 1a e 1b, genótipos 2, 3, 4, 5 e 6: Cirróticos e não cirróticos por 12 ou 24 semanas; Pacientes experimentados Subtipos 1a, 1b e genótipos 2 e 3: Cirróticos e não cirróticos por 24 semanas.

Legenda: Experimentados: tratamento prévio com Peg-IFN/RBV ou IPs; cp: comprimido.

Conforme evidenciado na tabela 1.5, os esquemas terapêuticos com DAAs e a duração da terapia antiviral podem variar de acordo com a condição clínica do paciente (*naives* ou experimentados; não cirróticos ou cirrróticos compensados ou descompensados) e com o genótipo/subtipo infectante do HCV. Zeuzem *et al.*

(2014) [112] realizaram um estudo clínico randomizado cuja coorte incluía cerca de 30% de indivíduos com fibrose F2 ou F3 (70% F0-F1) cuja administração da terapia 3D com veruprevir, ritonavir, ombitasvir e dasabuvir associado a RBV por 12 semanas resultou em taxas de RVS de 96% em indivíduos infectados pelo subtipo 1a e de 97% em infectados por 1b. Em pacientes com cirrose compensada (Child-Pugh A) a utilização desse regime por 12 semanas gerou taxa mais baixa para moninfectados pelo subtipo 1a e nulos de resposta a tratamento prévio com Peg-IFN e RBV (RVS na 12ª semana pós-tratamento (RVS12): 80%, n=50).

Em relação à eficácia do regime terapêutico com os DAAs sofosbuvir e ledipasvir com ou sem RBV, estudo clínico de fase III, multicêntrico, randomizado e aberto realizado por Afdhal *et al*. (2014) [113] incluiu 865 pacientes *naive* infectados pelo genótipo 1 do HCV, inclusive aqueles com cirrose compensada. De um total de 865 pacientes que receberam o esquema terapêutico entre sofosbuvir e ledipasvir 99% alcançaram RVS12 (sem RBV) e 97% (com RBV) com terapia por 12 semanas e 98% (sem RBV) e 99% (com RBV) com terapia por 24 semanas. Já em outro estudo clínico dos mesmos autores Afdhal *et al*. (2014) [114], porém com 440 pacientes experimentados previamente com IPs, as taxas de RVS12 foram de 94%, 96%, 99% e 99%, nos grupos que receberam sofosbuvir e lesipasvir sem ou com RBV por 12 ou 24 semanas, respectivamente.

Considerando tratamento com grazoprevir em associação a elbasvir com ou sem a adição de RBV, Lawitz *et al.* (2015) [115] descreveram um estudo de fase II, randomizado, *open-label* e multicêntrico. Um total de 253 indivíduos participaram do estudo, sendo 125 infectados pelo genótipo 1 do HCV e tratados por 12 ou 18 semanas, com ou sem adição de RBV. Dentre os pacientes cirróticos e *naive*, daqueles que utilizaram RBV, 90% alcançou RVS12. Contudo, para os que não utilizaram RBV esse resultado foi de 97%. Já dentre os pacientes previamente tratados, cirróticos ou não, 94% e 91% alcançaram RVS12 com o uso de RBV ou não, respectivamente.

Em referência ao esquema terapêutico entre os DAAs glecaprevir e pibrentasvir, Forns *et al.* (2017) [116] descreveram um ensaio clínico fase III (EXPEDITION-1), multicêntrico que avaliou a eficácia do tratamento em pacientes adultos (idade ≥ 18 anos), infectados pelos genótipos 1, 2, 4, 5 ou 6 do HCV e com cirrose compensada. Todos os pacientes eram virgens de tratamento para os

inibidores das proteínas virais NS3 e NS5A. Os pacientes receberam glecaprevir e pibrentasvir uma vez ao dia por 12 semanas. Entre 07/12/2015 e 04/05/2016 foram incluídos 146 pacientes com cirrose compensada, dos quais 48 (33%) foram infectados pelo subtipo 1a, 39 (27%) pelo subtipo 1b, 34 (23%) pelo genótipo 2, 16 (11%) pelo genótipo 4, 2 (1%) pelo genótipo 5 e 7 (5%) pelo genótipo 6 sendo a maioria virgem de tratamento (n=110, 75%). No grupo de pacientes que recebeu pelo menos uma dose do tratamento, 145 (99%) alcançaram a RVS12 com apenas uma (1%) recaída na semana 8 após o tratamento.

O regime terapêutico entre os DAAs SOF e velpatasvir foi avaliado por Feld *et al.* (2015) [117] em estudo clínico randomizado de fase III, duplo cego e controlado por placebo denominado ASTRAL-1. A pesquisa incluiu pacientes adultos infectados pelos genótipos 1, 2, 4, 5 e 6 do HCV, virgens ou não de tratamento, cirróticos ou não. Ao todo, 706 pacientes foram incluídos no estudo, sendo 624 no grupo intervenção e 116 no grupo placebo. A taxa de RVS12 entre os pacientes que receberam SOF e velpatasvir foi de 99%, sendo similar em todos os genótipos, com exceção para os pacientes infectados pelo genótipo 5 (< 97%). Dentre os pacientes cirróticos, 99% alcançaram RVS12, independentemente do genótipo. Nenhum dos pacientes do grupo placebo apresentou RVS12.

1.10.2 Coinfecção HCV/HIV

De acordo com dados da OMS, aproximadamente 2,3 milhões (6,2%) dos 3,7 milhões de indivíduos infectados pelo HIV globalmente apresentem evidência sorológica de infecção passada ou recente pelo HCV [118]. As infecções por ambos os vírus estão associadas a problemas de saúde pública com modos de transmissão sobrepostos e populações de risco semelhantes, como por exemplo, usuários de drogas intravenosas [119]. A infecção pelo HIV reduz a probabilidade de uma eliminação espontânea do HCV e, ainda, parece acelerar o curso da doença hepática associada ao HCV para cirrose e CHC [120]. Pacientes coinfectados HCV/HIV têm um risco maior de progressão para cirrose descompensada do que pacientes monoinfectados pelo HCV, principalmente àqueles com baixas contagens de linfócitos TCD4+ [121]. O regime terapêutico com IFN e RBV para pacientes coinfectados HCV/HIV promovia baixas taxas de RVS, além da ocorrência de eventos adversos. Entretanto, com a evolução no tratamento da hepatite C crônica

desencadeada pelo licenciamento dos DAAs, o mais recente PCDT disponibilizado pelo Ministério da Saúde indica a terapia antiviral para a hepatite C para todos os adultos (≥18 anos) coinfectados HCV/HIV, independentemente do estadiamento de fibrose hepática ou da contagem de células linfócitos TCD4+ [72]. Além disso, para os pacientes cujo diagnóstico de ambas as infecções ocorra simultaneamente, é aconselhável iniciar, primeiramente, o tratamento para o HIV e atingir a supressão viral antes de iniciar o tratamento para a hepatite C, em especial para aqueles pacientes com imunossupressão grave (contagem de linfócitos TCD4+ <200 células/mm^3) [72].

1.10.3 Resistência antiviral

Embora a evolução dos regimes terapêuticos livres de IFN tenha permitido o alcance de melhores taxas de RVS, o termo "resistência antiviral" aos DAAs tornou-se cada vez mais relevante no sentido da necessidade de desenvolvimeto de novas estratégias de tratamento, principalmente para os pacientes não respondedores a terapias com DAAs em geral. A elevada taxa de replicação viral (10^{12} vírions/dia) e a ausência de atividade de correção da RNA polimerase RNA dependente do HCV resulta na emergência de subpopulações virais (quasispécies) ao longo do processo replicativo do HCV propiciando uma condição de mutagênese aleatória [24]. A distribuição de quasispécies circulantes reflete um equilíbrio entre a aptidão replicativa de cada variante (*fitness* viral), a geração contínua de novas variantes e a pressão seletiva positiva aplicada pelo ambiente [122]. Além disso, a presença de subpopulações virais permite uma vantagem evolutiva considerável já que uma determinada variante viral pode se adaptar mais rapidamente a um ambiente [24].

As subpopulações virais diferem entre si por substituições de aminoácidos localizadas em uma região protéica viral que foram geradas durante a replicação. A seleção de estirpes virais com substituições associadas à resistência (RASs, do inglês *resistance-associated substituitions*) pode ser considerada um dos fatores limitantes para a ocorrência de falhas terapêuticas por conferir redução do efeito farmacológico do DAA [123]. Ao longo da infecção pelo HCV, a replicação de diferentes subpopulações virais depende da susceptibilidade aos DAAs administrados e, consequentemente, variantes resistentes são selecionadas positivamente [51]. Quando um DAA é administrado, a seleção positiva de variantes

virais com susceptibilidade reduzida a esse medicamento define o mecanismo de resistência viral. A emergência de mutações ao longo da replicação de variantes resistentes faz com que sua capacidade replicativa supere a de estirpes virais selvagens sensíveis ao efeito farmacológico do DAA [123].

A resistência do HCV aos DAAs é determinada por três fatores principais, sendo eles, a barreira genética das drogas, *fitness* replicativo das variantes resistentes e nível de exposição à droga *in vivo*. A barreira genética destes antivirais, relacionada ao número e tipo de mutações necessárias para a ocorrência de resistência a uma determinada droga, podendo variar de acordo com a classe da droga e o genótipo do HCV [123]. Cada composto ou família de medicamentos apresenta um perfil de RAS específico que vem a ser influenciado pelo genótipo/subtipo do HCV. Além disso, cada classe de DAA apresenta diferença na barreira genética à resistência [124]. Os inibidores análogos de nucleotídeos da proteína NS5B têm uma barreira alta à resistência, por outro lado, os inibidores da proteína NS5A têm baixas barreiras à resistência [123]. A resistência viral também é determinada pelo *fitness* viral das variantes resistentes que corresponde à probabilidade de adequação e persistência dessas estirpes virais ao longo da infecção. Além dos fatores citados, a resistência também é determinada pelo nível de exposição *in vivo* a uma determinada concentração do medicamento e por quanto tempo o mesmo é administrado para que ocorra a inibição da replicação das estirpes virais [123].

A resistência basal corresponde à ocorrência natural de RASs em regiões protéicas virais antes do início da terapia antiviral. Enquanro que RASs primárias podem influenciar no desfecho terapêutico com DAAs ao reduzir sua susceptibilidade *in vivo*, as RASs secundárias (também denominadas substituições associadas ao *fitness*) em diferentes posições de aminoácidos não altera a susceptibilidade à droga, porém atua de forma compensatória aumentando o *fitness* replicativo de variantes resistentes e, permitindo assim, uma vantagem replicativa ao longo da infecção [124-126]. Diversas RASs em posições específicas das proteínas NS3, NS5A e NS5B foram associadas *in vivo* e/ou *in vitro* com reduzida susceptibilidade aos DAAs [125]. No entanto, nem todas atuam de forma clinicamente relevante para um determinado genótipo/subtipo infectante do HCV ou para um DAA específico [127]. A **figura 1.9** demonstra as principais posições de

aminoácidos associadas a diferentes níveis de resistência aos DAAs que atuam bloqueando a função das proteínas não estruturais do HCV.

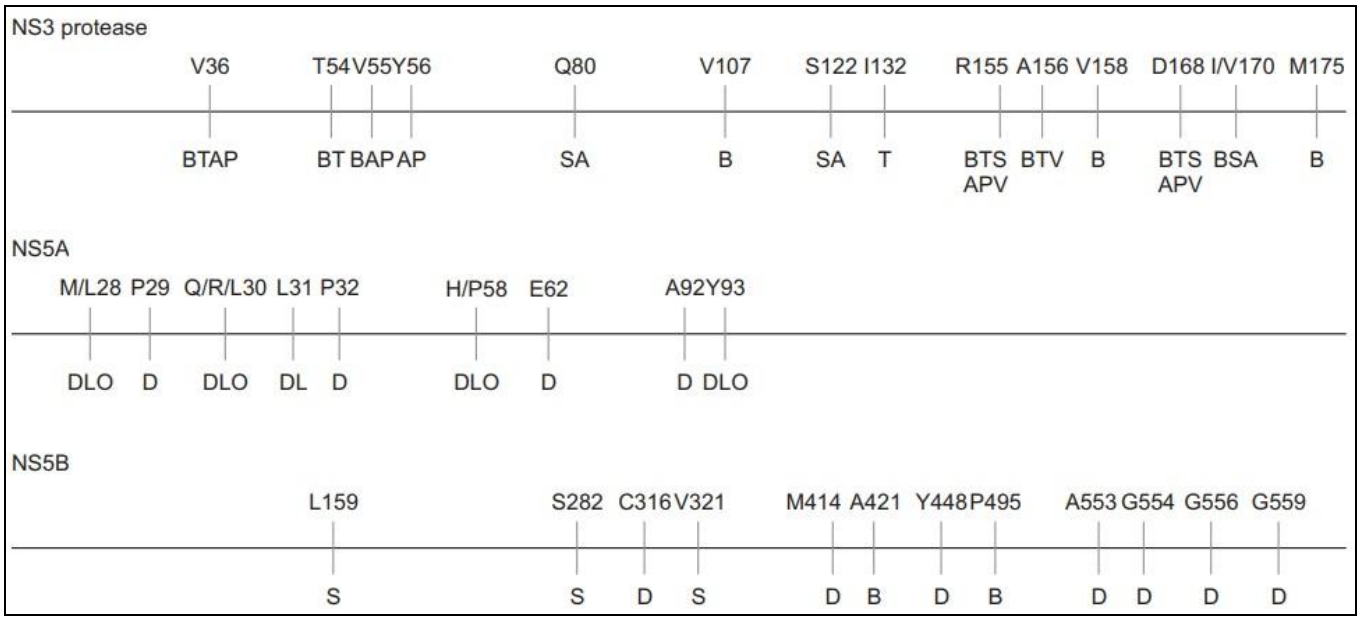

Figura 1.9. Posições de aminoácidos nas proteínas não estruturais NS3, NS5A e NS5B do genoma do HCV relacionadas a diferentes níveis de resistência *in vivo* e/ou *in vitro* aos DAAs. Fonte: Adaptado de Kim *et al.* 2016 [126]. Legenda: B, boceprevir; T, telaprevir; S, simeprevir; A, asunaprevir; P, paritaprevir; V, vaniprevir; D, daclatasvir; L, ledipasvir; O, ombitasvir; S, sofosbuvir; D, dasabuvir e B, beclabuvir

Referindo-se à ocorrência de RASs na proteína NS3 do HCV, a resistência a IPs de primeira geração é caracterizada pela seleção de mutações nas posições 36, 54, 55, 155, 156 e 170. Os pacientes infectados pelo subtipo 1a do HCV selecionam principalmente mutações nas posições 36 e 155. No entanto, considerando infecção pelo subtipo 1b do HCV as posições 54, 55, 156 e 170 são mais relevantes [124]. Importante ressaltar que o número de alterações nucleotídicas na posição 155 necessárias para conferir um perfil de resistência para os subtipos 1a e 1b do HCV é diferente. Por exemplo, para o subtipo 1b do HCV duas alterações de nucleotídeo na posição 155 são necessárias para produzir resistência (R155K: CGG para AAG), enquanto apenas uma é necessária para o subtipo 1a (R155K: AGG para AAG) [124]. Em relação à RAS Q80K, associada a uma redução de 10 vezes *in vitro* da susceptibilidade ao SMV, mas não a outros IPs da segunda onda [128], também já foi evidenciada *in vivo* em estudo clínico ASPIRE cujos pacientes infectados pelo subtipo 1a do HCV com Q80K tiveram uma diminuição significativa na taxa de RVS com o uso de SMV em comparação com pacientes com estirpe selvagem (22% *vs.* 70%, respectivamente) [129].

As RASs na proteína NS5A do HCV são as mais importantes clinicamente e as que persistem na maioria dos indivíduos por mais de dois anos após a suspensão da terapia antiviral. O impacto clínico das mutações basais varia de acordo com o genótipo/subtipo infectante do HCV, com o maior impacto nas infecções pelos genótipos 1a e 3 [125]. Além disso, fatores terapêuticos e do hospedeiro, incluindo a presença de cirrose e falha a tratamento prévio (não baseado em inibidores da NS5A) aumentam o impacto clínico das RASs no tratamento com DAAs [125]. Também é notável a presença de mutações que conferem resistência cruzada entre os inibidores de NS5A atualmente disponíveis. RASs em posições-chave na proteína NS5A (28, 30, 31 e 93) relacionadas ao subtipo 1a do HCV resultam em ampla resistência cruzada aos inibidores da NS5A. As exceções incluem a ausência de impacto da RAS L31M para o ombitasvir e da RAS M28V para o elbasvir ou ledipasvir. Embora o velpatasvir seja menos impactado pelas mutações na proteína NS5A, a RAS Y93H/N relacionada ao subtipo 1a do HCV ainda confere altos níveis de resistência a essa droga. Os inibidores de NS5A de última geração, como por exemplo, o pibrentasvir, mantém atividade contra todas as principais RASs da proteína NS5A para os subtipos 1a e 1b do HCV sendo, portanto, opções para uma possível terapia de resgate [125].

Os inibidores da polimerase NS5B do HCV incluem os NNIs que são um grupo heterogêneo de compostos antivirais com potência intermediária, que se liga a um dos quatro sítios alostéricos na superfície da enzima. Os NNIs, tais como o dasabuvir, utilizado em combinação com outros DAAs na terapia 3D, demonstram uma baixa barreira genética à resistência. Diversas RASs nas posições 316, 414, 448 e 556 estão associadas à falha virológica (*breakthrough* virológico durante o tratamento, ou seja, HCV-RNA indetectável no início da terapia, porém torna-se detectável até mesmo durante o tratamento) e falha terapêutica (HCV-RNA detectável 12 semanas após o fim do tratamento com DAAs) para regimes contendo dasabuvir [127]. Já os NIs mimetizam os substratos naturais da RNA polimerase RNA dependente do HCV e atuam como terminadores de cadeia. Geralmente demonstram uma atividade pangenotípica e uma barreira alta à resistência. O DAA SOF é um exemplo de NI aprovado no Brasil cujas RASs nas posições 159, 282, e 321 podem conferir redução de susceptibilidade *in vitro* e/ou *in vivo* [127].

1.11 Justificativa

A infecção pelo HCV é reconhecidamente um grave problema de saúde pública mundial devido ao alto índice de persistência encontrado nos indivíduos infectados. Estudos nesta área são necessários para observar se a taxa de RVS na prática de rotina clínica apresenta resultados tão satisfatórios quanto os observados nos estudos clínicos randomizados internacionais [130] que destacam a eficiência, segurança e tolerabilidade dos DAAs comparados à terapia dupla com IFN e RBV. Além disso, resultados promissores em pesquisas randomizadas foram obtidos para grupos considerados “difíceis de tratar", tais como, pacientes não respondedores às terapias prévias, cirróticos, transplantados de fígado ou rim, confectados HCV/HIV ou pacientes tratados com múltiplas drogas, aumentando o risco de interação medicamentosa [70]. O conhecimento prévio da presença de RASs em indivíduos virgens de tratamento pode ser um diferencial determinante para o sucesso terapêutico bem como respaldar decisões futuras do Ministério da Saúde na implementação de novos antivirais.

Diante do exposto, a análise de RASs no genoma do HCV em amostras de pacientes de diferentes grupos indicados para o tratamento com DAAs (monoinfectados por HCV e coinfectados HCV/HIV) é essencial para entender os mecanismos de resistência durante o tratamento [108]. Pacientes coinfectados HCV/HIV têm um risco três vezes maior de progressão para cirrose descompensada (cirrose com um escore de Child-Pugh>7 ou classe B de Child-Pugh) do que pacientes monoinfectados [131]. A evolução dos protocolos clínicos com a introdução de novas drogas está sendo determinante para a tomada de decisão entre os médicos, pois, inicialmente, a terapia era limitada aos pacientes com grau de fibrose maior que F2 e não permitia resgate de falha terapêutica [108]. Entretanto, poucos estudos nacionais avaliaram a real eficácia clínica do tratamento com novos DAAs voltados para as proteínas NS5A e NS5B do HCV para esses grupos de pacientes, cuja terapia dupla (Peg/IFN e RBV) e uso de IPs, disponíveis antes de 2015, em geral, apresentavam baixa eficácia, interações medicamentosas e restritas recomendações de uso diante do baixo número de estudos clínicos.

A evolução natural da infecção viral pelo HCV é definida pela dinâmica das quasispécies, que também determina a geração e manutenção de variantes

resistentes a medicamentos [86]. O nível de resistência a uma determinada droga será influenciado, principalmente, pela barreira genética à resistência determinada pelo número e tipo de mutações necessárias para atingir o fenótipo resistente, além do *fitness* replicativo de variantes resistentes e a concentração da droga *in vivo* que será capaz de inibir a replicação viral [123]. Os níveis de subpopulações virais são geralmente transitórios devido à emergência contínua de novas mutações e ao modo como uma variante resistente a medicamentos se replica em relação às demais estirpes da mesma população viral. Uma melhor compreensão da ocorrência de resistência basal no pré-tratamento com DAAs e da dinâmica de seleção de mutações nas subpopulações virais durante o tratamento antiviral para a hepatite C é fundamental na identificação de fatores virais de prognóstico negativo que poderão influenciar ou não o sucesso da terapia antiviral com DAAs. Estudos nesta área são importantes, pois podem fornecer informações úteis ao Ministério da Saúde sobre o entendimento epidemiológico das mutações de resistência e apresentar novos indícios moleculares ao identificar mutações que podem ser usadas para prognosticar a resposta do paciente.

De grande importância é a determinação da prevalência de mutações basais em amostras dos subtipos 1a, 1b e 3a já que são os principais subtipos do HCV circulantes no Brasil fornecendo assim dados científicos brasileiros fidedignos sobre a eficácia e alcance de DAAs na rotina prática terapêutica. A avaliação da presença de RASs no pré-tratamento e uma possível associação com o desfecho terapêutico poderia constituir uma alternativa para a predição de não resposta após terapias combinadas livres de IFN com DAAs. A contribuição científica com a temática de resistência antiviral e análise de RASs estimula o entendimento de informações genéticas do HCV para futuros guias terapêuticos combinados com novos DAAs aprovados pela Anvisa e incluídos em protocolos terapêuticos brasileiros. A manutenção de esquemas terapêuticos com boa relação custo-benefício deve ser respaldada por estudos científicos que relatem a real combinação de fatores determinantes para o sucesso terapêutico e com isso auxiliar na decisão de investimento financeiro do governo brasileiro em medicamentos eficientes no alcance da RVS.

2 OBJETIVOS

2.1 Objetivo Geral

- Determinar o perfil de resistência basal aos DAAs através da identificação de mutações de resistência nas regiões NS3, NS5A e NS5B do genoma do HCV de pacientes cronicamente monoinfectados ou coinfectados HCV/HIV (experimentados ou não) pelos genótipos 1 e 3 e suas possíveis implicações no prognóstico da terapia antiviral.

2.2 Objetivos Específicos

Artigo 1

- Avaliar a prevalência de RASs basais na região NS3 do HCV e sua possível influência no desfecho do tratamento com DAAs em pacientes cronicamente monoinfectados pelo genótipo 1 do HCV sem doença hepática avançada;

Artigos 2 e 3

- Determinar a taxa de RVS ao final do tratamento antiviral com DCV/SOF em pacientes brasileiros com hepatite C crônica buscando associações com os diferentes genótipos de HCV;

- Avaliar a prevalência de RASs basais nas regiões NS5A e NS5B do HCV no período pré-tratamento com DCV/SOF e sua possível influência no desfecho terapêutico em pacientes monoinfectados e coinfectados HCV/HIV pelos genótipos 1 e 3 do HCV.

3 RESULTADOS

Artigo 1. Costa VD, Delvaux N, Brandão-Mello CE, Nunes EP, de Sousa PSF, de Souza Rodrigues LLLX, Lampe E, do Amaral Mello FC. Prevalence of baseline NS3 resistance-associated substitutions (RASs) on treatment with protease inhibitors in patients infected with HCV genotype 1. *Clinics and Research in Hepatology and Gastroenterology* 2019 Nov;43(6):700-706.

Resumo

Introdução e objetivo: O tratamento da hepatite C evoluiu significativamente com o licenciamento dos DAAs. No entanto, um dos fatores limitantes da eficácia da terapia antiviral com IPs é o surgimento de resistência causada por mutações pontuais. O objetivo deste estudo foi determinar a prevalência de RASs na proteína NS3 do HCV em pacientes infectados pelo genótipo 1 antes da terapia com SMV.

Metodologia: Um total de 73 amostras de soro de 15 pacientes com tratamento prévio com BOC/TVR e 58 pacientes virgens de tratamento com DAAs foram coletadas antes da terapia com os DAAs SMV, DCV e/ou SOF. A presença de RASs basais no domínio da serino protease da proteína NS3 do HCV foi analisada por sequenciamento nucleotídico seguido por dedução de aminoácidos.

Resultados: A prevalência geral de RASs neste estudo foi de 13,7% (10/73). A prevalência de RASs para o subtipo 1b do HCV foi de 17,4% (4/23), enquanto para o subtipo 1a do HCV foi de 12% (6/50). As mutações primárias V36M/L e R155K foram observadas apenas no subtipo 1a do HCV, enquanto que T54S e Q80K foram identificados apenas no subtipo 1b do HCV. A RAS V36M, que está relacionada à redução da suscetibilidade aos IPs de segunda geração, foi a mais frequente no estudo (6,9%; 5/73).

Conclusões: Nossos resultados indicaram que os isolados brasileiros de HCV apresentam um padrão distinto de RASs dependendo do subtipo viral infectante. Em contraste com dados de outros países, a prevalência de RAS Q80K no Brasil é baixa no subtipo 1a do HCV. Este estudo aprimora o conhecimento da barreira genética para resistência a IPs envolvendo RASs em pacientes cronicamente infectados e seu possível impacto em um resultado de tratamento malsucedido, informação que pode ser crucial para as decisões futuras de incorporação de novos DAAs nas diretrizes brasileiras de terapia antiviral contra a infecção pelo HCV.

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ORIGINAL ARTICLE

Prevalence of baseline NS3 resistance-associated substitutions (RASs) on treatment with protease inhibitors in patients infected with HCV genotype 1

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KEYWORDS
HCV;
RAS;
Protease inhibitors

Summary
Background and aims: Treatment for hepatitis C has evolved significantly with the licensing of direct-acting antiviral drugs (DAAs). However, one of the limiting factors of the effectiveness of antiviral therapy with protease inhibitors (PIs) is the emergence of resistance caused by point mutations. The aim of this study was to determine the prevalence of resistance-associated substitutions (RASs) in HCV NS3 gene in patients infected with genotype 1 before therapy with simeprevir.
Methods: A total of 73 serum samples from 15 treatment-experienced patients with boceprevir/telaprevir and 58 DAA-naïve patients were collected before therapy with DAAs simeprevir, daclatasvir and/or sofosbuvir. Presence of baseline resistance-associated substitutions (RAS) in the serine protease domain of HCV NS3 was analyzed by nucleotide sequencing followed by amino acid deduction.
Results: Overall RAS prevalence in this study was 13.7% (10/73). RAS prevalence for HCV subtype 1b was 17.4% (4/23) while for HCV subtype 1a was 12% (6/50). Primary mutations V36M/L and

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R155K were observed only in HCV subtype 1a, whereas T54S and Q80K were identified only in HCV subtype 1b. RAS V36M, which is related to reduction of susceptibility to second-generation PIs, was the most frequent in the study (6.9%; 5/73).
Conclusions: Our results indicated that Brazilian isolates of HCV present a distinct pattern of RAS depending on the infecting viral subtype. In contrast to data from other countries, RAS Q80K prevalence in Brazil is low in HCV subtype 1a. This study improves the knowledge of genetic barrier for resistance to PIs involving RASs in chronically infected patients and its possible impact on an unsuccessful treatment outcome, information that might be crucial to upcoming decisions of incorporation of new DAAs in Brazilian guidelines of antiviral therapy against HCV infection.

Introduction

It is estimated that 71 million people worldwide are chronically infected with hepatitis C virus (HCV) and the number of deaths each year mostly from cirrhosis and hepatocellular carcinoma is approximately 399,000 [1]. An important clinical aspect of hepatitis C is the high rate of progression to chronicity observed in about 85% of individuals infected by HCV. A substantial fraction of these chronic carriers might develop progressive liver fibrosis, eventually leading to cirrhosis and hepatocellular carcinoma (HCC) [2]. HCV is classified in seven genotypes (1—7) and 67 subtypes [3]. The most common subtypes in Western countries are 1a and 1b [4].

Until 2011, the therapy for chronic hepatitis C was based in a combination of pegylated-interferon and ribavirin (peg-IFN/RBV), a long-term therapy that, besides the severe undesirable side effects [5], had not produced encouraging results in sustained virological response (SVR) mainly for patients infected with HCV genotype 1 [6]. Due to this unsatisfactory therapeutic approach, a regimen with fewer side effects, reduced rates of patient withdrawal and increased effectiveness in preventing the progression to decompensated cirrhosis and HCC has been the focus of several studies of drug development and clinical trials during the last decade. Advances in cell cultures lineages permissive to HCV infection had represented a milestone in understanding about viral life cycle. Along with the three-dimensional computational modelling of viral proteins, several molecules capable of a specific inhibition of proteins acting in different stages of virus replication have been developed and nominated as direct-acting antiviral drugs (DAAs) [7]. Among the targets for DAA is the HCV NS3 serine protease, a protein that forms a non-covalent complex with NS4A and is responsible for the cleavage of the non-structural portion of the translated viral polyprotein.

In 2013, first-wave, first-generation drugs telaprevir and boceprevir was the first protease inhibitors (PIs) incorporated in Brazilian Clinical Guidelines for the treatment of patients infected with HCV genotype 1. Based on this 2013 Clinical Guideline, telaprevir could be used for both naïve and experienced patients whereas boceprevir was only indicated for treatment-naïve patients with advanced fibrosis METAVIR F3 and F4. Its combination with peg-IFN/RBV yielded an improvement in the SVR rate up to 75% [8—12]. Nonetheless, significant side effects and unsatisfactory efficiency against genotype 1 highlighted the necessity of development of compounds targeting different viral proteins in order to achieve higher SVR rates and viral clearance. More recently, other HCV PIs have been incorporated to Clinical Guidelines and can be prescribed irrespective of patients' treatment records. Simeprevir, a second-wave, first-generation NS3/4A PI, daclatasvir (NS5A inhibitor) and sofosbuvir (NS5B polymerase inhibitor) were approved for clinical use in Brazil in 2015. Its genotypic coverage is broader than that of the first-wave drugs, including at least genotypes 1, 2, and 4 [13]. In 2017, paritaprevir in combination with ombitasvir, ritonavir and dasabuvir has been licensed in Brazil for subtype 1a patients without cirrhosis and for subtype 1b patients with compensated cirrhosis (Child—Pugh A). A second-generation PI, grazoprevir, co-administrated with elbasvir was incorporated in clinical protocol for patients infected with HCV genotype 1 and 4. Pangenotypic PIs, such as glecaprevir and voxilaprevir, are not yet registered in Brazilian regulatory agency however represents a promising alternative for patients with or without cirrhosis.

The occurrence of naturally HCV NS3 resistance-associated substitutions (RAS) affects virological outcome of DAA-based combination therapies [14—19]. For the majority of NS3 protease inhibitors the frequency of natural occurrence of single RASs in HCV genotype 1-infected patients is between 0.1% and 3.1% [20] and patients who failed to respond to simeprevir treatment had mutations at NS3 positions 80, 122, 155, and/or 168 [21]. Naturally occurring resistance have been reported in 4.1% to 18.9% of HCV infected patients with baseline NS3 mutations [22,23]. Detecting resistant variants at baseline in treatment-naïve patients infected with genotype 1 strains could represent an important background information to a more specific and efficient clinical conduct. The aim of this study was to determine the prevalence of naturally occurring RASs in the serine protease domain of HCV NS3 region in patients chronically infected with subtypes 1a and 1b.

Table 1 Patients characteristics according to HCV subtype.

Characteristics	Subtype 1a ($n = 50$)	1b ($n = 23$)	Total
Gender			
Male	27	8	35
Female	23	15	38
DAA-experienced patients (group 1) (telaprevir/boceprevir)	14	1	15
DAA-naïve patients (group 2)	36	22	58
Mean HCV viral load (IU/mL log10) ± SD	5.76 ± 0.63	5.77 ± 0.55	5.76 ± 0.6

Patients and methods

Study population

This study enrolled 73 individuals chronically infected with HCV genotype 1 who attended the Ambulatory of Viral Hepatitis (Oswaldo Cruz Institute) and Gaffrée & Guinle University Hospital between 2013 and 2016. Of the 73 individuals, 15 (1a: 14; 1b: 1) were treatment-experienced with first-wave, first-generation PIs boceprevir/telaprevir (group 1) and 58 (1a: 36; 1b: 22) have not been experienced with DAAs (group 2) (Table 1). Serum samples were collected before therapy with DAAs simeprevir, daclatasvir and/or sofosbuvir approved in 2015. The study included patients over 18 years old, both female and male, with positive diagnostic for chronic hepatitis C (Anti-HCV reagent for more than six months and confirmation with detectable HCV-RNA) and infected with HCV genotype 1 (subtypes 1a and 1b).

Ethical approval

Written informed consent was obtained from each patient before entering the study. This study was approved by the ethics committee from Oswaldo Cruz Foundation under number 142/01, and by the ethics committee of Gaffrée & Guinle University Hospital under number 204.445.

RNA extraction

RNA extraction from serum samples (200 μL) was done using High Pure Viral Nucleic Acid Kit (Roche Life Science, Mannheim, Germany) following manufacturer's instructions. Extracted RNA was eluted in 50 μL and stored at −70 °C until further analysis.

Reverse-transcription and PCR amplification

Partial NS3 region of HCV genome covering nucleotides 3465—3961 (~500 bp) was amplified by one-step reverse-transcription (RT) with polymerase chain reaction (PCR) followed by a second round of PCR (Nested-PCR) using specific primers for each subtype (Table 2). The first round PCR amplification was carried out using reagents from the Superscript™ III One Step RT-PCR system (ThermoFisher, Massachusetts, USA). RT-PCR mixture contained 10 μM of specific sense and antisense primers, 2X reaction mix, SuperScript™ III RT/Platinum® Taq DNA Polymerase (4 U/μL), RNaseOUT Recombinant Ribonuclease Inhibitor (ThermoFisher) and 5 μL of viral RNA. The conditions for RT-PCR were as follows: 45 °C for 30 min for reverse-transcription followed by an initial activation of DNA polymerase at 94 °C for 2 min, then 40 cycles at 94 °C for 15 sec, 60 °C for 30 sec and 68 °C for 120 sec and a final elongation at 68 °C for 5 min. Five microliters of the product was subjected to a second round of PCR that contained 10 μM of specific sense and antisense primers, 10× PCR buffer, 10 mM of dNTP, 50 mM of MgSO4 and Platinum® Taq High Fidelity (5 U/μL). After an initial denaturation at 94 °C for 2 min, DNA was amplified for 30 cycles at 94 °C for 15 sec, 54 °C for 30 sec and 68 °C for 120 seconds. PCR products of the expected length of 495 and 496 base pairs for subtypes 1a and 1b, respectively, were fractionated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

Table 2 Primers for amplification of NS3 region of HCV subtypes 1a and 1b.

Primers	Polarity	PCR	Position	Subtype	Sequences (5'—3')
1aF1	Sense	RT-PCR	3456—3475	1a	GCCTCYTAGGRTGYATARTYAC
1aR1	Antisense	RT-PCR	3948—3967	1a	ACCGGGGACCTCATRGTTGT
1aF2	Sense	Nested-PCR	3466—3484	1a	GYATARTCACCAGCYTRAC
1aR2	Antisense	Nested-PCR	3942—3961	1a	GACCTCATRGTTGTCTYTAG
1bF1	Sense	RT-PCR	3456—3475	1b	CTACTTGGYTGYATCRTCAC
1bR1	Antisense	RT-PCR	4071—4090	1b	TTGTACCCTTGGGCYGCRTA
1bF2	Sense	Nested-PCR	3465—3484	1b	TGYATCRTCACYAGCCTCAC
1bR2	Antisense	Nested-PCR	3942—3961	1b	GACCGCATRGTRGTYTCCAT

Table 3 Amino acid mutations in the NS3 protein and their association to protease inhibitors used in clinical therapy for subtypes 1a and 1b of HCV.

Amino acid position	RAS	Subtype		Drugs					
		1a (n = 50)	1b (n = 23)	TVR	BOC	SMP	PTV	ASU	GZV
V36	V36L	1	–	RS/RS	R/R	RS/RS	S/S	RS/RS	S/RS
	V36M	4	–	R/R	R/R	RS/RS	RS/S	RS/RS	RS/RS
F43	F43V	–	1	S/S	RS/RS	R/R	S/S	S/S	S/S
T54	T54S	–	1	RS/R	R/R	S/S	S/S	S/S	S/S
Q80	Q80K	–	1	S/S	S/S	R/R	S/S	S/RS	S/S
	Q80H	–	1	S/S	S/S	RS/RS	S/S	S/S	S/S
V36+R155	V36M + R155K	1	–	R/R	R/R	R/R	R/S	R/R	RS/S

S: susceptible; R: resistant; RS: reduced susceptibility (association to resistance, insufficient evidence for clinical outcome); TVR: telaprevir; BOC: boceprevir; SMP: simeprevir; PTV: paritaprevir; ASU: asunaprevir; GZV: grazoprevir.

Nucleotide sequencing

The NS3 nested-PCR products were purified using High Pure PCR Product Purification Kit (Roche Life Science) and DNA concentration of each sample was estimated with Low DNA Mass Ladder (ThermoFisher). Purified products were subjected to nucleotide sequencing reactions in both directions using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and analyzed on an ABI 3730 DNA automated sequencer (Applied Biosystems).

Mutation analysis

The obtained nucleotide sequences were assembled in MEGA version 6.0 [24] to obtain consensus and compared by alignment with NS3 sequences of representative reference strains of each HCV subtype obtained from the Los Alamos HCV Sequence Database [25]. To evaluate the presence of resistance mutations to DAAs, sequences from the HCV NS3 region were analyzed for substitutions in amino acid residues described in the literature associated or not with some degree of PI resistance: V36, Q41, F43, T54, Q80, S122, R155, A156, D168 and V170.

Statistical Analysis

Univariate analyses were used to associate hepatitis C subtypes and groups included in the study. Fisher's exact test and Pearson chi-square were chosen when appropriate to test the significance level of associations, which was assessed at the 0.05 probability level. All analyses were performed using software EpiInfo version 7.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Results

Overall RAS prevalence in this study was 13.7% (10/73). Among 73 patients enrolled in this study, 15 were treatment-experienced with first-wave PIs telaprevir/boceprevir (group 1) and 58 have not been treated with DAAs (group 2). The observation of amino acid residues of HCV NS3 in group 2 identified RASs at positions 36, 43, 54 and 80 in 6/58 (10.3%) patients. Regarding group 1 patients, RASs were identified in 4/15 (26.7%) at positions 36 (n = 3) and 155 (n = 1) in HCV 1a sequences.

Regarding HCV subtypes, the frequency of RAS in subtype 1b was 17.4% (4/23) while in subtype 1a was 12% (6/50). Primary mutations V36M and R155K were observed only in HCV subtype 1a, whereas T54S and Q80K were identified only in HCV subtype 1b. The positions 156 and 168, which are highly related to resistance to PIs, remained conserved in all 73 sequences. The association between amino acid mutations identified in NS3 region and resistance to protease inhibitors for HCV subtypes 1a and 1b is exposed in Table 3.

Regarding patients infected with HCV subtype 1a, 28.6% (4/14) from group 1 presented RASs while resistance strains were identified in 5.6% (2/36) of the individuals from group 2 (P = 0.044). For both groups, substitutions were observed at NS3 residue 36: 3 patients from group 1 presented RAS V36M and a combination of V36M+R155K was identified in another individual. For group 2, resistance strains V36L and V36M were found in two non-experienced patients.

In patients infected with HCV subtype 1b, RAS prevalence for group 2 was 18.1% (4/22). Identified mutations were F43V (1/22; 4.6%), T54S (1/22; 4.6%), Q80H (1/22; 4.6%) and Q80K (1/22; 4.6%). No mutations were found in the patient infected with HCV subtype 1b previously experienced with first-wave DAAs.

Discussion

In this study, 73 patients infected with HCV subtype 1a or 1b were evaluated to identify RASs previously reported in literature. Until the end of 2015, telaprevir and boceprevir were the unique PIs available for hepatitis C treatment in Brazil. However, the licensing of three drugs in 2015 (simeprevir, daclatasvir and sofosbuvir) allowed new combined therapeutic options. Simeprevir, a second-wave PI, was recommended for patients with non-advanced liver disease whereas the combination of DAAs daclatasvir (NS5A inhibitor) and sofosbuvir (NS5B nucleotide analogue inhibitor) was the main therapy for treatment-experienced patients especially those with decompensated cirrhosis. Recently, other PIs were included in clinical protocols for treating genotype 1-infected patients such as paritaprevir

combined with dasabuvir/ombitasvir and grazoprevir with elbasvir.

As expected, RAS prevalence for treatment-experienced patients who failed therapy with first-wave, first-generation drugs telaprevir/boceprevir was higher compared to non-experienced patients likely due to drug-selective pressure. For non-responders to previous therapy, resistance substitutions represent a negative prognosis factor for new treatments with DAAs [26—28]. For 4 patients analyzed in the present study who failed previous therapy, the presence of RAS might have been the cause of non-response while for other telaprevir/boceprevir non-responder patients in whom no RASs were detected, other host or virological factors could have contributed to unsuccessful treatment. Medical records did not describe how long, after the treatment with first-wave drugs, serum samples from non-responder patients were collected. Thus, since it would be expected that wild-type strains could re-emerge as the major viral population some time after the absence of selective pressure imposed by drugs, a therapeutic failure due to the presence of RAS could not be excluded. However, previous DAAs non-response could also be associated with other factors, such as infection with HCV subtype 1a, cirrhosis and high infective viral load [29].

NS3 RAS prevalence in group 2 was lower in subtype 1a (5.6%) compared to subtype 1b (18.1%). The presence of resistance in patients of subtype 1b not exposed to drug-selective pressure suggests a prime infection with RASs strains. Considering both groups, this study identified higher proportion of RASs in HCV subtype 1b sequences when compared to subtype 1a (17.4% vs. 12%), a distinct pattern from that observed in a previous Brazilian study enrolling blood donors, where the presence of RAS in subtype 1a was significantly higher than in subtype 1b (20% vs. 8%) [30]. This might be related to differences in the study population (more than 20% of our samples were from individuals previously experimented with DAAs) and/or which amino acid positions were evaluated and taken in consideration when calculation RAS proportion between subtypes.

According to literature, RASs V36M, T54S, Q80K and R155K are considered primary resistance mutations to different PIs. RASs V36M and R155K can reduce susceptibility to recently approved PIs simeprevir, paritaprevir and grazoprevir [18,31,32]. T54S is associated to resistance for first-wave telaprevir [33]. Q80K is highly associated with resistance to simeprevir [34].

The presence of RAS V36M in HCV strains could be related with therapeutic failure experimented by four patients in a previous treatment with telaprevir. The identification of this substitution in one patient from group 2 suggested a primary infection with a drug-resistant viral variant, an observation that warns for the circulation of resistant strains that could impact the effectiveness of DAAs in the near future. For all five patients with RAS V36M, combined therapy with new generation PIs should not be considered since V36M is associated with resistance to the majority of approved NS3 DAAs. Indeed, since it was already available in Brazil, treatment with sofosbuvir combined with daclatasvir was chosen and all five patients had similar treatment outcome which was undetectable HCV RNA after 12 weeks post-treatment. A study conducted a retrospective analysis to determine the prevalence of resistance mutations among telaprevir-treated patients [33] and V36M mutation was identified in 28/232 (12%) patients which failed telaprevir therapy, thus demonstrating its importance as a mutation indicative of resistance whose poor prognosis does not reveal reliability in the use of first-generation PI. Barnard et al. [35] identified resistance mutations in non-responders to triple therapy with boceprevir/peg-IFN/RBV infected with subtype 1a and concluded that V36M can be a major cause of therapeutic failure with the use of first-wave PIs. Results from the present study demonstrated that mutations at position 36 were found in both DAA-experienced and non-experienced patients included in the study, which indicated that treatment with DAAs other than PI should be considered in order to minimize risk of resistance and achieve SVR in these patients.

Among RASs observed in patients from group 2, RAS T54S was identified in one patient infected with subtype 1b. This mutation had been shown to cause resistance to boceprevir and telaprevir, but not to simeprevir [36]. This was confirmed here since this patient achieved SVR after 12 weeks of treatment with simeprevir. The low prevalence of RAS T54S (4.6%) in patients not treated with DAAs was also reported in previous Brazilian studies [30,37].

In the present study, RAS Q80K was not observed in isolates of subtype 1a and was only detected in one subtype 1b sample from a group 2 patient with compensated hepatic cirrhosis, type 2 diabetes mellitus and systemic arterial hypertension. In 2016, this patient was asymptomatic and decided not to continue with other available DAA therapeutic options. Q80K is most frequently observed in subtype 1a isolates and is rarely detected in HCV subtype 1b [20]. Studies had reported the high prevalence of Q80K mutation in USA (37—47%) [38,39]. Sarrazin et al. [40] evaluated NS3 baseline RASs from 467 patients and results for Q80 polymorphisms demonstrated high prevalence for PI treatment-experienced patients (110/265; 41.5%) and PI treatment-naive patients (93/202; 46%). In contrast to data from other countries, Q80K prevalence in Brazil is low [30,37,41]. Therefore, due to low prevalence of this mutation in Brazilian strains reported in previous studies and corroborated here, there is no need to incorporate pretreatment resistance tests for infected patients with subtypes 1a and 1b of HCV in Brazil. Even with the identification of this variant, the use of other PIs is not limited since there is no evidence with resistance.

RAS R155K is related to resistance to first and second-wave PIs. A study reported a higher frequency of treatment failure for subtype 1a due to low genetic barrier to viral resistance when compared to subtype 1b [13]. Sarrazin et al. [36] described that combination of substitutions V36M + R155K induces high resistance to telaprevir and may inhibit drug action. In the present study, combination of mutations at loci 36 (V36M) and 155 (R155K) was identified in one telaprevir-experienced patient infected with HCV subtype 1a. After 12 weeks of therapy with telaprevir, viral load was 4.74 $\log_{10}$ IU/mL and it was decided to suspend the treatment. RAS R155K confers resistance to all available PIs for subtype 1a strains and new therapeutic options for this patient should target other non-structural HCV proteins. Indeed, in this case, a rescue therapy with NS5B and NS5A inhibitors was selected and HCV RNA was undetectable after 4 weeks.

Substitutions V36L, F43V and Q80H were identified in the present study. RAS V36L is associated with resistance to boceprevir [18]. In 2015, Brazilian clinical practice guidelines on the management of hepatitis C no longer included combined therapies with boceprevir or telaprevir as a treatment option. RAS V36L was identified in one subtype 1a patient from group 2. A 12-week therapy with simeprevir was initiated. V36L was not related with less susceptibility to this drug and SVR was achieved post-treatment.

Analysis of mutations associated with resistance among patients infected with HCV subtype 1b indicated the presence of RAS F43V in a DAA treatment-naïve patient. To our knowledge, this is the first report of this mutation in vivo. Resistance profile for PIs was described in vitro and pointed out F43 locus as associated with resistance to simeprevir [18]. RAS Q80H, which can reduce susceptibility to simeprevir, was identified in one patient from group 2 infected with subtype 1b. Treatment with boceprevir was selected for this patient and Q80H did not influenced treatment response since SVR was achieved after treatment. No Brazilian data have described this mutation among treatment-naive patients.

In conclusion, genetic data from HCV strains circulating in Brazil reported in this study pointed out that the use of simeprevir, paritaprevir, asunaprevir and grazoprevir has a high probability of being effective in our country. The genetic barrier for resistance to PIs can vary according to different genotypes and its specific polymorphisms; hence, this study will contribute to the knowledge of the impact of RASs for HCV subtypes 1a and 1b and your relation to first and second-generation PIs in strains circulating among Brazilian HCV chronic carriers.

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Authors' contribution

V.D.C is the guarantor of the article.

V.D.C and F.C.A.M made the statistical analysis and wrote the paper; E.L. supervised the execution of the project and made critical reading of the manuscript; N.D. performed experiments; C.E.B.M and E.P.N. provided samples and data from patients; P.S.F.S. and L.L.L.X.S.R. provided clinical information. All co-authors approved the final version of the paper.

Disclosure of interest

The authors declare that they have no competing interest.

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Artigo 2. Costa VD, Brandão-Mello CE, Nunes EP, Dos Santos Silva PGC, de Souza Rodrigues LLLX, Lampe E, do Amaral Mello FC. Treatment of chronic HCV infection with DAAs in Rio de Janeiro/Brazil: SVR rates and baseline resistance analyses in NS5A and NS5B genes. *PLoS One*. 2019 May 7;14(5):e0216327.

Resumo

Introdução: A seleção de estirpes virais com RASs nas proteínas NS5A e NS5B do HCV é considerada um dos fatores limitantes para alcançar RVS com a combinação dos DAAs DCV e SOF. Desde 2015, este regime livre de interferon está disponível na rotina clínica brasileira para o tratamento de pacientes monoinfectados HCV e coinfetados HCV/HIV cronicamente pelos genótipos 1 e 3.

Objetivo: Avaliar a taxa de RVS em pacientes brasileiros cronicamente infectados pelos genótipos 1 e 3 do HCV após a terapia com DCV/SOF e a frequência de RASs basais nas proteínas NS5A e NS5B do HCV. Amostras de soro foram coletadas de 107 pacientes monoinfetados e 25 pacientes coinfetados HCV/HIV antes da terapia antiviral. A diversidade genética dos genes NS5A e NS5B foi avaliada por sequenciamento nucleotídico.

Resultados: No geral, a taxa de RVS foi de 95,4% (126/132) e a falha do tratamento ocorreu em cinco pacientes monoinfetados e um coinfetado HCV/HIV. A frequência de RASs na proteína NS5A foi maior para pacientes coinfectados (28%) do que para pacientes monoinfetados (16,8%). Nenhuma diferença foi evidenciada entre os grupos mono- e coinfectados (15% *vs.* 16%) em relação à proteína NS5B. As estirpes do subtipo 1b tiveram significativamente mais substituições basais na proteína NS5A (31,6%) do que os subtipos 1a e 3a. Pelo menos uma RAS primária na proteína NS5A descrita na literatura nos *lóci* 28, 30, 31 ou 93 foi identificada em estirpes referentes ao genótipo 1 para ambos os grupos. Quanto à NS5B, as RASs nas posições 159 e 316 foram observadas apenas nas estirpes do subtipo 1b. Este estudo destacou que a taxa de RVS na rotina clínica no Brasil foi semelhante aos ensaios clínicos randomizados (89–98%).

Conclusão: Nossa pesquisa forneceu dados genéticos sobre a circulação de variantes resistentes no Brasil. Apesar de sua presença, a maioria das mutações basais identificadas não impactou negativamente o resultado do tratamento. A diversidade genética das estirpes circulantes sugere que a maioria dos portadores crônicos do HCV no Brasil são suscetíveis a novos regimes terapêuticos, incluindo DAAs recentemente aprovados.

PLOS ONE

RESEARCH ARTICLE

Treatment of chronic HCV infection with DAAs in Rio de Janeiro/Brazil: SVR rates and baseline resistance analyses in NS5A and NS5B genes

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Abstract

The selection of viral strains with resistance-associated substitutions at hepatitis C virus (HCV) NS5A and NS5B genes is considered one of the limiting factors for achieving sustained virologic response (SVR) to combination of direct-acting antivirals daclatasvir (DCV) and sofosbuvir (SOF). Since 2015, this interferon-free regimen has been available in Brazilian clinical routine for treating mono- and HCV/HIV-coinfected patients chronically infected with genotypes 1 and 3. Our aim was to assess SVR rate for Brazilian patients chronically infected with genotypes 1 and 3 after DCV/SOF therapy and the frequency of baseline RASs in HCV NS5A and NS5B genes. Serum samples were collected from 107 monoinfected patients and 25 HCV/HIV co-infected patients before antiviral therapy with DCV/SOF. Genetic diversity of NS5A and NS5B genes was assessed by direct nucleotide sequencing. Overall, SVR rate was 95.4% (126/132), and treatment failure occurred in five monoinfected and one HCV/HIV co-infected patient. NS5A RASs frequency was higher for HCV/HIV patients (28%) than monoinfected patients (16.8%). No difference was evidenced between mono- and HCV/HIV-coinfected groups (15% vs. 16%) regarding NS5B gene. Genotype (GT) 1b strains had significantly more baseline substitutions in NS5A (31.6%) than GT 1a and 3a. At least one primary NS5A RAS described in literature at *loci* 28, 30, 31 or 93 was identified in HCV GTs 1 strains for both groups. As for NS5B, RASs at positions 159 and 316 was observed only in GT 1b strains. This study highlighted that SVR rate in clinical routine in Brazil was similar to randomized clinical trials (89–98%). Our research provided genetic data about the circulation of resistant variants in Brazil. Despite its presence, most of identified baseline mutations did not negatively impact treatment outcome. Genetic diversity of circulating strains suggested that most of the Brazilian HCV chronic carriers are susceptible to new therapeutic regimens including recently approved DAAs.

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Introduction

It is estimated that 71 million people worldwide are chronically infected with hepatitis C virus (HCV) and approximately 2.3 million individuals have HCV/HIV-coinfection [1]. From 2015, epidemiological data demonstrated that about 200,000 hepatitis C cases were notified in Brazil considering anti-HCV or HCV-RNA reactive [2].

The availability of all-oral direct-acting antivirals (DAAs) has further increased sustained virological response (SVR) rates, the primary objective for a successful therapy, usually evaluated 12 weeks after treatment conclusion [3]. These drugs target viral non-structural proteins NS3/4A, NS5A and NS5B. In 2015, Clinical Guidelines for the Treatment of Hepatitis C and Coinfections published by Brazilian Ministry of Health, included the administration of NS5A inhibitor daclatasvir (DCV) in combination with NS5B nucleotide analogue sofosbuvir (SOF) with or without ribavirina (RBV) in a daily regimen. The inclusion criteria for treatment with this DAA regimen was monoinfection with HCV GTs 1, 3 and 4 and an advanced liver fibrosis (treatment-naïve or – experienced) and HIV co-infection in patients infected with HCV GT 1 regardless of liver fibrosis stage [4]. Recently, an update in treatment guidelines has determined the incorporation of new DAAs options for patients infected with GT 1a, such as: (1) NS3/4A protease inhibitor (PI) paritaprevir boosted with ritonavir (PTV/r) plus NS5A inhibitor ombitasvir (OBV) in combination with a non-nucleoside polymerase inhibitor dasabuvir (DSV); (2) SOF plus ledipasvir (LDV) and (3) elbasvir (EBV) and grazoprevir (GZV) [5].

Despite the promising results, the selection of viral strains with resistance-associated substitutions (RASs) at HCV NS5A and NS5B genes can be considered one of the limiting factors for failures to DAA combinations. Viral resistance is characterized by positive selection of viral variants that carry amino acid substitutions responsible to reduce susceptibility to certain drug [6]. Each family of drug exhibits a specific RAS profile that is influenced by HCV GT and is characterized by a difference in the genetic barrier to resistance [7]. Naturally occurring primary RASs in viral subpopulations can affect therapy effectiveness after drug selective pressure [8]. Despite primary RASs usually compromise viral fitness in comparison to wild-type strains, compensatory amino acid substitutions that enhance or restore replication capacity might be selected in resistant variants leading to a viral breakthrough and treatment failure [6].

Considering drug-specific RASs, mutations with high fold change seem to have increased clinical relevance in inducing treatment failure when associated with drug- and host-related factors (presence of cirrhosis and prior HCV treatment) [9]. RASs in HCV NS5A have the major impact on clinical routine and persist for years after treatment [10]. Considering NS5A protein, RASs at amino acid positions M/L28, Q/R30, L31, H/P58, E62 and Y93 can reduce susceptibility to DCV. In relation to the NS5B protein, substitutions at residues L159, S282, V321 and C316 can determine resistance to SOF [7, 9, 11, 12].

Few studies in Brazil have evaluated the effectiveness of DAA therapeutic regimens in Brazilian patients (represented by SVR rate achieved in clinical routine) and the role of RASs in a flaw treatment outcome [13]. Researches in this area can provide background information to Brazilian Ministry of Health on the actual response in practice clinical routine of chronic carriers in the face of financial efforts to provide DAAs regimen as a public health policy. In addition, they provide an evaluation of the circulation of resistant variants in Brazil and its possible impact in therapeutic failure with licensed DAAs and those in phase III clinical trials. The aim of this study was to assess SVR rate after SOF/DCV therapy and the frequency of RASs in HCV NS5A and NS5B genes for mono- and HCV/HIV-coinfected patients chronically infected with GTs 1 and 3a.

Materials and methods

Patients

Based on inclusion criteria indicated in 2015 clinical guidelines for using therapeutic regimen with DCV and SOF, this study enrolled 132 NS5A and NS5B inhibitors-naïve individuals chronically infected with HCV GTs 1 or 3a with advanced fibrosis (METAVIR score F3) or cirrhosis (F4), which attended the National Institute of Infectology Evandro Chagas (INI) and Gaffrée & Guinle University Hospital (UNIRIO). Between 2015 and 2017, serum samples were collected from 107 HCV monoinfected patients (1a: 46; 1b: 45; 3a: 16) and 25 from HCV/HIV co-infected patients (1a: 9; 1b: 12; 3a: 4) before antiviral therapy with SOF/DCV with or without RBV (12 or 24 weeks). As determined in 2015 Brazilian Clinical Guideline, RBV addition may be performed especially in patients with cirrhosis, with no response to prior therapy and patients with HCV/HIV co-infection regardless of the degree of fibrosis. Regarding therapy duration, a 12-week regimen was standardized for treatment-naïve GT 1 patients while a 24-week regimen was indicated for GT 1 mono- and HCV/HIV-coinfected patients which had already been experimented with DAAs and/or individuals with cirrhosis Child-Pugh B and C. All GT 3 patients received a 12-week therapeutic regimen. All mono- and HCV/HIV-coinfected patients have completed therapy with SOF/DCV with or without RBV. Fourteen of the monoinfected patients and one HCV/HIV coinfected patient had already been experienced with first-generation PIs boceprevir or telaprevir. Serum samples from six non-responder patients with detectable viral load after the end of therapy period were included in our analyses. SVR rates were accessed 12 weeks after treatment conclusion. HCV viral loads was measured by Abbott Real Time HCV assay (Abbott Laboratories, Chicago, Illinois, USA) with a limit of detection of HCV RNA > 12 IU/mL or > 1.08 Log IU/mL.

Ethical approval

Samples were collected after obtaining written informed consent from each patient. This study was approved by the ethics committee from Oswaldo Cruz Foundation (CAAE 68116417.2.0000.5248) and by the ethics committee of Gaffrée & Guinle University Hospital (Number 204.445).

RNA extraction, reverse-transcription and PCR amplification

Viral RNA was extracted from serum samples (200 μL) using High Pure Viral Nucleic Acid Kit (Roche Life Science, Mannheim, Germany) following manufacturer's recommendations. HCV NS5A (~1600 bp) and NS5B (~1500 bp) genes were amplified by one-step reverse-transcription (RT) with polymerase chain reaction (PCR) followed by a second round of PCR (nested-PCR) using specific primers designed for each subtype (Tables 1 and 2). For first round PCR amplification, reagents from Superscript III One Step RT-PCR system (Thermo Fisher Scientific, Waltham, MA, USA) were used. Second round PCR was accomplished with reagents from Platinum *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific).

For HCV NS5A gene, PCR products were obtained with the following conditions: 30' at 45°C for the reverse transcription followed by 2' at 94 °C, and then 35 cycles at 94 °C for 15", 61 °C for 30" and 68 °C for 90", with an extension at 68 °C for 5' for GT 1a; for GTs 1b and 3a annealing temperature was 56 °C and 62 °C, respectively. Five microliters of RT-PCR were used in second round PCR with following conditions: initial denaturation at 94 °C for 2' followed by 30 cycles at 94 °C for 15", 60 °C for 30" and 68 °C for 2' for GT 1a; for GTs 1b and 3a annealing temperature was 54 °C and 62 °C, respectively.

Table 1. Oligonucleotides for NS5A gene amplification of HCV GTs.

Genotype	Technique	NS5A Primers	Sequence (5'–3')	Genome position	Reference
1a	PCR 1	NS5A_F1	CAGTGCARTGGATGAACCG	6076 – 6094	[14]
		NS5A_R1	CGAGTTGCTCAGTGCGTT	7671 – 7688	- -
	PCR 2	NS5A_F1	CAGTGCARTGGATGAACCG	6076 – 6094	[14]
		NS5A_R2	TARGACATYGAGCARCACAC	7593 – 7612	[14]
1b	PCR 1	NS5A_F1	CAGTGCARTGGATGAACCG	6076 – 6094	[14]
		NS5A_R3	GTCTGTCAAATGTGACTTTCTTCT	7747 – 7770	[14]
	PCR 2	NS5A_F2	CGGCTGATAGCGTTCG	6093 – 6108	[14]
		NS5A_R2	TARGACATYGAGCARCACAC	7593 – 7612	[14]
3a	PCR 1	NS5A_F4	CAGTGGATGAACAGGCTCAT	6097 – 6116	- -
		NS5A_R4	CCTCAGCACTACATGGTGT	7663 – 7681	- -
	PCR 2	NS5A_F5	GTGGATCAATGAAGACTACCC	6243 – 6263	- -
		NS5A_R5	CCTCAGCACTACATGGTGT	7663 – 7681	- -

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For HCV NS5B gene, first and second rounds PCR conditions were the same to HCV NS5A region, except for annealing temperatures which were 53 ˚C, 54 ˚C and 62 ˚C (first PCR) and 53 ˚C, 53 ˚C and 62 ˚C (second PCR) for GTs 1a, 1b and 3a, respectively. PCR products were submitted to 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

Nucleotide sequencing

NS5A and NS5B products were purified using High Pure PCR Product Purification Kit (Roche Life Science) and concentration was estimated with Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) for each sample. Purified products were subjected to nucleotide sequencing reactions in both directions using Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and analyzed on ABI 3730 DNA automated sequencer (Applied Biosystems). After assembly of overlapping contigs, HCV NS5A and NS5B nucleotide sequences were submitted to GenBank database under accession numbers MK135170-MK135301 (NS5A gene) and MK135302-MK135433 (NS5B gene).

Table 2. Oligonucleotides for NS5B gene amplification of HCV GTs.

Genotype	Technique	NS5B Primers	Sequence (5'–3')	Genome position	Reference
1a	PCR 1	NS5B_F1	CTYAGCGACGGRTCRT	7539 – 7554	[15]
		NS5B_R1	TCACGGGTRAGGTARTAGAC	8742 – 8761	[15]
	PCR 2	NS5B_F2	TCGTGTGYTGCTCRATG	7591 – 7607	[15]
		NS5B_R2	TACCTGGTCATAGCCTCC	8621 – 8638	[15]
1b	PCR 1	NS5B_F3	TCYTGGTCTACYGTRAG	7551 – 7567	[15]
		NS5B_R3	AGGARCATGATGTTATCARCTC	8679 – 8700	[15]
	PCR 2	NS5B_F3	TCYTGGTCTACYGTRAG	7551 – 7567	[15]
		NS5B_R4	CCTAGTCATAGCCTCCGT	8616 – 8633	[15]
3a	PCR 1	NS5B_F5	TCTATGTCGTACTCTTGGACCG	7630 – 7651	- -
		NS5B_R5	GGAGTAGGCAAAGCAGCAAAT	9341 – 9361	- -
	PCR 2	NS5B_F5	TCTATGTCGTACTCTTGGACCG	7630 – 7651	- -
		NS5B_R6	CGATCAAGTATCTCCTGGGATTG	8929 – 8951	- -

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Mutation analyses

Nucleotide sequences were aligned in MEGA version 7.0 [16] together with HCV NS5A and NS5B reference sequences of each HCV GT obtained from the Los Alamos HCV Sequence Database. To evaluate the presence of RASs, it was considered substitutions in amino acid residues described in the literature associated or not with some degree of resistance: M/L28, Q/R30, L31, H/P58, E62 and Y93 for HCV NS5A and L159, S282, V321 and C316 for HCV NS5B protein [7, 9, 11, 12].

Statistical analyses

Univariate analyses were used to associate the presence of baseline NS5A RASs between HCV GTs. Fisher's exact test and Pearson chi-square were selected to test the significance level of associations, which was assessed at the 0.05 probability level. Statistical analyses were performed using software Epi Info version 7.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Results

Patients characteristics

Clinical, virological and therapeutically baseline data of 132 patients studied are provided in Table 3.

Post-treatment SVR

Overall, SVR rate in both groups was 95.4% (126/132) after 12 or 24-weeks of treatment. The majority of patients had non-specific symptoms during therapy, such as headache, anemia, nausea and fatigue. Therapeutic failure with SOF/DCV occurred in six (4.6%) individuals, five monoinfected and one HCV/HIV co-infected patient. Clinical and resistance data of non-responders are described in Table 4. Considering monoinfected patients, 102/107 (95.3%) did respond to antiviral therapy. Of these, SVR was achieved by 92.9% (13/14) of the experimented

Table 3. Therapeutic data for mono- and HCV/HIV-coinfected patients.

Characteristics		Groups			
		Monoinfected (n = 107)	SVR (%)	Coinfected (n = 25)	SVR (%)
Mean age (years) ± SD		63.3 ± 9.9	95.3	56.4 ± 10.4	96
Gender	Female	62 (58%)	96.8	5 (20%)	100
	Male	44 (42%)	93.1	20 (80%)	95
DAA-naïve patients		93 (87%)	95.7%	24 (96%)	95.8%
DAA-experienced patients		14 (13%)	92.9	1 (4%)	100
Genotype	1a	46 (43%)	91.3	9 (36%)	100
	1b	45 (42%)	100	12 (48%)	100
	3a	16 (15%)	93.8	4 (16%)	75
Therapeutic regimen/duration (weeks)	SOF + DCV / 12	24 (22.4%)	91.7	9 (36%)	88.9
	SOF + DCV / 24	1 (0.9%)	100	12 (48%)	100
	SOF + DCV + RBV / 12	62 (58%)	98.4	3 (12%)	100
	SOF + DCV + RBV / 24	20 (18.7%)	90	1 (4%)	100
Mean HCV viral load (IU/mL log10) ± SD		5.7 ± 0.86	95.3	5.9 ± 0.56	96
Hepatic condition	Cirrhotic	90 (84%)	94.4	5 (20%)	80
	Non-cirrhotic	17 (16%)	100	20 (80%)	100

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Table 4. Clinical and resistance data for non-responders patients after DAAs therapy.

Characteristics	Patients					
	1	2	3	4	5	6
Age	56	61	76	57	65	56
Genotype	1a	1a	1a	1a	3a	3a
Group	HCV	HCV	HCV	HCV	HCV	HCV/HIV
Therapeutic regimen	S/D/R	S/D/R	S/D/R	S/R	S/R	S/R
Therapy duration (weeks)	24	24	12	12	12	12
Baseline viral load (Log UI/mL)	5.0	6.8	5.7	4.85	6.86	6.36
Hepatic condition	Cirrhotic	Cirrhotic	Cirrhotic	Cirrhotic	Cirrhotic	Cirrhotic
Baseline NS5A RASs	-	-	-	Q30Y[◆◆]	-	A30S[◆◆]
Post-treatment NS5A RASs	-	Y93N	M28T, Q30R, E62D[◆]	Q30Y[◆◆]	-	A30S[◆◆]
Baseline NS5B RASs	-	-	-	-	-	-
Post-treatment NS5B RASs	-	-	-	-	-	-

S: sofosbuvir; D: daclatasvir; R: ribavirina

[◆]Secondary RASs

[◆◆]RASs not demonstrated to be clinically relevant due to low evidence *in vivo*

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and 95.7% (89/93) of the treatment-naïve patients. For HCV/HIV co-infected patients, SVR rate was 96% (24/25).

SVR rates in each HCV GTs for both groups were 92.7% (51/55) for GT 1a, 100% (57/57) for GT 1b and 90% (18/20) for GT 3a. For patients with HCV monoinfection, GT 1b had a higher response rate (100%; 45/45) followed by GT 3a (93.8%; 15/16) and GT 1a (91.3%; 42/46). Regarding co-infected individuals, GT 3a had a response rate of 75% (3/4), while for GTs 1a and 1b SVR was 100%.

Prevalence of pretreatment NS5A and NS5B RASs

In the present study, NS5A RASs frequency was higher in HCV/HIV patients (28%; 7/25) than in monoinfected patients (16.8%; 18/107). Regarding NS5B RASs, similar frequency was found for both groups (16% vs 15%). Filtering the presence of NS5A RASs by HCV GTs, GT 1b strains (NS5A: 31.6%; 18/57) had significantly more resistance mutations when compared to GTs 1a (NS5A: 7.3%; 4/55) and 3a (NS5A: 15%; 3/20) together ($p = 0.0040$). Similarly, resistance analyses for HCV NS5B gene indicated a frequency of amino acid substitutions in GT 1b strains of 35% (20/57) whereas for GTs 1a and 3a no mutations were identified for both groups.

Considering monoinfected group, GT 1b strains had significantly more substitutions than other GTs ($p = 0.0076$). Similarly, it was observed a statistically significant association for the presence of baseline NS5A RASs between GT 1b and 1a ($p = 0.0164$) and for GTs 1b, 1a and 3a ($p = 0.0171$).

Tables 5 and 6 indicate all identified amino acid substitutions in HCV NS5A and NS5B regions for mono- and HCV/HIV-coinfected patients.

Discussion

In 2015, DAAs SOF and DCV were included in Brazilian clinical guidelines for the treatment of HCV chronic infection. This combined therapy had been the most used by hepatologists in clinical routine, due to its effectiveness to different patient profiles: treatment-naive, non-responders to previous therapies and HCV/HIV co-infected individuals. Until November

Table 5. Baseline amino acids substitutions in HCV NS5A gene.

Genotype	Wild-type amino acid	NS5A RASs[◆]		Total	*p*
		HCV	HCV/HIV		
1a	Q30	30Y (1)	-	4/55 (7.3%)	
	L31	31M (1)	-		
	E62	62D (2)	-		
1b	L31	31M (3)	-	18/57 (31.6%)	0.0040
	L28+R30	28M+30Q (5)	28M+30Q (4)		
	L28+R30+L31	28M+R30Q+31M (1)	-		
	R30+ Y93	30Q+93H (1)	-		
	R30+P58	30H+58S (1)	-		
	P58	-	58S (1)		
	Y93	Y93H (2)	-		
3a	A30	-	30S (1)	3/20 (15%)	
	A30+S62	30V+62T (1)	30S+62T (1)		

[◆]Values in brackets represent the number of single or combined RASs found in the study population

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2015, treatment options available in Brazil were limited to a protocol with "first-wave" PIs directed to patients infected with HCV GT 1. Those who experienced previous failure did not have other therapeutic alternatives since at that period no other DAAs options were available. In general, the real efficacy of DAAs and SVR assessment rates in non-clinical studies in Brazil could only be accessed since 2016. Consequently, the low number of national scientific studies evaluating and comparing their findings with controlled clinical trials whose SVR rates ranged from 89% to 98% depending on the infecting GT and treatment with previous drugs [17]. In the current study, SVR rate at week-12 post-treatment with SOF/DCV with or without RBV was 95.4% (126/132) which means that therapeutic response for patients from two ambulatories in Rio de Janeiro was similar to results from a previous Brazilian research by Cheinquer *et al*. (2017) [13] and a randomized trial performed by Zhang *et al*. (2016) [18] where SVR values higher than 95% were observed indicating that response rates of Brazilian chronic carriers in practical routine were similar to controlled clinical trials. No statistical analyses were done in our study to compare different groups and genotypes as the samples were biased by the inclusion criteria defined by Brazilian clinical guidelines.

Studies indicated that previous treatment with DAAs for a specific gene may represent a negative factor for a future therapeutic success with a DAA directed to the same target likely due to drug-selective pressure [7, 19]. Here, all patients were treated with NS5A/NS5B inhibitors and those experienced were previously treated exclusively with DAAs directed to NS3 region. Due to this, no impact on SVR rates would be expected between naïve or experienced

Table 6. Baseline amino acids substitutions in HCV NS5B gene.

Genotype	Wild-type amino acid	NS5B RASs[◆]		Total
		HCV	HCV/HIV	
1b	L159	159F (1)	159F (1)	20/57 (35%)
	L159+C316	159F+316N (14)	159F+316N (2)	
	C316	316N (1)	316N (1)	

[◆]Values in brackets represent the number of single or combined RASs found in the study population

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patients, as reflected in our results considering monoinfected group, where no statistically significant difference was observed (95.7% vs. 92.9%, respectively). As for HCV/HIV coinfected patients, response rate observed in the present study (96%) was close to that reported in ALLY-3 clinical trial where SVR was 97% (133/137) for patients treated for 12 weeks with SOF/DCV [20]. The licensing of new DAAs has improved response rates in HCV/HIV co-infected individuals and SVR values became similar to that observed for monoinfected patients [21]. Here, no noticeable SVR difference was identified between monoinfected and co-infected groups (95.3% vs 96%), showing that introduction of more effective DAAs has considerably increased response rates in co-infected individuals, which were around 50% of SVR in previous therapy with pegylated interferon/RBV [22]. Our results highlighted that HIV co-infection no longer represent a limiting factor for therapy success, with current DAAs regimens being very effective in yielding SVR, the major aim of HCV therapy.

SVR rates observed for each GT was 92.7% (51/55), 100% (57/57) and 90% (18/20) for GTs 1a, 1b and 3a, respectively. Our results are similar to that reported by Sulkowski *et al*. (2014) [17] in a randomized multicenter trial where SVR rates for monoinfected patients (naive or experienced) with GTs 1b or 3 was 100% (35/35) and 89% (16/18), respectively.

In the non-responder GT 1a monoinfected patient who failed a 12-week treatment with SOF/DCV/RBV, the primary (M28T and Q30R) and secondary (E62D) mutations observed could be associated to the treatment failure. M28T mutation confers high-level resistance to DCV (fold change > 100) and OBV (fold change > 1000), while Q30R confers a clinical impact due to the high fold-change to DCV, EBV, LDV and OBV [10]. RAS E62D, although not acting in a primary way, may compensate decreased viral fitness of resistant variants enhancing replication levels that might have influenced negative post-treatment outcome. Importantly, other negative predictive factors related to therapeutic failure, such as decompensated liver cirrhosis and infection with GT 1a strain, may also have contributed with the non-response.

Here, we report the presence of RAS Y93N in a HCV NS5A sequence from a non-responder GT 1a monoinfected patient whose serum sample was isolated after treatment with SOF/DCV/RBV. A study from Wyles DL *et al*. (2017) [10] had reported that this substitution led to a 10000-fold reduced susceptibility to DCV, LDV and OBV, three of the main DAAs currently in use in interferon-free combined therapies. Additionally, therapy duration for this patient was 24 weeks due to unsuccessful previous treatment with interferon/RBV and first-wave PI telaprevir. Baseline sample from this patient indicated that mutation Y93N was not identified in HCV strain before therapy suggesting that the presence of selective pressure imposed by DCV might have influenced the emergence of viral populations with RAS Y93N. In addition to the presence of RASs after treatment, other factors such as high infective viral load (6.8 Log UI/mL), cirrhotic condition and infection with GT 1a might have contributed to a negative therapy outcome.

Sarrazin *et al*. (2016) [23] have identified Q30Y variant in 2.0% of HCV GT 1a sequences analyzed prior to NS5A inhibitor treatment. A similar frequency (2.2%) of this mutation was found in our study. The amino acid substitution from glutamine (Q) to tyrosine (Y) has been rarely described *in vivo* for non-responders patients, therefore, additional studies will be needed to evaluate a possible new resistance profile associated with this mutation.

In regard to NS5A sequence from HCV GT 3a, substitution A30S has previously been reported by Malta F. *et al*. (2017) [11] in a viral sequence of a HCV GT 3a/HIV non-responder coinfected patient (baseline prevalence of 6.7%; 1/15) and in an international case report [24]. In the present study, we found RAS A30S in one GT 3a non-responder patient, however, the inexpressive number of GT 3a patients available in our study (n = 4) prevented us to verify whether this substitution is common in HCV strains circulating in Brazil. The amino acid

substitution at position 30 could have been related to the therapeutic failure experienced by the patient, but few *in vivo* reports so far limited the possibility of association of this RAS with non-response. The patient also presented other negative predictive factors for non-response to treatment: (1) viral factors: high infective basal viral load (log 6.36) and HCV GT 3a infection; (2) host factors: male and chronic cirrhotic profile; and (3) therapeutic factor: reduced treatment duration (12 weeks) [19]. An update in Brazilian clinical guidelines published in 2018 increased the duration of treatment for cirrhotic patients infected with HCV GT 3 from 12 to 24 weeks [5].

According to previous reports, baseline RASs frequency in HCV NS5A region determined by conventional and next-generation sequencing techniques presented values between 6% and 16% [25 - 27]. Here, we observed statistically significant association for the presence of NS5A mutations in GT 1b in contrast to GTs 1a and 3a. These mutations, however, did not impact treatment effectiveness since all patients infected with GT 1b strains achieved SVR. This finding could be explained by differences in genetic barrier to resistance for DCV depending on the infecting HCV GT and/or due to concomitantly administration of SOF with DCV in a combined DAA regimen. A previous clinical report suggested that GT 1b strains might accumulate more resistance mutations than GTs 1a and 3a to overcome DAA action [17]. Here, baseline NS5A RASs was found in 16.8% (18/107) of the monoinfected group, a percentage slightly higher than reported in a study whose prevalence of NS5A mutations was 11.5% (18/156) [11]. Our study has found the presence of RASs in 6.5% (4/46) and 28.9% (13/45) for HCV GTs 1a and 1b, respectively. Our findings differed from the proportion of RASs between GTs 1a and 1b reported by Zeuzem *et al*. (2017) [28] in a study with 5397 samples from North America, Europe, Oceania and Asia where a prevalence of 13% for HCV GT 1a and 17.6% for HCV GT 1b was found. Nonetheless, similar to results from Paolluci *et al*. (2013) [29] (GT 1a: 12.5%; GT 1b: 53.3%), here, the frequency of RASs in GT 1b strains was significantly higher than in GT 1a strains ($p < 0.05$). Although its higher frequency in GT 1b, impact of the presence of RASs in treatment outcome is considered more evident in patients infected with GTs 1a and 3a [20, 30]. In our study group, NS5A RASs did not seem to have influenced treatment outcome since all HCV GT 1b monoinfected patients have responded to SOF/DCV therapy.

According to Wyles *et al*. (2017) [10] amino acid substitution L31M confers a clinical impact due to the high fold-change observed *in vitro* for DAAs DCV and LDV (fold change > 100). However, our analyses *in vivo* indicated that baseline presence of RAS L31M in HCV strains of GTs 1a (2.2%) and 1b (6.7%) did not influenced treatment outcome since all five patients achieved SVR. Additionally, low prevalence for L31M variant was also observed by Zeuzem *et al*. (2017) [28] (2.3%) and Paolluci *et al*. (2013) [29] (3.1%). Besides RAS L31M, substitution Y93H has also been highly associated with resistance to DCV [31]. Our findings were similar to previous published data since substitution Y93H was found in 6.7% of GT 1b samples [31, 32]. Likewise RAS L31M, presence of substitution Y93H did not affected treatment efficacy in the three patients infected with this variant strain.

Regarding HCV NS5B RASs for monoinfected patients, the high frequency of combination L159F + C316N (31.1%, 14/45) found in strains of HCV GT 1b was similar to results reported by Noble C.F. *et al*. (2017) [33] and Peres-da-Silva A. *et al*. (2017) [15] whose prevalence was 14.2% (8/56) and 25% (13/52), respectively. The occurrence of these RASs did not appear to have influenced therapeutic response after treatment, since all HCV GT 1b-monoinfected patients has achieved SVR. Substitution at residue 282, mainly identified *in vitro* and highly associated with SOF resistance, was not observed for isolates of HCV GT 1b in our study as well as in findings from Castilho M.C. *et al*. (2011) [34] and Costatino A. *et al*. (2015) [35].

Few Brazilian studies have analyzed NS5A and NS5B genes resistance profile for HCV/HIV co-infected patients. Further studies are needed to better understand the circulation of

resistant viral subpopulations in this group. Our analyses indicated that NS5A RASs frequency for referred group was 28% (7/25) while for NS5B RASs was 16% (4/25). HCV GT 1b strains had higher frequency of NS5A (41.7%; 5/12) and NS5B (33.3%; 4/12) RASs than GTs 1a and 3a. According to Plaza Z. *et al.* (2012) [36], none of GT 1a NS5A sequences carried substitutions while for HCV GT 1b, double mutation L31M + Y93H, highly associated with resistance to DCV, were detected in the proportion of 1/15 (6.7%). In contrast to these results, RASs combination L28M + R30Q was identified in a greater proportion in HCV GT 1b isolates. Resistance analyses for HCV NS5B region revealed similarity in RAS C316N frequency reported by Plaza *et al.* (2011) [37] (13.3%), Trevino A. *et al.* (2011) [38] (10%) since 8.3% GT 1b sequences had this mutation in the current study.

In conclusion, this study demonstrated that SVR rate in Brazilian patients chronically infected with HCV treated with DAA regimen DCV/SOF (95.4%) was similar to results from randomized clinical trials. Our research also sought to identify the circulation of resistant variants in Brazil and if emerging RASs could have influenced or not in treatment outcome. Here, amino acid substitutions already described in literature and with limited clinical reports *in vivo* were observed. However, most of these baseline substitutions did not seem to negatively impact treatment outcome, especially for GT 1b since all patients achieved SVR, demonstrating the importance of a combined therapy directed to different viral proteins. Our observations about circulation of resistant variants in Brazil suggested that new DAAs combination, such as OBV/DSV/PTV/r, SOF/LED and EBV/GZV, whose efficacy is not diminished by these substitutions, could be licensed and included in future clinical protocols for Brazilian chronic HCV carriers.

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Artigo 3. Costa VD, Pellegrini P, Rotman V, Pittella AM, Nunes EP, Lago BV, Lampe E, Mello FCA. Resistance Mutations A30K and Y93N Associated with Treatment Failure with Sofosbuvir and Daclatasvir for Hepatitis C Virus Infection Non-Responder Patients: Case Reports. *Viruses*. 2019 Oct 31;11(11). pii: E1004.

Resumo

No Brasil, o tratamento da hepatite C vem evoluindo significativamente com o licenciamento dos DAAs. No entanto, determinantes virais (substituições de aminoácidos no genoma do HCV e genótipo infeccioso do HCV) associados a fatores do hospedeiro (condição hepática e terapia anterior) podem limitar a obtenção da RVS. Neste trabalho, descrevemos dois relatos de caso em que a ocorrência das mutações na proteína NS5A do HCV A30K (subtipo 3a) e Y93N (subtipo 1a) pode ter influenciado a não resposta à terapia combinada com DCV/SOF. Apesar das altas taxas de resposta para as terapias combinadas com DAAs no Brasil, esses relatos de caso afirmaram a importância de uma investigação sobre como gerenciar uma falha no tratamento com DAAs, uma vez que uma combinação de fatores, especialmente a ocorrência de substituições de resistência, poderia impactar uma terapia de resgate com novos antivirais disponíveis na rotina clínica.

viruses

MDPI

Communication

Resistance Mutations A30K and Y93N Associated with Treatment Failure with Sofosbuvir and Daclatasvir for Hepatitis C Virus Infection Non-Responder Patients: Case Reports

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Abstract: In Brazil, hepatitis C treatment has been evolving significantly with the licensing of direct-acting antivirals (DAAs). However, viral determinants (amino acid substitutions in hepatitis C virus (HCV) genome and infective genotype) associated with host factors (hepatic condition and prior HCV therapy) might limit the achievement of sustained virologic response (SVR). Here, we described two case reports in which the occurrence of HCV NS5A mutations A30K (subtype 3a) and Y93N (subtype 1a) might have influenced daclatasvir (DCV)/sofosbuvir (SOF) combined therapy non-response. Despite high response rates for DAA combined therapies in Brazil, these case reports stated the importance of an investigation about how to manage a DAA treatment failure since a combination of factors, especially the occurrence of resistance substitutions, could impact a rescue therapy with new available antivirals in clinical routine.

Keywords: hepatitis C virus (HCV); DAA; resistance

1. Introduction

It is estimated that 71 million people worldwide are chronically infected with hepatitis C virus (HCV) [1]. Genotype distribution in Brazil indicated a prevalence of HCV subtypes 1a, 1b and 3a [2]. In 2015, oral combinations of direct-acting antivirals (DAAs) targeting HCV non-structural proteins NS5A and NS5B were included in Clinical Guidelines for the Treatment of Hepatitis C and Coinfections [3] published by Brazilian Ministry of Healthy. Daclatasvir (DCV), a NS5A inhibitor, in combination with sofosbuvir (SOF), a nucleotide analogue inhibitor of NS5B, is a daily regimen administrated with or without ribavirin for Brazilian patients infected with HCV genotypes 1 and 3. Despite its high rate of effectiveness in viral clearance, host and virus factors associated with treatment failure, which include liver fibrosis, negative response to previous therapy and resistance associated-substitutions (RASs), could limit the effectiveness of these drugs in achieving sustained virological response (SVR) [4]. NS5A amino acid substitutions A30K (subtype 3a) and Y93N (subtype 1a) were described in literature

in vitro [5,6] and in vivo [7–10] as responsible for reducing DCV action. Regarding NS5B gene, previous studies reported that substitutions on residues S282 and V321 can reduce susceptibility to SOF [11,12]. We report here two cases of chronic hepatitis C patients infected with HCV subtypes 1a and 3a who failed DCV/SOF combined therapeutic regimen. Treatment failure might have been related to the occurrence of NS5A RASs Y93N and A30K which were identified in persistent HCV strains recovered after the end of unsuccessful treatment.

2. Materials and Methods

Two patients who failed to achieve viral clearance 12 weeks after the end of DCV/SOF combined therapy were attended at Ambulatory of Viral Hepatitis/FIOCRUZ, Rio de Janeiro. Serum sample from patient 1 and patient 2 were collected in October 2016 and November 2017, respectively. A written informed consent was obtained from patients as established in the ethics statement approved by the ethics committee from Oswaldo Cruz Foundation (CAAE 68116417.2.0000.5248). Molecular tests included HCV RNA extraction using commercial reagent High Pure Viral Nucleic Acid Kit (Roche Life Science, Mannheim, Germany) following manufacturer's recommendations. After, HCV NS5A and NS5B genes were amplified by one-step reverse-transcription (RT) with polymerase chain reaction (PCR) followed by a second round of PCR (nested-PCR) through reagents from Superscript III One Step RT-PCR system (Thermo Fisher Scientific, Waltham, MA, USA) and Platinum *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific). Specific primers used for each subtype and PCR conditions were described in previous study by Costa et al. (2019) [10]. Amplified products were submitted to purification using High Pure PCR Product Purification Kit (Roche Life Science) and double-stranded DNA concentration was estimated by Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). Additionally, nucleotide sequencing was performed with Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and analyzed on ABI 3730 DNA automated sequencer (Applied Biosystems). Reference HCV sequences from subtypes 1a and 3a were obtained from the Los Alamos HCV Sequence Database [13]. Nucleotide sequences retrieved from both strands were assembled to generate a consensus sequence and a multiple sequence alignment was analyzed in MEGA version 7.0 [14]. Deduced amino acid composition of NS5A and NS5B proteins was evaluated for the presence of RASs at residues previously reported as associated to drug resistance (NS5A: M28, Q/A30, L31 and Y93; NS5B: L159, S282 and V321) [15].

3. Results

3.1. Case Report 1

Patient 1 was a cirrhotic 65-year-old woman from Northeast Brazil (Alagoas State). The anamnesis revealed that patient 1 was diagnosed with chronic hepatitis C (HCV subtype 3a) in 2011. In 2013, patient 1 was submitted to antiviral therapy with pegylated interferon and ribavirin. Treatment was discontinued due to occurrence of thrombocytopenia. In 2016, a retreatment with DCV/SOF and ribavirin for 12 weeks was conducted in a private hospital in Rio de Janeiro. Patient adherence to treatment was satisfactory as she concluded the 12-week period of therapy. Molecular diagnosis by real-time PCR indicated that post-treatment HCV viral load was 9360 IU/mL (3.97 Log IU/mL). Laboratory biochemical tests showed ALT of 18 IU/L, AST of 49 IU/L and gamma glutamyl transpeptidase (GGT) of 134 IU/L. In order to investigate viral molecular factors that could have influenced non-response status, resistance analyses were requested by physicians after referring patient 1 to our Ambulatory of Viral Hepatitis in FIOCRUZ. The presence of HCV RNA was confirmed by qualitative NS5A gene RT-PCR amplification and posterior nucleotide sequencing [10]. Sequence assembly and deduced amino acid residues alignment with reference HCV subtype 3a sequence (Ref.CON_3a) identified the presence of RAS A30K. No RASs in NS5B deduced amino acid residues were observed. Besides resistance analysis after 12-week therapy with DCV/SOF in 2016, patient 1 had two other serum samples collected in 2017 and 2018. Nucleotide sequencing results from both samples

demonstrated the persistence of RAS A30K in NS5A protein two years after the end of treatment (Figure 1).

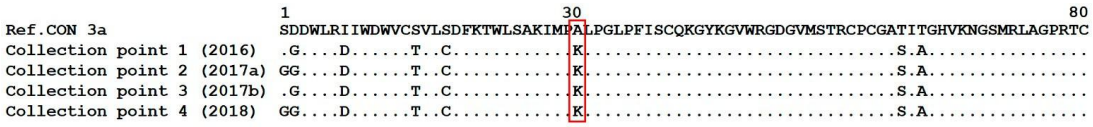

Figure 1. NS5A deduced amino acid sequence alignment comparing a consensus hepatitis C virus (HCV) subtype 3a sequence and four post-treatment collection points of patient 1. Substitution in residue 30 (A30K) is indicated in a red box.

3.2. Case Report 2

Patient 2 was a 67-year-old Brazilian male who had been diagnosed with HCV genotype 1a infection in 2011. In anamnesis, patient reported type 2 mellitus diabetes (MD). Glibenclamide and metformin was administrated for treating MD. In June 2012, a liver biopsy revealed advanced fibrosis (METAVIR score F3). Serological and biochemical tests performed in May 2013 showed that patient 2 was anti-HBc positive, HBsAg and anti-HBs negative (serological evidence of past hepatitis B infection), alanine aminotransferase (ALT) of 64 IU/L and aspartate aminotransferase (AST) of 59 IU/L. In December 2013, patient 2, until then therapy-naive, started antiviral treatment with DAA telaprevir 375 mg (3 tablets every 12 h) plus peginterferon alfa-2a (180 µg/week) and ribavirin (250 mg). HCV RNA viral load before this triple therapy was 1,820,817 IU/mL (6.26 Log IU/mL). Treatment was well-tolerated in the first three weeks; however, at week 4, patient 2 had episodes of myalgia and asthenia. HCV RNA viral load evaluated at week 12 and week 16 was 446 IU/mL (2.65 Log IU/mL) and 95,904 IU/mL (log 4.98 Log IU/mL), respectively. Due to the increase in HCV viral load, therapy was suspended and patient 2 was considered non-responder (absence of 2 log decline in HCV RNA levels between weeks 12 and 16). Laboratory biochemical tests performed after the treatment cessation showed ALT levels of 70 IU/L and AST levels of 45 IU/L.

In May 2016, after the inclusion and availability of new DAAs in Brazil, physicians prescribed a retreatment for patient 2. In October 2016, patient 2 began a combined 24-week interferon-free therapy with DCV (60 mg/daily), SOF (400 mg/daily) and ribavirin (5 tablets daily). After 24 weeks of combined therapy, HCV viral load was undetectable; however, SVR 12 weeks after the end of treatment was not achieved, configuring a virologic relapse. In order to verify the occurrence of drug resistance profile in ongoing HCV infection, physicians had requested molecular tests in a serum sample collected seven months after the end of treatment. After nucleotide sequencing of HCV strain infecting patient 2, deduced NS5A amino acid sequence was aligned against HCV genotype 1a reference sequence H77 and primary RAS Y93N was identified (Figure 2). No RASs in NS5B amino acid sequence were observed. In spite of treatment failure with viral persistence, overall clinical condition of patient 2 was considered healthy by physicians after therapeutic regimen with DCV/SOF. Based on this, medical staff decided that patient 2 should wait for the upcoming DAAs before attempting a retreatment to clear HCV viral infection.

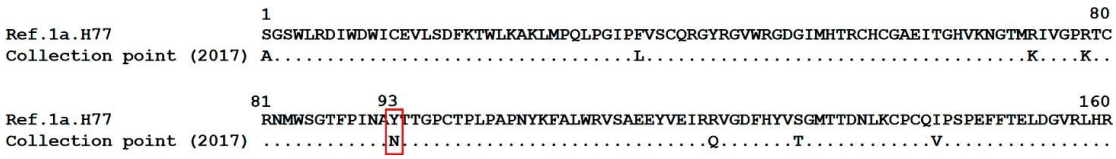

Figure 2. NS5A deduced amino acid sequence alignment comparing a consensus HCV subtype 1a sequence and a post-treatment collection point of patient 2. Substitution in residue 93 (Y93N) is indicated in a red box.

4. Discussion

In Brazil, routine clinical practices in hepatitis C treatment had reported SVR rates higher than 95% after the availability of interferon-free combined DAAs therapies [10,16]. Despite the high efficacy rate of new DAAs, previous studies had pointed out that a combination of viral factors such as HCV infecting genotype and drug-specific mutations in HCV genome could have major influence in therapeutic response. According to Buti et al. (2016) [17], infection with HCV subtypes 1a and 3a strains and the presence of NS5A RASs in specific amino acid residues are associated with reduction in DAAs susceptibility. Based on data from previous clinical trials from Nelson et al. (2015) [18] and Poordad et al. (2016) [19], presence of baseline NS5A RASs in viral subpopulations seems to reduce DCV effectiveness in patients with advanced cirrhosis.

Regarding case report 1, negative predictive factors such as HCV subtype 3 infection and cirrhotic condition could have influenced an unsatisfactory therapy outcome. Quantitative molecular analysis and biochemical tests at the end of DCV/SOF treatment indicated detectable HCV viral load (3.97 Log UI/mL) with elevated hepatic enzymes AST and GGT. In addition to HCV infecting genotype and hepatic condition, resistance analyses demonstrated the presence of amino acid substitution A30K in NS5A protein. Hernandez et al. (2013) [7] reported that viral strains with amino acid substitution from alanine (A) to lysine (K) were 44-fold more resistant to DCV inhibition than the wild-type in vitro. A previous Brazilian research study from Malta et al. (2017) [9] identified the presence of substitution A30K in 16.1% (5/31) of monoinfected patients; however, its clinical impact in the outcome of treatment was not described. In addition, analyses of subsequent samples of patient 1 have demonstrated the persistence of mutation at residue 30 in NS5A gene for two years after the end of therapy with DCV/SOF. Other studies have also reported the persistence of NS5A RASs after treatment cessation [12,20]. These reports suggested that the occurrence of some NS5A substitutions might have conferred an evolutionary advantage for resistant variants since they are not affected by NS5A inhibitors and, even in the absence of the selective pressure imposed by antiviral drugs, these substitutions persist and do not affect viral replication level, as evidenced for viral strain studied in case report 1. This might be a matter of concern since recent publications indicate that newly approved DAAs elbasvir, ledipasvir, pibrentasvir and velpatasvir might have limited efficacy when HCV infecting strain presents substitution A30K in NS5A protein [7,21–23]. In conclusion, case report 1 has shown a particular case in which a molecular viral factor (RAS A30K) in combination with other factors, such as HCV subtype 3a infection and cirrhosis, might have contributed to non-response and might limit the efficacy of retreatment with other NS5A inhibitors.

In case report 2, side effects had limited the therapy conclusion with DAA telaprevir. Until now, no association of MD or past hepatitis B infection and alteration in response to treatment was reported. In addition to treatment cessation, the high viral load (6.26 Log IU/mL) could also have influenced the negative outcome. Along with high HCV RNA levels, non-response condition was corroborated by augmented liver biochemical markers, indicating active hepatic damage. Although encouraging results were seen in overcoming viral infection with a 24-week rescue therapy combining DAAs targeting different HCV proteins, SVR12 was not achieved by patient 2. Based on our resistance analyses findings, the selection of viral strains with amino acid substitution in residue 93 of NS5A protein from tyrosine (Y) to asparagine (N) could have represented a major factor associated to therapeutic failure in patient 2. Considering in vitro experiments from Wyles et al. (2017) [12], RAS Y93N could lead to a 10,000-fold reduction in susceptibility to DCV. In a previous study, our group reported the emergence of this mutation in a non-responder patient monoinfected with subtype 1a whose sample was collected after the end of therapy, suggesting that emergence of RAS Y93N after DCV-selective pressure in combination with host and viral negative predictive factors contributed to treatment failure, as well as observed here with patient 2 [10]. In summary, despite hepatitis C treatment with DAAs being considered well-tolerated and efficient in Brazil, our case report 2 suggested that identification of mutations in HCV genome, especially in NS5A gene, should be considered in particular cases

when SVR rates were not achieved even with favorable factors, such as longer duration of treatment, non-cirrhotic hepatic condition and non-previous therapy with same DAA class.

Here, two cases of DAA-failure in which the presence of RASs A30K and Y93N in HCV NS5A protein might have been an important factor for patients' non-response were presented. Unfortunately, we were not able to perform pretreatment resistance tests since, in Brazil, physicians only request resistance analysis after DAA-therapy failure. Due to few reports to date, the clinical impact of the association between the presence of NS5A RASs A30K and Y93N in vivo and DAA-therapy non-response remains to be clarified. Nonetheless, here we report two cases of treatment failure where identifying the presence and persistence of mutant strains with NS5A RASs represented a crucial background information to guide physicians' decision to wait for the availability of upcoming DAAs in Brazil before prescribing a rescue therapy in order to prevent the occurrence of cross-resistance to the NS5A inhibitors currently available.

5. Conclusions

The availability of all-oral DAA-combined therapies in Brazilian clinical guidelines for the treatment of hepatitis C represented a breakthrough in the clearance of viral infection and achievement of SVR. In counterpoint, there are still cases of treatment failure that reinforce the evaluation of viral resistance to support physicians' conduct in a rescue therapy with different DAAs. In this report we highlighted that a combination of viral and host factors might represent negative predictive determinants in hepatitis C DAA treatment outcomes. Non-responders should be carefully evaluated, including considering resistance associated-substitution analysis, before retreatment.

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4 DISCUSSÃO

Até o final de 2015, telaprevir e boceprevir eram os únicos IPs disponíveis para o tratamento da hepatite C crônica no Brasil. O tratamento limitava-se a um protocolo com IPs de "primeira onda" voltados somente para o tratamento de pacientes infectados pelo genótipo 1 e que não abrangia opções terapêuticas de resgate para aqueles com falha prévia à terapia dupla com Peg/IFN e RBV. No entanto, o licenciamento de três medicamentos em 2015 (SMV, SOF e DCV) permitiu novas opções terapêuticas combinadas livres de IFN e visando diferentes alvos terapêuticos da replicação viral. O SMV, um IP de "segunda onda", foi recomendado para pacientes com doença hepática não avançada, enquanto a combinação de DAAs DCV (inibidor da NS5A) e SOF (NI da NS5B) foi a principal alternativa terapêutica para pacientes experimentados previamente à terapia antiviral com DAAs, especialmente aqueles com cirrose descompensada. Em geral, a eficácia dos novos DAAs em estudos de rotina clínica e a avaliação das taxas de RVS para pacientes com resultado METAVIR F2 há mais de 2 anos e fibrose hepática avançada (METAVIR F3 ou F4) somente pôde ser elaborada de 2016 até o presente momento. Isso justifica o baixo número de trabalhos científicos nacionais que avaliam e comparam seus achados com ensaios clínicos controlados cujas taxas de RVS variam entre 89% e 98% dependendo do subtipo infectante e, do tratamento ou não com drogas prévias [111, 132].

O uso terapêutico dos IPs telaprevir e boceprevir era vinculado à ocorrência de efeitos colaterais que dificultavam a adesão dos pacientes com hepatite C crônica à terapia antiviral. Com isso, o SMV poderia ser uma alternativa terapêutica para pacientes com variantes resistentes cujas RASs na proteína NS3 do HCV reduziram a susceptibilidade ao uso dos IPs de "primeira onda". O primeiro manuscrito, intitulado "Prevalence of baseline NS3 resistance-associated substitutions (RASs) on treatment with protease inhibitors in patients infected with HCV genotype 1", descreveu as mutações de resistência basais na proteína NS3 antes da terapia de resgate com SMV para pacientes com falha terapêutica para Peg/IFN/RBV e/ou telaprevir/boceprevir. Para este fim, um estudo transversal foi conduzido com 73 pacientes cronicamente infectados pelo HCV, sendo 15 tratados previamente com os IPs telaprevir ou boceprevir e 58 virgens de tratamento para

DAAs. O objetivo do estudo foi determinar a prevalência de RASs basais no domínio serino-protease da proteína NS3 do HCV em pacientes cronicamente infectados pelos subtipos 1a e 1b do HCV previamente ao tratamento com os DAAs SMV, SOF ou DCV.

Conforme esperado, a prevalência de RASs basais na proteína NS3 do HCV antes de uma possível terapia de resgate com os novos DAAs aprovados em 2015 para pacientes previamente que falharam a terapia com telaprevir ou boceprevir foi maior em comparação com a prevalência dos pacientes não experimentados, provavelmente devido à pressão seletiva imposta pela droga. Para aqueles que não responderam à terapia com os IPs de “primeira onda”, as RASs representaram um fator prognóstico negativo para novos tratamentos com DAAs [123, 124, 133]. Em quatro pacientes analisados no estudo que falharam a terapia anterior, a presença de RASs pode ter sido a causa da não resposta ao tratamento, enquanto que em outros pacientes não respondedores a telaprevir ou boceprevir, nos quais não foram detectadas RASs, outros fatores considerados de pior prognóstico terapêutico, como infecção pelo subtipo 1a do HCV, cirrose e alta carga viral infectante podem ter contribuído para o tratamento malsucedido [134]. Os prontuários médicos não permitiram a obtenção de dados de quanto tempo, após o tratamento com medicamentos de “primeira onda”, foram coletadas amostras de soro de pacientes não respondedores. Assim, como seria de se esperar que as estirpes do tipo selvagem pudessem reemergir como a principal população viral em poucas semanas ou meses após a ausência de pressão seletiva imposta pelos medicamentos até o retratamento com novas drogas, uma falha terapêutica devido à presença de RASs não pôde ser excluída.

A prevalência de RASs no grupo 2 (virgens de tratamento) foi menor para o subtipo 1a (5,6%) em comparação ao subtipo 1b (18,1%). A presença de RASs em pacientes infectados pelo subtipo 1b do HCV, cuja barreira genética para resistência é mais alta quando comparada ao subtipo 1a [124], não expostos à pressão seletiva de medicamentos sugere uma infecção primária por estirpes virais com RASs. Considerando os dois grupos, este estudo identificou maior proporção de RASs nas sequências referentes ao subtipo 1b quando comparadas ao subtipo 1a (17,4% *vs.* 12%), um padrão distinto do observado em estudo brasileiro anterior realizado com doadores de sangue, onde a presença de RASs no subtipo 1a foi significativamente

maior que no subtipo 1b (20% *vs.* 8%) [135]. Isso pode estar relacionado a diferenças na população estudada (mais de 20% de nossas amostras eram de indivíduos previamente experimentados com DAAs) e/ou quais posições de aminoácidos foram avaliadas e levadas em consideração no cálculo da proporção de RASs entre os subtipos do HCV. No presente estudo foram consideradas as RASs nas posições V36, Q41, F43, T54, Q80, S122, R155, A156, D168 e V170, enquanto que Nishiya *et al.* (2014) [135], além das já mencionadas, também incluíram outras posições de RASs em sua análise, são elas, V55, A87, R109, R117, N174 e S138.

De acordo com a literatura, as RASs V36M, T54S, Q80K e R155K são consideradas mutações primárias de resistência a diferentes IPs. As RASs V36M e R155K podem reduzir a susceptibilidade a IPs aprovados recentemente como SMV, paritaprevir e grazoprevir [136-138]. A T54S está associada à resistência ao telaprevir [139] enquanto que a Q80K está altamente associada à resistência ao SMV [129].

A presença da RAS V36M em estirpes do HCV pode estar relacionada à falha terapêutica experimentada por quatro pacientes em um tratamento anterior com telaprevir. A identificação dessa substituição em um paciente do grupo 2 sugere uma infecção por variante viral resistente a IPs, uma observação que alerta para a circulação de estirpes resistentes que podem afetar a eficácia dos DAAs no futuro próximo. A identificação da substiuição de aminoácido na posição 36 da proteína NS3 em análise de amostra coletada pouco tempo antes do início da terapia antiviral com os novos DAAs sugere que a terapia combinada com IPs de nova geração não deve ser considerada para os cinco pacientes, pois a V36M está associada à resistência à maioria dos IPs aprovados no Brasil à época. De fato, uma vez disponível no Brasil, o tratamento com SOF combinado com DCV foi escolhido para o tratamento de todos os cinco pacientes, que tiveram resultado de tratamento semelhante, que foi o RNA do HCV indetectável 12 semanas após o tratamento. Um estudo conduziu uma análise retrospectiva para determinar a prevalência de mutações de resistência entre pacientes tratados com telaprevir [139] e a mutação V36M foi identificada em 28/232 (12%) pacientes que falharam terapia com telaprevir, demonstrando sua importância como uma mutação indicativa de resistência cujo mau prognóstico revela não confiabilidade no uso de IPs de primeira geração. Barnard *et al.* (2013) [140] identificaram mutações de resistência em

pacientes não respondedores à terapia tripla com boceprevir/Peg-IFN/RBV infectados pelo subtipo 1a e concluíram que a V36M pode ser uma das principais causas de falha terapêutica com o uso de IPs de “primeira onda”. Os resultados do presente estudo demonstraram que mutações na posição 36 foram encontradas tanto para pacientes virgens de tratamento quanto experimentados, indicando que o tratamento com DAAs sem uso de IPs deve ser considerado para minimizar o risco de resistência e alcançar RVS para esses pacientes.

Entre as RASs observadas para pacientes do grupo 2, a T54S foi identificada em um paciente infectado pelo subtipo 1b do HCV. Essa mutação demonstrou causar resistência ao boceprevir e telaprevir, mas não ao SMV [141]. Isso foi confirmado no presente estudo, pois esse paciente alcançou a RVS após 12 semanas de tratamento com SMV. A baixa prevalência da RAS T54S (4,6%) em pacientes não tratados com DAAs também foi relatada em estudos brasileiros anteriores [135, 142].

Em nosso estudo, a RAS Q80K não foi observada em isolados do subtipo 1a do HCV e só foi detectada em uma amostra do subtipo 1b referente a um paciente do grupo 2 com cirrose hepática compensada, diabetes mellitus tipo 2 e hipertensão arterial sistêmica. Em 2016, esse paciente era assintomático e decidiu não continuar tratamento, mesmo com outras opções terapêuticas de DAAs disponíveis. A Q80K é mais frequentemente observada em isolados do subtipo 1a e raramente é detectada no subtipo 1b do HCV [143]. Estudos relataram a alta prevalência da mutação Q80K nos EUA (37-47%) [128, 144]. Sarrazin *et al.* (2016) [145] avaliaram as RASs basais da proteína NS3 do HCV para 467 pacientes e os resultados dos polimorfismos Q80 demonstraram alta prevalência em pacientes experimentados com IPs (110/265; 41,5%) e pacientes virgens de tratamento (93/202; 46%). Ao contrário dos dados de outros países, a prevalência de Q80K no Brasil é baixa [135, 142, 146]. Portanto, devido à baixa prevalência dessa mutação em estirpes brasileiras relatadas em estudos anteriores e corroboradas no presente estudo, não há necessidade de incorporar testes de resistência ao pré-tratamento para pacientes infectados pelos subtipos 1a e 1b do HCV no Brasil. Mesmo com a identificação dessa variante, o uso de outros IPs não é limitado, pois não há evidências de resistência.

A RAS R155K está relacionada à resistência aos IPs de primeira e segunda onda. Um estudo relatou uma maior frequência de falha ao tratamento em indivíduos

que apresentavam esta RAS no subtipo 1a do HCV devido à baixa barreira genética à resistência viral quando comparado ao subtipo 1b [147]. Sarrazin *et al.* (2007) [141] descreveram que a combinação das substituições V36M+R155K induz alta resistência ao telaprevir e pode inibir a ação do medicamento. No presente estudo, a combinação de mutações nos *loci* 36 (V36M) e 155 (R155K) foi identificada para um paciente previamente tratado com telaprevir e infectado pelo subtipo 1a do HCV. Após 12 semanas de terapia com telaprevir, a carga viral foi de log 4,74 e houve a suspensão do tratamento. A RAS R155K confere resistência a todos os IPs disponíveis para as estirpes do subtipo 1a e as novas opções terapêuticas para esse paciente devem ter como alvo outras proteínas não estruturais do HCV. De fato, neste caso, uma terapia de resgate com inibidores da NS5B e NS5A foi selecionada e o RNA-HCV foi indetectável após 4 semanas de tratamento.

As substituições V36L, F43V e Q80H foram identificadas no presente estudo. A RAS V36L está associada à resistência ao boceprevir [136]. Em 2015, as diretrizes terapêuticas brasileiras sobre o manejo da hepatite C não incluíram terapias combinadas com boceprevir ou telaprevir como opções de tratamento. A RAS V36L foi identificada em um paciente infectado pelo subtipo 1a do grupo 2. Foi iniciada uma terapia de 12 semanas com SMV. A V36L não foi relacionada com menor susceptibilidade a esse medicamento e a RVS foi alcançada após o tratamento.

A análise das RASs entre os pacientes infectados pelo subtipo 1b do HCV indicou a presença de RAS F43V em um paciente virgem de tratamento. Considerando pesquisa na literatura, este é o primeiro relato dessa mutação *in vivo*. Um perfil de resistência para IPs foi descrito *in vitro* e apontou o *lócus* F43 como associado à resistência ao SMV [136]. A RAS Q80H, que pode reduzir a susceptibilidade ao SMV, foi identificada em um paciente do grupo 2 infectado pelo subtipo 1b do HCV. O tratamento com boceprevir foi selecionado para esse paciente e a Q80H não influenciou a resposta ao tratamento, uma vez que a RVS foi alcançada após o tratamento. Nenhum dado brasileiro descreveu essa mutação *in vivo* entre pacientes que não receberam tratamento prévio.

Em 2015, os DAAs SOF e DCV foram incluídos nas diretrizes clínicas brasileiras para o tratamento da infecção crônica pelo HCV. Essa terapia combinada foi a mais utilizada pelos hepatologistas na rotina clínica, devido à sua eficácia em

diferentes perfis de pacientes: virgens de tratamento, não respondedores às terapias prévias e indivíduos coinfectados HCV/HIV. Aqueles que apresentaram falha não tinham alternativas terapêuticas, pois naquele período não havia outras opções de DAAs disponíveis. Em geral, a eficácia clínica dos DAAs e as taxas de RVS em estudos não clínicos no Brasil só puderam ser acessadas a partir de 2016. Logo, no segundo manuscrito, intitulado “Treatment of chronic HCV infection with DAAs in Rio de Janeiro/Brazil: SVR rates and baseline resistance analyses in NS5A and NS5B genes”, foi possível acessar a taxa de RVS após terapia combinada com uso de SOF/DCV com ou sem RBV (12 ou 24 semanas) e a frequência de RASs basais nas proteínas NS5A e NS5B do HCV para 25 pacientes coinfectados HCV/HIV e 107 pacientes monoinfectados cronicamente pelo genótipo 1 e subtipo 3a do HCV.

A taxa de RVS na semana 12 pós-tratamento com SOF/DCV com ou sem RBV foi de 95,4% (126/132), o que significa que a resposta terapêutica para pacientes atendidos em dois ambulatórios do Rio de Janeiro foi semelhante aos resultados de uma pesquisa brasileira de Cheinquer *et al.* (2017) [132] e um estudo randomizado realizado por Zhang *et al.* (2016) [130], onde foram observadas taxas de RVS acima de 95%, indicando que as taxas de resposta de portadores crônicos brasileiros na prática de rotina clínica foram semelhantes a de ensaios clínicos controlados.

O tratamento prévio com DAAs voltados para uma região protéica viral específica pode representar um fator negativo para o sucesso terapêutico futuro com um DAA direcionado para o mesmo alvo, provavelmente devido à pressão seletiva de drogas [124, 134]. No presente estudo, todos os pacientes foram tratados com inibidores da NS5A/NS5B e os previamente experimentados foram tratados exclusivamente com DAAs direcionados para a proteína NS3 do HCV já que os IPs representavam a única opção terapêutica licenciada para o tratamento da hepatite C crônica antes de 2015. Por esse motivo, não seria esperado nenhum impacto nas taxas de RVS entre pacientes virgens ou experimentados, conforme refletido nos resultados para pacientes monoinfetados, onde não foi observada diferença estatisticamente significativa (95,7% *vs.* 92,9%, respectivamente). Quanto aos pacientes coinfectados HCV/HIV, a taxa de resposta observada no presente estudo (96%) foi próxima da relatada no ensaio clínico ALLY-3, em que a RVS foi de

97% (133/137) para pacientes tratados por 12 semanas com SOF/DCV [148]. O licenciamento de novos DAAs melhorou as taxas de resposta em indivíduos coinfectados HCV/HIV sendo as taxas de RVS semelhantes aos observados para pacientes monoinfectados [131]. Nenhuma diferença de RVS foi identificada entre os grupos monoinfectados e coinfectados (95,3% *vs* 96%), demonstrando que a introdução de DAAs mais efetivos aumentou consideravelmente as taxas de resposta em indivíduos coinfectados, que era de cerca de 50% para a terapia dupla com peg-IFN e RBV [149]. A coinfecção HCV/HIV não é mais considerada uma condição crítica para o sucesso da terapia diante da eficiência dos regimes atuais com DAAs nas taxas de RVS.

As taxas de RVS observadas para cada subtipo do HCV foram de 92,7% (51/55), 100% (57/57) e 90% (18/20) para os subtipos 1a, 1b e 3a, respectivamente. Nossos resultados foram semelhantes aos relatados por Sulkowski *et al.* (2014) [111] em um estudo multicêntrico randomizado, em que as taxas de RVS para pacientes monoinfectados (virgens ou experimentados) pelo subtipo 1b ou genótipo 3 foram 100% (35/35) e 89% (16/18), respectivamente.

Seis pacientes falharam ao tratamento com SOF/DCV com ou sem RBV (4,6%) e amostras foram coletadas no fim do tratamento a fim de avaliar uma provável emergência de RASs em variantes resistentes ao longo da infecção com a utilização dos inibidores da NS5A/NS5B. Para paciente monoinfectado pelo subtipo 1a do HCV que falhou ao tratamento de 12 semanas com SOF/DCV/RBV, as RASs primárias M28T e Q30R e secundária E62D não foram observadas e podem estar associadas a não resposta. A mutação M28T confere um alto nível de resistência *in vitro* ao DCV (100 vezes maior que a estirpe selvagem) e ombitasvir (1000 vezes maior que a estirpe selvagem), enquanto a Q30R confere um impacto significativo *in vitro* para DCV, elbasvir, ledipasvir e ombitasvir [125]. A RAS E62D, embora não atuante de forma primária, pode compensar o *fitness* viral de variantes resistentes, melhorando os níveis de replicação. É importante ressaltar que outros fatores preditivos negativos relacionados à falha terapêutica, como cirrose hepática descompensada e infecção pelo subtipo 1a do HCV, também podem ter contribuído para a não resposta.

A RAS Y93N em uma sequência de aminoácidos da proteína NS5A do HCV cuja amostra de soro de paciente monoinfectado pelo subtipo 1a do HCV foi coletada após a não resposta ao tratamento com SOF/DCV/RBV foi identificada. Estudo de Wyles DL *et al.* (2017) [125] relatou que essa RAS proporcionou uma redução de 10.000 vezes a susceptibilidade aos DAAs DCV, ledipasvir e ombitasvir, três dos principais inibidores da NS5A atualmente em uso em terapias combinadas ausentes de IFN. Além disso, a duração da terapia para esse paciente foi de 24 semanas devido ao tratamento anterior sem sucesso com IFN/RBV e telaprevir. Em sequência de aminoácidos referente à amostra coletada antes do tratamento com SOF/DCV, a mutação Y93N não foi identificada para esse paciente, sugerindo que a presença de pressão seletiva imposta pelo DCV pode ter influenciado a emergência de subpopulações virais com a RAS Y93N. Além da presença da RAS na posição 93, outros fatores como alta carga viral infecciosa (log 6,8), presença de cirrose e infecção com pelo subtipo 1a do HCV também podem ter contribuído para um resultado negativo da terapia.

Sarrazin *et al.* (2016) [145] identificaram a variante Q30Y em 2,0% das sequências do subtipo 1a do HCV analisadas antes do tratamento com inibidor da NS5A. Frequência semelhante (2,2%) dessa mutação foi identificada no presente estudo. A substituição de aminoácidos de glutamina (Q) para tirosina (Y) tem sido raramente descrita *in vivo* para pacientes não respondedores; portanto, estudos adicionais serão necessários para avaliar um possível novo perfil de resistência associado a essa mutação.

A substituição A30S foi relatada anteriormente por Malta *et al.* (2017) [150] para paciente coinfectado HCV/HIV não respondedor (prevalência basal de 6,7%; 1/15) e em um relato de caso internacional [151]. No presente estudo, a RAS A30S foi identificada para paciente não respondedor infectado pelo subtipo 3a do HCV, no entanto, o número inexpressivo de pacientes infectados por esse subtipo em nosso estudo (n=4) nos impediu de determinar se essa substituição é comum nas estirpes de HCV que circulam no Brasil. A substituição de aminoácidos na posição 30 poderia estar relacionada à falha terapêutica experimentada pelo paciente, no entanto, os poucos relatos *in vivo* até o momento limitaram a possibilidade de associação dessa RAS com a não resposta. Além da mutação,

paciente também apresentou outros fatores preditivos negativos para a não resposta ao tratamento: (i) fatores virais como alta carga viral basal (log 6,36) e infecção pelo subtipo 3a do HCV; (ii) fatores do hospedeiro como gênero masculino e presença de cirrose; e (iii) fator terapêutico como a duração reduzida do tratamento (12 semanas) [134]. Uma atualização nas diretrizes clínicas brasileiras publicadas em 2018 aumentou a duração do tratamento para pacientes cirróticos infectados pelo genótipo 3 do HCV de 12 para 24 semanas, o que poderia influenciar positivamente no *clearance* de variantes virais suscetíveis aos DAAs ou resistentes que apresentem RASs com baixa redução de susceptibilidade *in vitro* ou pouca evidência clínica *in vivo* [72].

A frequência de RASs basais na região NS5A do HCV, determinada por técnicas de sequenciamento convencionais e de alta vazão, apresentou valores entre 6% e 16% [144, 152, 153]. No presente estudo, observamos uma associação estatisticamente significativa para a presença de mutações da proteína NS5A no subtipo 1b, em contraste com os subtipos 1a e 3a. Essas mutações, no entanto, não afetaram a eficácia do tratamento, uma vez que todos os pacientes infectados pelo subtipo 1b do HCV alcançaram a RVS. Esse achado pode ser explicado por diferenças na barreira genética à resistência ao DCV, dependendo do genótipo infectante e/ou devido à administração concomitante de SOF com DCV em um regime combinado de DAA. Um relato clínico anterior sugeriu que as estirpes do subtipo 1b do HCV podem acumular mais mutações de resistência do que os subtipos 1a e 3a para superar a ação do DAA [111]. Nosso estudo demonstrou que as RAS basais na proteína NS5A do HCV foram identificadas em 16,8% (18/107) do grupo de pacientes monoinfetados, uma porcentagem ligeiramente superior à relatada em um estudo cuja prevalência de mutações na NS5A foi de 11,5% (18/156) [150]. Além disso, observou-se a presença de RASs em 6,5% (4/46) e 28,9% (13/45) das sequências referentes aos subtipos 1a e 1b do HCV, respectivamente. Nossos achados diferiram da proporção de RASs entre os subtipos 1a e 1b relatados por Zeuzem *et al.* (2017) [154] em estudo com 5397 amostras da América do Norte, Europa, Oceania e Ásia, onde foi encontrada uma prevalência de 13% para o subtipo 1a e 17,6% para o subtipo 1b. No entanto, semelhante aos resultados de Paolluci *et al.* (2013) [155] (subtipo 1a: 12,5%; subtipo 1b: 53,3%), no presente estudo, a frequência de RASs nas estirpes do subtipo 1b foi significativamente maior que nas estirpes do subtipo 1a ($p<0,05$). Embora com maior

frequência no subtipo 1b, o impacto da presença de RASs no resultado do tratamento foi mais evidente para os pacientes infectados pelos subtipos 1a e 3a [148, 156]. Em nosso grupo de estudo, as RASs na proteína NS5A do HCV não parecem ter influenciado o resultado do tratamento, pois todos os pacientes monoinfectados pelo subtipo 1b do HCV responderam à terapia com SOF/DCV.

De acordo com Wyles *et al.* (2017) [125], a substituição de aminoácidos L31M confere um impacto de resistência *in vitro* para os DAAs DCV e ledipasvir (100 vezes maior que a estirpe selvagem). No entanto, nossas análises *in vivo* indicaram que a presença basal da RAS L31M em estirpes dos subtipos 1a (2,2%) e 1b (6,7%) do HCV não influenciou o resultado do tratamento, uma vez que os cinco pacientes com estirpes mutantes atingiram a RVS. Além disso, a baixa frequência da variante L31M também foi observada por Zeuzem *et al.* (2017) [154] (2,3%) e Paolluci *et al.* (2013) [155] (3,1%). Além da RAS L31M, a substituição Y93H também tem sido altamente associada à resistência ao DCV [157]. A baixa frequência da substituição Y93H (6,7%) identificada em nosso estudo entre as sequências do subtipo 1b foi semelhante a dados publicados anteriormente por McCormick *et al.* (2015) o qual a Y93H foi observada com uma frequência de 4,3% [157, 158]. Da mesma forma que a RAS L31M, a presença da substituição Y93H não afetou a eficácia do tratamento nos três pacientes infectados com esta estirpe variante.

Em relação às RASs identicadas na proteína NS5B do HCV para pacientes monoinfectados, a alta frequência da combinação L159F+C316N (31,1%, 14/45) observadas nas estirpes do subtipo 1b do HCV foi semelhante aos resultados relatados por estudos brasileiros de Noble *et al.* (2017) [159] e Peres-da-Silva *et al.* (2017) [160] cuja prevalência foi de 14,2% (8/56) e 25% (13/52), respectivamente. A ocorrência dessss RASs não parece ter influenciado a resposta terapêutica após o tratamento, uma vez que todos os pacientes infectados pelo subtipo 1b do HCV atingiram a RVS. Em nosso estudo, não foi observada substituição no resíduo 282, identificada principalmente *in vitro* e altamente associada à resistência ao SOF para os isolados do subtipo 1b, bem como nos achados de Castilho *et al.* (2011) [161] e Costatino *et al.* (2015) [162].

Poucos estudos brasileiros analisaram o perfil de resistência dos genes NS5A e NS5B em pacientes coinfectados HCV/HIV. Mais estudos serão necessários para entender melhor a circulação de subpopulações virais resistentes nesse grupo.

Nossas análises indicaram que a frequência de RASs na proteína NS5A do HCV para o referido grupo foi de 28% (7/25), enquanto que para a proteína NS5B do HCV foi de 16% (4/25). As estirpes do subtipo 1b apresentaram maior frequência de RASs na NS5A (41,7%; 5/12) e NS5B (33,3%; 4/12) quando comparada aos subtipos 1a e 3a. De acordo com Plaza *et al.* (2012) [163], nenhuma das sequências do subtipo 1a apresentou substituições na proteína NS5A, enquanto que para o subtipo 1b, a combinação de mutações L31M+Y93H, altamente associada à resistência ao DCV, foi detectada na proporção de 1/15 (6,7%). Em contraste com esses resultados, a combinação de RASs L28M+R30Q foi identificada em uma proporção maior nos isolados do subtipo 1b. As análises de resistência para a proteína NS5B do HCV revelaram similaridade na frequência da RAS C316N relatada por Plaza *et al.* (2011) [164] (13,3%) e Trevino *et al.* (2011) [165] (10%), uma vez que 8,3% das sequências referentes ao subtipo 1b apresentaram essa mutação no presente estudo.

A aprovação de novas terapias combinadas livres de IFN voltadas para as regiões NS5A e NS5B do HCV ao longo dos últimos anos permitiu um aumento nas taxas de RVS, principalmente, para aqueles pacientes considerados “difíceis de tratar” (pacientes cirróticos, coinfectados HCV/HIV e experimentados com falha terapêutica prévia). Entretanto, o terceiro manuscrito intitulado “Resistance Mutations A30K and Y93N Associated with Treatment Failure with Sofosbuvir and Daclatasvir for Hepatitis C Virus Infection Non-Responder Patients: Case Reports”, relatou dois casos de pacientes cronicamente infectados pelos subtipos 1a e 3a do HCV que não alcançaram a RVS12 e representaram casos excepcionais que contrariam as altas taxas de resposta terapêutica. A demanda pelo licenciamento e incorporação de novos medicamentos nos protocolos clínicos brasileiros ainda é uma realidade, visto que terapias de resgate serão necessárias para alguns pacientes que tenham uma combinação de fatores prognósticos negativos que comprometam um desfecho de resposta aos DAAs disponíveis atualmente para uso clínico no Brasil.

Segundo Buti *et al.* (2016) [134], a infecção pelos subtipos 1a e 3a do HCV e a presença de RASs em resíduos de aminoácidos específicos na proteína NS5A do HCV estão associados à redução da susceptibilidade aos DAAs. Com base em dados de ensaios clínicos anteriores de Nelson *et al.* (2015) [148] e Poordad *et al.*

(2016) [166], a presença de RASs basais na NS5A parece reduzir a eficácia do DCV em pacientes com cirrose descompensada.

Em relação ao relato de caso 1, fatores preditivos negativos, tais como infecção pelo subtipo 3a do HCV e presença de cirrose, podem ter influenciado o resultado terapêutico insatisfatório de falha terapêutica ao final do tratamento com SOF/DCV. A análise molecular quantitativa e os testes bioquímicos no término da terapia antiviral indicaram carga viral detectável (log 3,97) com enzimas hepáticas aspartato aminotransferase (AST) e gama glutamil transferase (GGT) elevadas. Além do genótipo infectante e da condição hepática, as análises de resistência demonstraram a presença da RAS A30K na proteína NS5A do HCV. Hernandez *et al.* (2013) [167] relataram que as estirpes virais com substituição de aminoácidos de alanina (A) por lisina (K) eram, *in vitro*, 44 vezes mais resistentes à inibição do DCV do que as estirpes selvagens. Estudo brasileiro de Malta *et al.* (2017) [150] identificou a presença da substituição A30K em 16,1% (5/31) dos pacientes monoinfectados, no entanto, seu impacto clínico no resultado do tratamento não foi descrito. Além disso, análises de amostras subsequentes da paciente 1 demonstraram a persistência da mutação no resíduo 30 por dois anos após o final da terapia com DCV/SOF. Outros estudos também relataram a persistência de RASs na proteína NS5A do HCV após a interrupção do tratamento [125, 168]. Esses achados sugeriram que a ocorrência de algumas substituições na proteína NS5A pode ter conferido uma vantagem evolutiva para variantes resistentes, uma vez que não são afetadas pelos inibidores da NS5A e, mesmo na ausência da pressão seletiva imposta por medicamentos antivirais, essas substituições persistem e não afetam o nível de replicação do vírus, conforme evidenciado para a estirpe viral estudada no relato de caso 1. Isso pode ser motivo de preocupação, uma vez que publicações recentes indicam que os DAAs elbasvir, ledipasvir, pibrentasvir e velpatasvir podem ter eficácia limitada quando a estirpe infectante apresenta substituição A30K na proteína NS5A [167, 169-171].

Já o relato de caso 2 (homem de 67 anos com fibrose avançada), a ocorrência de efeitos colaterais limitou a conclusão da terapia com o IP telaprevir. Embora a terapia de resgate com a utilização dos DAAs SOF/DCV em combinação com RBV tenha apresentado resultado promissor na 24ª semana de tratamento (HCV-RNA indetectável), a RVS12 não foi alcançada pelo paciente 2.

Com base em nossos resultados de análises de resistência, a seleção de estirpes virais com substituição de aminoácidos no resíduo 93 da proteína NS5A do HCV de tirosina (Y) para asparagina (N) pode ter representado um fator importante associado à falha terapêutica. Considerando experimentos *in vitro* de Wyles et al. (2017) [125], a RAS Y93N pode levar a uma redução de 10.000 vezes na susceptibilidade ao DCV. Um estudo anterior do nosso grupo relatou a ocorrência dessa mutação em um paciente não respondedor monoinfetado pelo subtipo 1a do HCV cuja amostra foi coletada após o término da terapia, sugerindo que o surgimento da RAS Y93N após pressão seletiva de DCV em combinação com fatores preditivos negativos do hospedeiro e virais contribuiu para a falha do tratamento, bem como observado também para o paciente 2 [172]. Em resumo, apesar do tratamento da hepatite C crônica com DAAs ser considerado bem tolerado e eficiente no Brasil, o relato de caso 2 sugere que a identificação de mutações de resistência no genoma do HCV, especialmente na proteína NS5A, deve ser considerada em casos particulares.

Além do exposto, em 2016, a OMS divulgou o documento “Global Health Sector Strategy on Viral Hepatitis 2016–2021: Towards Ending Viral Hepatitis” cujo objetivo é a eliminação das hepatites virais como ameaça à saúde pública até 2030 [173]. Considerando as hepatites B e C, a implementação de estratégias no acesso à prevenção, ao diagnóstico e ao tratamento poderia evitar aproximadamente 7,1 milhões de mortes entre 2015 e 2030 de acordo com a OMS [173]. O aumento do acesso ao diagnóstico, principalmente para portadores do HCV de difícil acesso, tais como, usuários de drogas intravenosas, tornou-se essencial para o cumprimento da meta estabelecida pela OMS. Além disso, a disponibilidade de novos medicamentos pangenotípicos para o tratamento da hepatite C crônica ainda é uma necessidade, visto que a presença e persistência de variantes resistentes do HCV ao longo da infecção pode influenciar negativamente no desfecho terapêutico e, assim, dificultar o alcance da meta de eliminação da hepatite C.

5 CONCLUSÕES

- O estudo referente ao impacto das RASs da proteína NS3 para os subtipos 1a e 1b do HCV mostrou que o uso dos IPs SMV, paritaprevir e grazoprevir tem uma alta probabilidade de ser eficaz na rotina clínica brasileira para o tratamento de portadores crônicos do HCV;

- A ocorrência de RASs na proteína NS3 do HCV foi maior para o grupo de pacientes previamente experimentados com os IPs de "primeira onda" telaprevir e boceprevir (26,7%), o que já era esperado;

- A mutação V36M, associada ou não com a RAS R155K, foi a mais prevalente no estudo e estava em estirpes virais do subtipo 1a do HCV para ambos os grupos;

- A mutação Q80K, associada à resistência ao SMV, foi observada em apenas uma sequência de aminoácidos da proteína NS3, portanto, sua baixa prevalência em amostras brasileiras sugere que não há necessidade de teste de resistência antes do início do tratamento com esta droga;

- O estudo de RASs nas proteínas NS5A e NS5B, demonstrou que a taxa de RVS em pacientes brasileiros infectados cronicamente pelo HCV tratados com o regime terapêutico entre DCV e SOF (95,4%) foi semelhante aos resultados de ensaios clínicos randomizados;

- Identificamos substituições de aminoácidos já descritas na literatura, algumas, porém, com limitações de ocorrência *in vivo*, o que dificulta uma conclusão mais fidedigna a respeito do seu impacto clínico. No entanto, a

maioria das RASs basais observadas nas proteínas NS5A e NS5B não impactou negativamente o resultado do tratamento, especialmente para o subtipo 1b;

- As RASs detectadas não estão associadas com a diminuição da eficácia de novas combinações de DAAs, como a terapia 3D, SOF/ledipasvir e elbasvir/grazoprevir, podendo assim, ser eficientes no tratamento de portadores crônicos do HCV;

- Fatores virais como a ocorrência de RASs específicas na proteína NS5A e a presença de subtipos do HCV mais correlacionados com não resposta ao tratamento e do hospedeiro (cirrose) influenciou na falha terapêutica;

- A identificação das RASs A30K (subtipo 3a) e Y93N (subtipo 1a) na proteína NS5A para amostras de falha terapêutica foi fundamental na prescrição de uma terapia de resgate que evitasse a ocorrência de resistência cruzada aos inibidores da NS5A atualmente disponíveis;

- A realização de testes moleculares de resistência quando ocorrer a seguinte combinação de fatores de prognóstico negativo: pacientes infectados pelos subtipos 1a e 3a, falha terapêutica prévia após uso de DAAs, cirróticos (principalmente os descompensados) e alta carga viral basal.

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Nationwide overview of the distribution of hepatitis B virus genotypes in Brazil: a 1000-sample multicentre study

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Abstract

The influence of hepatitis B virus (HBV) genotypes in the natural history of the disease and its response to antiviral treatment have been addressed in many studies. In Brazil, studies on HBV genotype circulation have been restricted to specific population groups and states. Here, we have conducted a nationwide multicentre study with an unprecedented sample size representing all Brazilian regions in an effort to better understand the viral variants of HBV circulating among chronic carriers. Seven HBV genotypes were found circulating in Brazil. Overall, HBV/A was the most prevalent, identified in 589 (58.7%) samples, followed by HBV/D (23.4%) and HBV/F (11.3%). Genotypes E, G, C and B were found in a minor proportion. The distribution of the genotypes differed markedly from the north to the south of the country. While HBV/A was the most prevalent in the North (71.6%) and Northeast (65.0%) regions, HBV/D was found in 78.9% of the specimens analysed in the South region. HBV/F was the second most prevalent genotype in the Northeast region (23.5 %). It was detected in low proportions (7 to 10%) in the North, Central-West and Southeast regions, and in only one sample in the South region. HBV/E was detected in all regions except in the South, while monoinfection with HBV/G was found countrywide, with the exception of Central-West states. Our sampling covered 24 of the 26 Brazilian states and the Federal District and is the first report of genotype distribution in seven states. This nationwide study provides the most complete overview of HBV genotype distribution in Brazil to date and reflects the origin and plurality of the Brazilian population.

INTRODUCTION

Chronic hepatitis B still represents a significant global public health problem despite the availability of an effective preventive vaccine against the aetiologic agent of the infection, hepatitis B virus (HBV). The World Health Organization (WHO) estimates that more than 240 million people worldwide are chronic carriers of HBV, an infection that represents an increasing risk of liver complications such as cirrhosis and hepatocellular carcinoma [1, 2].

HBV features a unique replication strategy in which the production of new genomic DNA molecules is preceded by an RNA intermediate synthesized by reverse transcription. The lack of proofreading activity during this replication step yields high substitution rates in the genome, conferring an increased genetic variability to HBV than would be expected for a DNA virus [3].

Based on this genetic variability, HBV has been classified into eight genotypes (A–H) by divergences of more than 7.5 % in the entire genome sequences within each genetic group [4, 5]. More recently, two additional genotypes (I and J) were tentatively proposed [6, 7]. Although recent studies have shown that the distribution pattern of HBV genotypes is changing, especially in regions of the world with elevated migratory waves, in general these ten genetic groups have distinct geographical distributions [8, 9]. Genotype A has a worldwide distribution but is primarily found

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Five supplementary figures are available with the online Supplementary Material.

in Northern Europe, North America and sub-Saharan Africa. Genotypes B and C are preferably found in East and Southeast Asia, and Oceania. A worldwide distribution is also a characteristic of the genotype D strains, with higher prevalence being reported in European, Middle Eastern and Mediterranean countries. Genotype E is endemic to West Africa and is rarely found outside Africa, a pattern similar to that observed for genotypes F and H, whose geographic distributions are almost restricted to Central and South America. Genotype G is not restricted to a specific location and has been reported in samples from the USA, Mexico, France, Germany, Turkey, Brazil and Japan. The newly designated genotypes I and J were only detected in individuals from Vietnam and Japan, respectively [4, 10].

Brazil is a federal republic composed of a Federal District and 26 states that are distributed within five geographic regions (North, Northeast, Central-West, Southeast and South), each presenting socio-demographic, economic and cultural particularities. One of the main characteristics of the population as a whole is its aetiological plurality, with great miscegenation among the native population, European colonizers, descendants of African slaves and more recent immigration of individuals from European, Asian and African countries. Regarding the prevalence of HBV infection, different rates can be observed throughout the country. While Brazil is considered a country with low to intermediate prevalence of HBsAg carriers (less than 1 %) [11, 12], there are some areas of high endemicity for HBV infection like the Amazon basin and villages located in the South and Southeast regions [13].

Although studies regarding HBV genotype circulation in Brazil have been published, most are restricted to a particular state and to specific population groups. In addition, HBV genotype distribution is unknown in seven of the 26 Brazilian states, namely Amapá, Espírito Santo, Ceará, Minas Gerais, Paraíba, Sergipe and Roraima. This multicentre study included samples from 24 of the 26 Brazilian states and the Federal District, including samples from capitals and from hundreds of cities countrywide, being the most comprehensive and representative overview of the circulation of HBV genotypes among chronic carriers in Brazil to date.

RESULTS

Sampling, demographic characteristics and overall HBV genotype distribution

Among the 1004 samples included in the study, the proportion of samples from each Brazilian geographical region included in this nationwide overview of HBV genotype distribution roughly reproduces the proportion of the population living in the corresponding regions, with the exception of the South region, which was slightly under-represented in the total sample size. Regarding all 26 Brazilian states, only two states, Piauí and Rio Grande do Norte, both located in the Northeast region, had no samples included in the study. In relation to gender, of the 915 samples with available information, 58.7 % were from men. The mean age of the patients was 43.4±13.7 years with more than 50 % of the samples with available information being from individuals between 30 and 50 years old. Viral load was available for 919 samples and the majority (37.2 %) of them had values between 3.3 and 4.3 log, a range that requires continuous monitoring of the individual in order to evaluate the necessity of starting antiviral treatment. The distributions of demographic and virological characteristics according to geographic region of the study population are listed in Table 1.

A total of 1004 serum samples were genotyped by INNO-LiPA HBV Genotyping (INNO-LiPA) assay (n=835) and by direct nucleotide sequencing (n=169). Seven HBV genotypes (A–G) were found circulating in Brazil. Overall, HBV genotype A was the most prevalent, being identified in 589 (58.7 %) samples, followed by genotypes D (235; 23.4 %) and F (114; 11.3 %). Genotypes E, G, C and B were found in minor proportions in 18, 13, 9 and 1 sample(s), respectively. Among the samples genotyped by INNO-LiPA assay, infection with more than one genotype (mixed infection) was detected in 25 samples (2.5 %).

Statistical analyses to evaluate the relationship between the most prevalent genotypes (A, D and F) and gender, age groups and viral load did not reveal any significant relationship.

A distinct genotype distribution was found in the different states of the country (Fig. 1). The distribution of HBV genotypes A, D and F in the five Brazilian geographic regions indicated that HBV/A was the most prevalent in the North (151/211; 71.6 %), Northeast (141/217; 65.0 %), Central-West (50/87; 57.5 %) and Southeast (232/394; 58.9 %) regions (Table 1). An exception to this higher prevalence of genotype A was observed in the South region, where genotype D was found in nearly 80 % of the specimens analysed. HBV/D was the second most prevalent in the North, Central-West and Southeast regions, accounting for 14.2, 27.6 and 22.6 %, respectively, of total sampling in these regions. In relation to genotype F, a similarly low proportion (7 to 11 %) was found in samples from the North, Central-West and Southeast regions. This genotype was found in only one sample from the South region and had a high proportion (23.5 %) in the Northeast region, being the second-most prevalent after genotype A.

A detailed distribution of the genotypes found in each state is presented in Table 2. The regional distribution of HBV genotypes per state indicated that differences in genotype frequency could be remarkable between states within the same geographic region. In the North region, HBV/A was the most prevalent in all states (Fig. S1, available in the online Supplementary Material), achieving proportions greater than 78 % in samples from Pará, Amazonas and Tocantins. Unlike other states, Amapá showed a high proportion of genotype D (40 %) and had a single case of monoinfection with genotype G. Genotype F was the second most common in Amazonas, Pará, Rondônia and Roraima.

Table 1. Demographic and virological characteristics of the study population and distribution of HBV genotypes by geographic region

	Brazilian regions					
	North (*n*=211)	Northeast (*n*=217)	Central-West (*n*=87)	Southeast (*n*=394)	South (*n*=95)	Total (*n*=1004)
Gender*						
Female	103 (52.3 %)	78 (52.3 %)	24 (33.8 %)	136 (34.7 %)	37 (38.9 %)	378 (41.3 %)
Male	94 (47.7 %)	82 (47.7 %)	47 (66.2 %)	256 (65.3 %)	58 (61.1 %)	537 (58.7 %)
Age (years)*						
<20	4 (2.3 %)	4 (6.1 %)	0 (0 %)	7 (1.9 %)	1 (1.2 %)	16 (2.1 %)
20–30	38 (22.0 %)	14 (21.2 %)	15 (21.4 %)	54 (14.7 %)	8 (9.4 %)	129 (16.9 %)
31–40	42 (24.3 %)	23 (34.8 %)	19 (27.1 %)	95 (25.9 %)	11 (12.9 %)	190 (25.0 %)
41–50	49 (28.3 %)	11 (16.7 %)	18 (25.7 %)	91 (24.8 %)	31 (36.5 %)	200 (26.3 %)
51–60	20 (11.6 %)	10 (15.1 %)	9 (12.9 %)	75 (20.4 %)	17 (20.0 %)	131 (17.2 %)
>60	20 (11.6 %)	4 (6.1 %)	9 (12.9 %)	45 (12.3 %)	17 (20.0 %)	95 (12.5 %)
Viral load*, †						
<3.3	87 (44.4 %)	54 (28.1 %)	14 (22.9 %)	71 (18.8 %)	26 (28.3 %)	252 (27.4 %)
3.3–4.3	69 (35.2 %)	75 (39.1 %)	19 (31.2 %)	137 (36.2 %)	42 (45.6 %)	342 (37.2 %)
>4.3	30 (15.3 %)	47 (24.5 %)	20 (32.8 %)	132 (34.9 %)	22 (23.9 %)	251 (27.3 %)
>8	10 (5.1 %)	16 (8.3 %)	8 (13.1 %)	38 (10.1 %)	2 (2.2 %)	74 (8.1 %)
Genotype						
A	151 (71.6 %)	141 (65.0 %)	50 (57.5 %)	232 (58.9 %)	15 (15.8 %)	589 (58.7 %)
D	30 (14.2 %)	17 (7.8 %)	23 (26.4 %)	89 (22.6 %)	75 (78.9 %)	235 (23.4 %)
F	23 (10.9 %)	51 (23.5 %)	9 (10.3 %)	29 (7.4 %)	1 (1.0 %)	114 (11.3 %)
Others	3 (1.4 %)	6 (2.8 %)	2 (2.3 %)	31 (7.9 %)	1 (1.0 %)	41 (4.1 %)
Mixed	4 (1.9 %)	2 (0.9 %)	3 (3.4 %)	13 (3.3 %)	3 (3.2 %)	25 (2.5 %)

*Data considering available information for each category.

†Log.

Two chronic carriers infected with genotype E were identified in Amazonas and Rondônia.

A high predominance of genotype A could be observed in all Northeast states, with the exception of Ceará (Fig. S2). This genotype was found exclusively among samples from Alagoas and had substantial frequencies in Sergipe (91.7 %), Bahia (84.1 %) and Pernambuco (74.2 %). Genotype D was not frequently found in the Northeast region as a whole, having a considerable proportion only in Maranhão, infecting 36.7 % of the analysed samples. Genotype F was the major genotype circulating in Ceará (52.6 %) and found in 35.5 % of the samples from Paraíba. HBV genotypes C, E and G, not commonly detected in Brazil, were found in Ceará. Bahia had one case of genotype E infection and an HBV/G monoinfection was observed in Paraíba.

Regarding the Central-West region, in the Federal District and in Goiás, the great majority of samples was classified as HBV/A (71.4 and 86.2 %, respectively) while genotype D prevailed in Mato Grosso do Sul (54.5 %). Frequencies under 15 % were observed for HBV/F in the states from this region and only one infection with genotype E was found in Mato Grosso do Sul (Fig. S3).

In São Paulo, Brazil's most populated state, seven HBV genotypes (A–G) were detected circulating among chronic carriers. Genotypes A, D and F were most frequently observed (46.1, 30.4 and 7.4 %, respectively). São Paulo had 10 cases of HBV/E, eight monoinfections with HBV/G, seven HBV/C and one HBV/B. In Rio de Janeiro and Minas Gerais, genotype A represented nearly 80 % of the analysed samples and Espírito Santo had the greatest proportion of genotype D (33.3 %) among the states of the Southeast region (Fig. S4).

In the South region, a high predominance of genotype D strains among the study population was observed (Fig. S5). This genotype was very frequent in all three states, representing 74.3 % of the samples from Paraná, 79.1 % from Rio Grande do Sul and 88.2 % from Santa Catarina. HBV/A prevalence ranged from 11.8 to 17.1 % and HBV/F was found in only one sample from Paraná. In Rio Grande do Sul, one sample characterized as a monoinfection with genotype G was detected.

Confirmation of INNO-LiPA results by direct sequencing and genotype-specific PCR assay

In order to confirm the reliability of HBV genotyping by LiPA, 85 samples genotyped using this methodology, including randomly selected samples and those characterized as genotypes not commonly reported in Brazil (genotypes B, C, E and H), were submitted to direct nucleotide sequencing to compare the results. Comparison between both methodologies indicated a 97.6 % (83/85) concordance

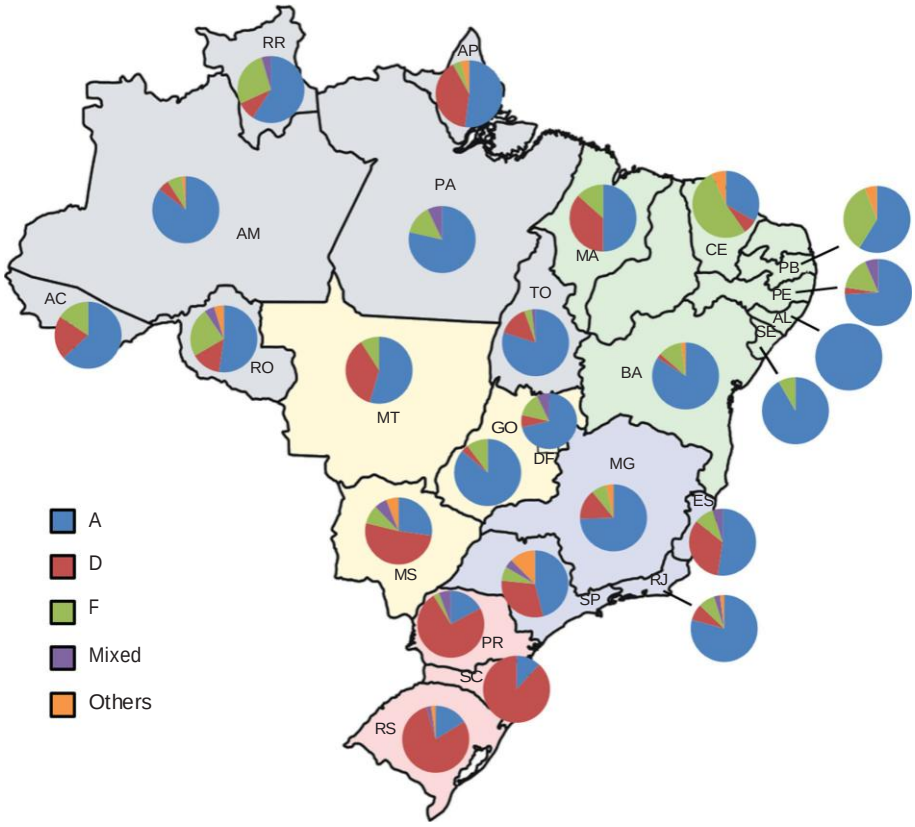

Fig. 1. Distribution of HBV genotypes in Brazilian states. State abbreviations: North – AC, Acre; AM, Amazonas; AP, Amapá; PA, Pará; RO, Rondônia; RR, Roraima; and TO, Tocantins. Northeast – AL, Alagoas; BA, Bahia; CE, Ceará; MA, Maranhão; PB, Paraíba; PE, Pernambuco; and SE, Sergipe. Central-West – DF, Federal District; GO, Goiás; MT, Mato Grosso; and MS, Mato Grosso do Sul. Southeast – ES, Espírito Santo; MG, Minas Gerais; RJ, Rio de Janeiro; and SP, São Paulo. South – PR, Paraná; SC, Santa Catarina; and RS, Rio Grande do Sul.

of the results. The two samples with discordant results had been characterized by LiPA as genotype H. The phylogenetic analysis performed after the determination of the nucleotide sequences of these samples did not confirm this result, indicating that these samples were phylogenetically related to genotype D and F strains.

Also, a semi-nested PCR assay using HBV genotype G-specific primers was performed to confirm the high proportion (68/835; 8.1 %) of mixed infection with genotype G indicated by the INNO-LiPA assay results, especially the mixed infection of genotypes D/G and F/G, an unusual infection profile. Co-infection with genotypes D/G represented almost half (28/68; 41.2 %) of the total mixed infections found while co-infection with genotypes F/G was the second most commonly observed in 22.1 % of samples considered infected with more than one genotype. This result should be analysed with caution since further tests to confirm the presence of HBV/G using PCR primers specific for the characteristic 36 bp insertion in the genome of genotype G strains were negative. Furthermore, analysis of the electropherograms generated by nucleotide sequencing of available samples considered D/G or F/G by LiPA did not show any evidence of overlapping peaks that could suggest the occurrence of a mixed infection. Due to these observations, mixed infections D/G and F/G determined by INNO-LiPA seem to represent a methodology flaw with unspecific binding of the amplified PCR product of certain subgenotypes to the genotype G-specific probe, as discussed below. Aiming at a more accurate and likely scenario of HBV genotype distribution in Brazil, samples classified as mixed infection D/G or F/G were considered as monoinfection with genotypes D or F. Apart from these two mixed infections, co-infection with genotypes A and G was the most prevalent overall, identified in 11 samples. Further investigation by clonal analysis or next-generation sequencing (NGS) to confirm the mixed genotype infection determined by INNO-LiPA assay is warranted.

DISCUSSION

The efforts of the Brazilian Ministry of Health in establishing a nationwide cooperation of nine public laboratories (Brazilian Hepatitis B Research Group) in a project conceived to trace an updated map of the genetic profile of HBV circulating among chronic carriers across Brazil resulted in a

Table 2. HBV genotype distribution in HBsAg-positive samples according to locality

Location		*n*	Genotype distribution (%)							
			A	B	C	D	E	F	G	Mix*
Brazil		1004	589 (58.7)	1 (0.1)	9 (0.9)	235 (23.4)	18 (1.8)	114 (11.3)	13 (1.3)	25 (2.5)
Region	State									
North		211	151 (71.6)	–	–	30 (14.2)	2 (0.9)	23 (10.9)	1 (0.5)	4 (1.9)
	Acre (AC)	19	12 (63.2)	–	–	4 (21.0)	–	3 (15.8)	–	–
	Amapá (AP)	25	13 (52.0)	–	–	10 (40.0)	–	1 (4.0)	1 (4.0)	–
	Amazonas (AM)	55	47 (85.4)	–	–	3 (5.4)	1 (1.8)	4 (7.3)	–	–
	Pará (PA)	14	11 (78.6)	–	–	–	–	2 (14.3)	–	1 (7.1)
	Rondônia (RO)	21	11 (52.4)	–	–	3 (14.3)	1 (4.8)	5 (23.8)	–	1 (4.8)
	Roraima (RR)	22	13 (59.1)	–	–	2 (9.1)	–	6 (27.3)	–	1 (4.5)
	Tocantins (TO)	55	44 (80.0)	–	–	8 (14.5)	–	2 (3.6)	–	1 (1.8)
Northeast		217	141 (65.0)	–	2 (0.9)	17 (7.8)	2 (0.9)	51 (23.5)	2 (0.9)	2 (0.9)
	Alagoas (AL)	26	26 (100)	–	–	–	–		–	–
	Bahia (BA)	44	37 (84.1)	–	–	1 (2.3)	1 (2.3)	5 (11.3)	-	-
	Ceará (CE)	57	19 (33.3)	–	2 (3.5)	4 (7.0)	1 (1.7)	30 (52.6)	1 (1.7)	–
	Maranhão (MA)	30	15 (50.0)	–	–	11 (36.7)	–	4 (13.3)	–	–
	Paraíba (PB)	17	10 (58.8)	–	–	–	–	6 (35.3)	1 (5.9)	–
	Pernambuco (PE)	31	23 (74.2)	–	–	1 (3.2)	–	5 (16.1)	–	2 (6.4)
	Sergipe (SE)	12	11 (91.7)	–	–	–	–	1 (8.3)	–	–
Central-West		87	50 (57.5)	–	–	24 (27.6)	1 (1.1)	9 (10.3)	–	3 (3.4)
	Distrito Federal (DF)	14	10 (71.4)	–	–	1 (7.1)	–	2 (14.3)	–	1 (7.4)
	Goiás (GO)	29	25 (86.2)	–	–	1 (3.4)	–	3 (10.3)	–	–
	Mato Grosso (MT)	11	6 (54.5)	–	–	4 (36.7)	–	1 (9.1)	–	–
	Mato Grosso do Sul (MS)	33	9 (27.3)	–	–	18 (54.5)	1 (3.0)	3 (9.1)	–	2 (6.1)
Southeast		394	232 (58.9)	1 (0.2)	7 (1.8)	89 (22.6)	13 (3.3)	30 (7.6)	9 (2.3)	13 (3.3)
	Espírito Santo (ES)	21	11 (52.4)	–	–	7 (33.3)	–	2 (9.5)	–	1 (4.8)
	Minas Gerais (MG)	55	41 (74.5)	–	–	8 (14.5)	2 (3.6)	4 (7.3)	–	–
	Rio de Janeiro (RJ)	101	80 (79.2)	–	–	8 (7.9)	1 (1.0)	8 (7.9)	1 (1.0)	3 (3.0)
	São Paulo (SP)	217	100 (46.1)	1 (0.5)	7 (3.3)	66 (30.4)	10 (4.6)	16 (7.4)	8 (3.7)	9 (4.1)
South		95	15 (15.8)	–	–	75 (78.9)	–	1 (1.0)	1 (1.0)	3 (3.2)
	Paraná (PR)	35	6 (17.1)	–	–	26 (74.3)	–	1 (2.9)	–	2 (5.7)
	Rio Grande do Sul (RS)	43	7 (16.3)	–	–	34 (79.1)	–	–	1 (2.3)	1 (2.3)
	Santa Catarina (SC)	17	2 (11.8)	–	–	15 (88.2)	–	–	–	–

*Mixed genotype infection: 11 A/G; 5 A/D; 3 D/F; 2 A/F; 2 A/B; 2 D/E.

broad overview of the current situation in virtually all Brazilian states.

To our knowledge, this is the first report of HBV genotype distribution in seven Brazilian states: Amapá, Roraima, Ceará, Paraíba, Sergipe, Espírito Santo, Minas Gerais and in the Federal District of Brazil. With the exception of Ceará, genotype A was the major circulating strain in these states, with prevalence ranging from 52.0 % in Amapá to 91.7 % in Sergipe. A pronounced presence of HBV/F was observed in Ceará (52.6 %) and in Paraíba (35.3 %). Circulation of genotypes not commonly reported in Brazil was detected in Amapá (HBV/G), Minas Gerais (HBV/E), Paraíba (HBV/G) and Ceará (genotypes C, E and G). The knowledge of the circulation of HBV genotypes in these states provides relevant background information for future molecular epidemiological surveillance policies to identify and control viral dispersion, especially importation and dissemination of 'foreign' genotypes.

The overall HBV genotype distribution in Brazil reported here confirmed the preponderant circulation of genotypes A, D and F indicated by previous studies [14–18]. However, the overall prevalence of genotypes A (58.7 %), D (23.4 %) and F (11.3 %) found in the current study differed significantly ($P<0.00001$) from the proportion reported by Mello *et al.* (A: 48.5 %; D: 38.5 %; F: 13.0 %) in a study that also included samples from all Brazilian regions [17]. This discrepancy, which is more than that associated with a shift in the circulation of HBV strains in Brazil during the decade separating sampling, may reflect differences between the two studies in the study population (blood donors versus

chronic carriers) and, mainly, the wide coverage of the present work with regard to sampling. Mello ***et al.*** [17] had limited access to samples from some geographic regions, which led to an extrapolation of results found in a single state capital to a region as a whole. Moreover, the previous study included samples from only nine Brazilian states. Here, we provide a robust sampling encompassing 24 of the 26 Brazilian states and the Federal District, including not only samples from state capitals but also from hundreds of cities countrywide, making the results presented here the most accurate picture of the distribution of HBV genotypes described so far.

In addition to genotypes A, D and F, another four HBV genotypes were found to be circulating in Brazil in minor proportions. To our knowledge, only one previously published work reported the co-circulation of the main eight HBV genotypes in a single country. Osiowy ***et al.*** [19] identified, in a study with acute and chronic hepatitis B cases, HBV genotypes A–I circulating in Canada, a country that shares some similarities with Brazil, such as the continental dimensions of its territory and having immigrants of different nationalities [19]. It is interesting to note that, in our study, genotypes A–G were detected in a single state, São Paulo. It is Brazil's most populated state, has a large number of immigrant communities and is one of the main destinations of internal migratory flux in Brazil. The circulation of genotypes B, C and E is usually restricted to specific geographic regions (HBV/B and HBV/C in Eastern Asia and HBV/E in West Africa) [20] and there are only a few reports of its circulation in Brazil [15, 18, 21]. It is likely that these 'foreign' genotypes reflect the origin of the immigrants or the country where a Brazilian citizen acquired the virus, reinforcing the concept that HBV genotype might act as a valuable tool in the surveillance of viral spread. Regarding HBV genotype G, the least common throughout the world and usually found in co-infection with another genotype, our findings indicated the presence of 13 samples in mono-infection, a condition rarely reported in studies worldwide [22–24], which deserves further investigation to confirm the single genotype character of the infection.

Analysing the results of genotype distribution focusing on states per geographic region, previous studies carried out with samples from the North region reported a predominance of HBV/A in Acre, Amazonas, Pará and Tocantins [25–28], which was corroborated by our findings. A higher frequency of genotype D (45 %) compared to other states in the North region was observed in a study with HBsAg-positive pregnant women in Acre [29]. Here, although in a lower prevalence likely due to differences in the studied population, we also found a higher proportion of HBV/D samples in this state (21 %). However, it was in Amapá that a remarkable presence of genotype D (40 %) could be noticed. Since this higher frequency differs from that observed in neighbouring states, a refined characterization of the subgenotype level of D strains circulating in Amapá would be interesting to elucidate if its origin is related to internal migratory flux or the influence of its bordering country, French Guiana.

In Northeastern states, we found exclusively HBV/A in samples from Alagoas and frequencies over 74 % in Bahia, Sergipe and Pernambuco. A major circulation of this genotype had already been reported in previous studies with chronic carriers in Alagoas (92.5 %) [30] and Bahia (85.5 %) [21]. Heading to the northern portion of this region, we noticed an increase in the detection of HBV/F strains in Paraíba and especially in Ceará. A remarkably high prevalence of genotype F, a genotype typically found in Brazil's indigenous population, was found in Ceará state, where constant movement of individuals towards the Amazon region in search of temporary work in isolated areas can be noticed historically and could suggest greater contact with the Brazilian indigenous population. A recent manuscript describing HBV genotype distribution in Piauí reported genotype F as the second most prevalent genotype in the state, highlighting the tendency of finding this genotype in greater proportions in this part of the country [31]. Regarding genotype D, although its detection was low in the Northeast region as a whole (7.8 %), we found it circulating in 36.7% of the samples from Maranhão. The presence of HBV/D in Maranhão had been reported earlier [32, 33] with a predominance of subgenotype D4 strains, considered an ancient and rarely reported subgenotype possibly related to an African origin [32, 34]. Interestingly, samples from Maranhão available in our study were genotyped by direct sequencing and demonstrated being closely related with D4 reference sequences, presumably related to the strong influence of African descendants in Maranhão's population.

Most of the studies conducted in the states in the Central-West region have been targeted to specific population groups such as injecting drug users [35], men who have sex with men [36], waste collectors [37] and HIV-positive patients [38, 39], impairing an appropriate comparison with our sampling. Despite this, these previous studies reported a greater incidence of HBV/A in Goiás and HBV/D in Mato Grosso do Sul, which was confirmed here. Genotype distribution in Federal District, geographically located inside the territory of Goiás, followed the findings from this state. The Central-West region is considered an area of late occupation in Brazil that received migratory fluxes from the South, Southeast and Northeast regions after the federal capital of Brazil moved to Brasília in 1960. Our results indicated that Goiás and the Federal District had a genotype distribution similar to that observed in the Southeast region while the higher frequency of HBV/D found in Mato Grosso do Sul might be related to its geographical proximity to the states in the South region.

The circulation of three main genotypes, A, D and F, has been demonstrated in the Southeast region since the first report on HBsAg subtype diversity in 1987 [40]. The higher prevalence of HBV/A, followed by HBV/D and HBV/F in smaller proportions, has been confirmed in several studies since then [14, 17, 41–47]. Here, we show for the first time

the distribution of genotypes in two other states in the Southeast region, Minas Gerais and Espírito Santo. The main presence of the three genotypes was observed, with a significantly higher proportion of genotype D found in Espírito Santo when compared to Rio de Janeiro ($P<0.005$). This difference might be explained by the historical process of territorial occupation in Espírito Santo, where a large migratory contingent, mainly composed of Italians, has settled in the state since the 19th century [48]. In São Paulo, our findings indicated the circulation of HBV genotypes typically found in other parts of the world, as is the case for genotypes B and C in Asia and genotype E in West Africa. The identification of 12.4 % of samples with genotypes rarely found in Brazil agrees with the cosmopolitan character of São Paulo, a state that is home to diverse foreign communities of different ethnicities.

The relationship between HBV genotype and the ancestral origin of an infected individual is very evident when analysing samples from states in the South region. All three states were the destination of a large contingent of European immigrants, coming especially from Italy and Germany in the mid-20th century. This ancestral background is likely responsible for the very high prevalence of HBV/D found in Paraná (74.3 %), Rio Grande do Sul (79.1 %) and Santa Catarina (88.2 %), which is corroborated in the studies available in the literature [15, 49–51]. A genetic study based on mtDNA heritage also confirmed a greater influence of European origins in the population from the South region [52].

The method chosen for this study was the commercial assay 'INNO-LiPA HBV Genotyping' which has automated hybridization, scanner reading and strip interpretation, and standardization and reproducibility that allow the execution of tests in different laboratories around the country. This methodology is considered relatively simple, rapid and reliable, and has been used in other large-scale studies [19, 53]. One of the advantages highlighted by the commercial line probe assay manufacturer is the greater sensitivity of the test when compared to direct sequencing, which makes it capable of identifying minority viral populations. However, a limitation of methods based on reverse hybridization is that polymorphisms in the genomic region amplified for the hybridization with the immobilized probes may lead to unspecific binding and consequent misinterpretation of the result. Although previous studies had reported that INNO-LiPA was sensitive in detecting infection with more than one HBV genotype (mixed infection), especially genotypes A/B, A/C, A/G and B/C co-infection [53–55], our results showed an unexpectedly high proportion (8.1 %) of mixed infection determined by INNO-LiPA assay. Interestingly, the most detected co-infection was between genotypes D/G (28/68; 41.2 %) and F/G (15/68; 22.1 %), two genotype mixtures that have not been reported yet. Preliminary investigation using PCR amplification with genotype G-specific primers indicated that there was no evidence for the presence of HBV/G isolates co-infecting these samples. A possible unspecific ligation site in the INNO-LiPA probes, particularly the genotype G probe, with Brazilian strains of genotype D and F may have occurred. Knowing about such misinterpretation of the INNO-LiPA assay in certain HBV subgenotypes is important in diagnosis and clinical practice since some studies reported that the occurrence of a mixed genotype infection is related to a worse outcome and progression of the disease than a single genotype infection [56, 57]. In terms of genotype circulation, however, the assumption that mixed infections D/G and F/G were methodological artefacts would not greatly affect the overall genotype distribution.

This large-scale study had some limitations. Information about the ethnic background of the studied individuals would have been useful to establish a relation with the different genotypes found. Furthermore, demographic data was not available for all the samples and the cross-sectional nature of our study meant that the sampling included multiple scenarios regarding the stage of disease chronicity and management. The presence of untreated individuals along with patients under antiviral therapy for years or starting a rescue treatment with a new drug prevented the application of statistical analysis aiming to identify a relationship between certain genotypes and the outcome of disease.

The influence of the different HBV genotypes in the natural history of the disease and the response to antiviral treatment remains unclear [10]. Nonetheless, there is increasing evidence that HBV genotypes may be associated with a higher rate of progression to chronic infection and hepatocellular carcinoma, spontaneous HBeAg seroconversion, mutations in the precore and/or in basal core promoter, and response to interferon-based therapy [9]. Moreover, the genetic variability of HBV expressed in the different genotypes and its distinct distribution around the world and in relation to ethnic background make genotype classification a useful tool in epidemiological and transmission studies.

In this nationwide, multicentre study, our findings provide the most complete overview of HBV genotype distribution in Brazil to date, spanning virtually all Brazilian states and hundreds of cities countrywide. These data might contribute to molecular epidemiological surveillance of strains circulating in the country, assisting and supporting the strategic actions of the Ministry of Health to monitor and control viral dissemination.

METHODS

Study population

In order to access the distribution of HBV genotypes across Brazil, a joint effort of nine public institutions representing all five regions of the country (North, Northeast, Central-West, Southeast and South) analysed serum samples from 1004 HBsAg-positive chronic hepatitis B carriers who attended public health care services between the years 2013 and 2015. Federal District and all Brazilian states except Piauí and Rio Grande do Norte, located in Northeast region, were represented in sampling that included

individuals not only from state capitals but also several cities countrywide. The number of samples analysed in each state is given in Table 2.

Viral DNA extraction

HBV DNA was isolated from serum samples using the Biopur Mini Spin Viral DNA Extraction Kit (Biometrix Diagnostica, Paraná, Brazil) according to the manufacturer's instructions. Viral DNA was recovered in 50 µL of elution buffer and stored for further application to HBV genotyping using two strategies: (i) commercial line probe assay INNO-LiPA HBV Genotyping (Fujirebio Europe, Gent, Belgium) and (ii) direct nucleotide sequencing along with phylogenetic analysis.

Genotyping by line probe assay

A total of 835 extracted viral DNA samples were submitted to nested PCR amplification using specific biotinylated primers directed to the HBsAg genomic region supplied by the INNO-LiPA HBV Genotyping Kit. The PCR cycle conditions were based on the manufacturer's protocol and amplicons were visualized in a 2% agarose gel for certification of proper product length. PCR product denaturation, hybridization to genotype-specific probes immobilized on membrane strips and posterior processing of the strips in a streptavidin-based chromogenic reaction were performed using the automated workstation AutoBlot3000H (MedTec, North Carolina, USA). Reading and interpretation of strips were also automated using a scanner and LiRAS report software (Fujirebio Europe, Gent, Belgium). All participating laboratories processed the samples using this equipment and following the same protocol.

Genotyping by direct nucleotide sequencing and phylogenetic analysis

A total of 169 extracted HBV DNA samples were submitted to PCR amplification and nucleotide sequencing of a genomic region containing portions of both ORFs P and S with primers and thermal cycling profiles as described by Mallory ***et al.*** [58]. The generated PCR products of approximately 1 kb were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and prepared for sequencing using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) along with internal sequencing primers as described by Mallory ***et al.*** [58]. Sequencing products were electrophoresed on an ABI 3500 Genetic Analyzer (Applied Biosystems).

HBV genotyping was performed by phylogenetic analysis of the DNA sequences determined in this study compared with a multiple sequence alignment of HBV sequences representing all known genotypes available in GenBank. Phylogenetic analysis was carried out using the maxi- mum-likelihood method (bootstrap resampling test with 1000 replicates) in MEGA version 6.0 software [59] under the model of nucleotide substitution GTR+G+I, which was selected as the best-fit model by the jModeltest program [60].

Confirmation of INNO-LiPA results by direct sequencing and genotype-specific PCR assay

To confirm the reliability of genotyping results of the INNO-LiPA HBV Genotyping Kit, 10.2 % (85/835) of the samples were randomly selected for direct nucleotide sequencing. In addition to this randomly selected percentage of samples, those characterized by INNO-LiPA as genotypes B, C, E and H, rarely reported in Brazil, were also submitted to direct sequencing, upon availability of the specimen. Also, a semi-nested PCR assay using HBV genotype G-specific primers was performed as previously described [61] to confirm the high proportion of mixed infection with genotype G indicated by the INNO-LiPA assay results, especially mixed infection of genotypes D/G and F/G, an unusual profile of infection.

Statistical analysis

Univariate analyses were used to describe the distribution of hepatitis B genotypes and categorical variables gender, age group and viral load range. Differences in genotype prevalence in each region and its comparison with previously reported data were also accessed by univariate analysis. Fisher's exact test and Pearson's chi-squared test were used to test the significance level of associations, which was assessed at the 0.05 probability level. All analyses were conducted using the software Epi Info version 7.1 (Centers for Disease Control and Prevention, Atlanta, USA).

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

Ethical statement
The study was approved by the Ethical Committee of the Oswaldo Cruz Foundation (FIOCRUZ) on 16 December 2013 (protocol number: 495.687) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

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Genetic Diversity of the Hepatitis B Virus Subgenotypes in Brazil

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Abstract: Hepatitis B virus (HBV) subgenotypes may be related to clinical outcomes and response to antiviral therapy. Most Brazilian studies on HBV subgenotypes are restricted to some regions and to specific population groups. Here, we provide an insight about genetic diversity of HBV subgenotypes in 321 serum samples from all five geographical regions, providing a representative overview of their circulation among chronic carriers. Overall, HBV/A1 was the most prevalent subgenotype, being found as the major one in all regions except in South Brazil. Among HBV/D samples, subgenotype D3 was the most prevalent, found in 51.5%, followed by D2 (27.3%) and D4 (21.2%). D2 and D3 were the most prevalent subgenotypes in South region, with high similarity with European strains. D4 was found in North and Northeast region and clustered with strains from Cape Verde and India. For HBV/F, the most frequent subgenotype was F2 (84.1%), followed by F4 (10.1%) and F1 (5.8%), closely related with strains from Venezuela, Argentina and Chile, respectively. Phylogeographic analyses were performed using an HBV full-length genome obtained from samples infected with genotypes rarely found in Brazil (B, C, and E). According to Bayesian inference, HBV/B2 and HBV/C2 were probably introduced in Brazil through China, and HBV/E from Guinea, all of them mostly linked to recent events of human migration. In conclusion, this study provided a comprehensive overview of the current circulation of HBV subgenotypes in Brazil. Our findings might contribute to a better understand of the dynamics of viral variants, to establish a permanent molecular surveillance on the introduction and dispersion patterns of new strains and, thus, to support public policies to control HBV dissemination in Brazil.

Keywords: hepatitis B virus; subgenotypes; Brazil

1. Introduction

Despite the implementation of a successful vaccine in several countries, eradication of hepatitis B virus (HBV) is still a challenge. It is estimated that 2 billion people have been exposed to HBV worldwide and 240 million are currently at risk of developing cirrhosis and hepatocellular carcinoma due to chronic infection. Chronic carriers are the main reservoirs of HBV and probably act as a major driven force on HBV evolution. During a long-lasting chronic infection, different selective pressures, as immune system and/or antiviral therapy, shape viral populations. HBV evolution occurs inter and intra-hosts and is punctuated by the geographic distribution of HBV variants. HBV evolution

rate has been estimated varying from 2.2 × 10^{-6} to 7.7 × 10^{-4} nucleotide substitutions/site/year [1–5]. These discrepancies may be due to differences in the methodology employed for HBV evolution rate calculations. Whereas long-term studies employed internal node calibrations on phylogenetic trees using conserved regions of the HBV genome [3], the short-term studies have been performed on HBV evolution within a single patient or are based on family history or pedigree [2,5–7].

Due to its unique life cycle, in which an error-prone reverse transcriptase is employed for genome replication, HBV presents a large genetic variability, resulting in at least 9 genotypes (A–I), almost 40 subgenotypes, several recombinants, clades and quasispecies [8]. Genotype A is currently divided in 4 subgenotypes, named A1–A4. Subgenotypes A1, A4, and quasi-subgenotype A3 are endemic in Africa, while A2 prevails in Europe and North America. Genotypes B and C are prevalent in East Asia, Indonesia and Oceania. Genotype B have been divided into 6 subgenotypes: B1, B2, B4–B6, and quasi-subgenotype B3, whereas genotype C, the oldest HBV genotype, has the highest number of subgenotypes: C1, quasi-subgenotype C2, C3–C16. Genotype D has a worldwide distribution and is currently divided in 9 subgenotypes/recombino-subgenotypes: D1–D9. Genotype E is found almost exclusively in West Africa and is not divided into subgenotypes. Its low genetic diversity is consistent to a short evolutionary history in humans. Genotype F is classified into 4 subgenotypes, F1–F4, and are more frequent in Amerindian populations of Central and South America. This geographic distribution is also shared by genotype H, which is genetically related to genotype F. Genotype G does not have a distinct geographic distribution and has been related to a specific group as men who have sex with men in samples from Europe, USA, Mexico, Brazil and Japan. Genotype I was first reported in a single sequence from Vietnam. Subsequently, sequences found in Laos, India and China allowed the classification into two subgenotypes: I1 and I2 [8–13]. More recently, a putative subgenotype J was proposed from a single sample found in Japan [14].

Genotypes A, D and F are the most prevalent in Brazil, reflecting Brazilian population origins, descendant mainly from African slaves, European colonizers and native Amerindians [15–17]. Moreover, the number of immigrants is increasing considerably, leading to demographic changes and introduction of foreign viral variants.

Recently, a large-scale study with more than one thousand sample spanning virtually all Brazilian States and hundreds of cities countrywide, revealed a markedly difference in genotype distribution in the distinct geographic regions. While HBV/A was the most prevalent in the North and Northeast regions, HBV/D in South region, the HBV/F was the second most prevalent genotype in the Northeast region. Genotypes B, C, E and G were found in a minor proportion, so that in total, seven HBV genotypes were found circulating in Brazil [18]. There are increasing evidences that HBV subgenotypes may be related to clinical outcomes, as progression to hepatocarcinoma, and the response to antiviral therapy [9,12,13]. Despite several studies on HBV subgenotypes have been performed in Brazil, most are restricted to some regions and to specific population groups [17,19,20]. In this study, we analyzed the genetic diversity of HBV subgenotypes from all five geographical regions, providing a representative overview of subgenotypes circulation among chronic carriers. Moreover, Bayesian evolutionary analyses were conducted for the subgenotypes rarely found in Brazil to estimate the most probable dissemination routes.

2. Materials and Methods

2.1. Study Population

Serum samples from 321 chronic hepatitis B carriers (HBsAg positive and HBV DNA load > 200 IU/mL) were included in this study. All samples were collected between 2013–2015, from capitals and countryside cities of the five Brazilian geographical regions, and previously genotyped by "INNO-LiPA HBV Genotyping assay" [18]. Except for HBV/F, the proportion of samples from each genotype selected for subgenotype characterization was similar to the proportion of genotype distributions observed in the correspondent regions, as stated in the nationwide multicenter study

representing all Brazilian regions [18]. Therefore, 177 samples of genotype A (55.1%), 66 samples of genotype D (20.6%) and 69 of genotype F (21.4%) were included in this study. All samples from HBV/A, /D, and /F were selected using the random sample selection tool in our Excel datasheet. In addition, 9 samples (2.8%) from the genotypes rarely found in Brazil were also selected: B (n = 1), C (n = 2), E (n = 3) andG (n = 3). Genotype F sampling was overrepresented as a way of providing molecular data about this less studied genotype as required by other researchers [8,12].

2.2. Ethics Statement

The study was approved by the Ethical Committee of the Oswaldo Cruz Foundation (FIOCRUZ) in December 16th, 2013 under protocol number 495.687 and it is in agreement to the ethical guidelines of the 1975 Declaration of Helsinki.

2.3. Viral DNA Extraction

HBV DNA was isolated from serum samples using the Biopur Mini Spin Viral DNA Extraction Kit (Biometrix Diagnostica, Paraná, Brazil) according to the manufacturer's instructions. HBV DNA was stored for later use for genome amplification and direct nucleotide sequencing followed by phylogenetic analyses.

2.4. HBV DNA Sequencing and Subgenotype Characterization

All 321 samples were submitted to PCR amplification of partial S/Pol genes containing 805 bp, using primers and thermal cycling conditions as described by Mallory et al. (2011) [21]. Genotypes rarely found in Brazil as B (n = 1), C (n = 2), E (n = 1) and G (n = 1) were submitted to full-length genome amplification and sequencing (~3200 bp). Another 14 Brazilian samples from different HBV genotypes endemic in Brazil were also submitted to full-length amplification, as described by Günther et al. (1995) [22], in order to verify the accuracy of HBV subgenotypic classification of the partial S/Pol fragment (~800 bp) compared to the full-length genome. Then, phylogenetic analyses for both complete and partial sequences were conducted with additional 65 sequences from all HBV subgenotypes available in GenBank (see list and origin of all reference sequences used in this study in Table S1). Multiple sequence alignment was performed by using Clustal W program implemented in MEGA software version 7.0 [23].

Phylogenetic analysis was carried out using the maximum likelihood method, bootstrap resampling test with 1000 replicates. The dispersal pattern of the rare/unusual genotypes as B, C and E was accessed by Bayesian Inference using the Bayesian Markov Chain Monte Carlo (MCMC) statistical framework implemented in the BEAST v1.10 package [24] under GTR (General Time Reversible) + G + I, which was selected as the best-fit model. Phylogeographic datasets were performed according to the following criteria: non-recombinant human full genome sequences with known country and collection date, whose nucleotide sequences did not present any insertion. The number of sequences from the same locality was proportionally adjusted in order to avoid bias. MCMC analysis was run for 1 + E08 generations. Calculation of the effective sample size (ESS) was performed using TRACER v1.7. All parameters showed ESS values >200 and their uncertainties were reflected in the 95% Highest Posterior Density intervals. The maximum clade credibility was visualized with FigTree v1.4.2 program.

3. Results

Nucleotide sequences of the partial S/Pol fragment (805 bp) were obtained from 321 Brazilian HBV positive samples. Full-length genome sequences (~3200 bp) were successfully obtained from the rare genotypes B, C and E, as well as from other 14 samples of genotypes A, D and F circulating in Brazil. Phylogenetic analysis was performed along with 65 sequences from all HBV subgenotypes (complete genomes) available in GenBank.

A comparative analysis of the topologies of full-length and partial S/pol phylogenetic trees was performed aiming to verify the accuracy of HBV subgenotypic classification using partial S/Pol fragment. The Brazilian HBV strains clustered with the sequences of reference subgenotypes with high bootstrap value (**>** 80%) in both full-length (Figure 1A) and partial S/Pols phylogenetic analysis (Figure 1B). The classification of HBV strains into subgenotypes was the same in both phylogenetic trees. Since no significant di**ff**erences between trees topologies were observed, the analyses of HBV subgenotype prevalence and phylogenetic relationships were based on the partial S/Pol fragment.

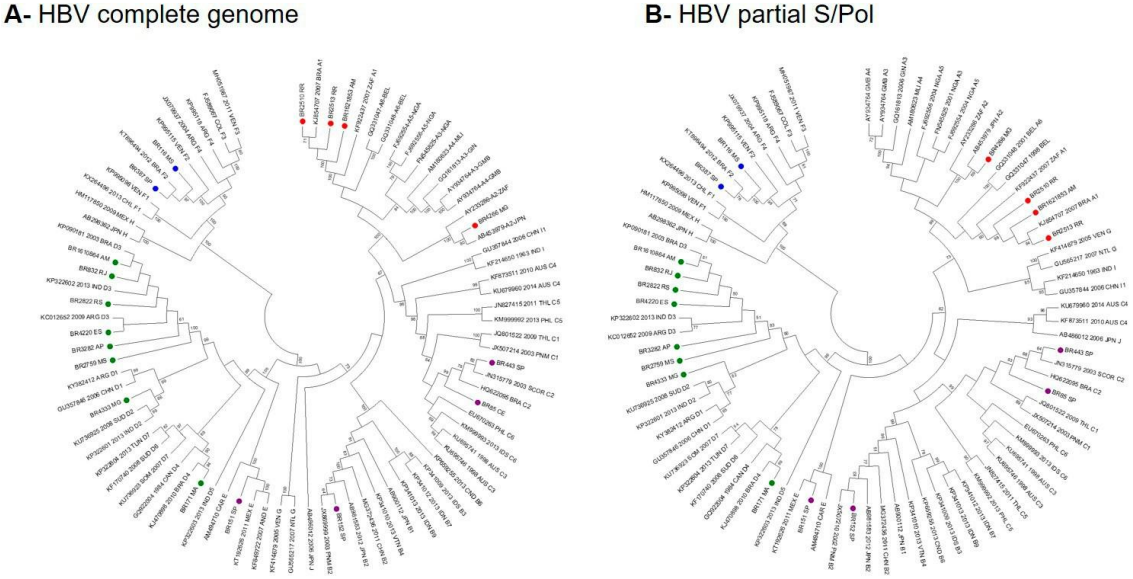


Figure 1. Comparison of the full-length and partial S/pol phylogenetic trees topologies. Red dots: HBV/A; purple dots: HBV/B, C and E; green dots: HBV/D; blue dots: HBV/F genotypes/subgenotypes.

Phylogenetic analyses of partial S/Pol gene sequences (805 bp) from all 321 HBV-DNA positive samples revealed that, overall, HBV/A1 was the most prevalent subgenotype (Figure 2). From HBV/A samples selected for subgenotyping (n **=** 177), subgenotype A1 was detected in 87.6% and A2 in 11.3%. Two samples (1.1%) belonging to HBV/A could not be subgenotyped, clustering close to sequences for the quasi-subgenotype A3, however without a strong bootstrap support (Figures 2 and 3). Among HBV/D samples (n **=** 66), subgenotype D3 was the most prevalent, found in 51.5%, followed by D2 (27.3%) and D4 (21.2%). For HBV/F (n **=** 69), the most frequent subgenotype was F2, in 84.1% samples, followed by F4 (10.1%) and F1 (5.8%).

HBV/B full-length genome (n **=** 1) was classified as subgenotype B2 and HBV/C (n **=** 2) as *quasi*-subgenotype C2. One genotype E full-length sequence was obtained and genetically analyzed. Unfortunately, genotype G could not be successfully full-length amplified and reamplification was not possible due to sample volume limitation. Figure 2 show the maximum likelihood phylogenetic tree of partial S/Pol gene sequences (805 bp) from all 321 HBV samples (subgenotype-specific clades are indicated by colours).

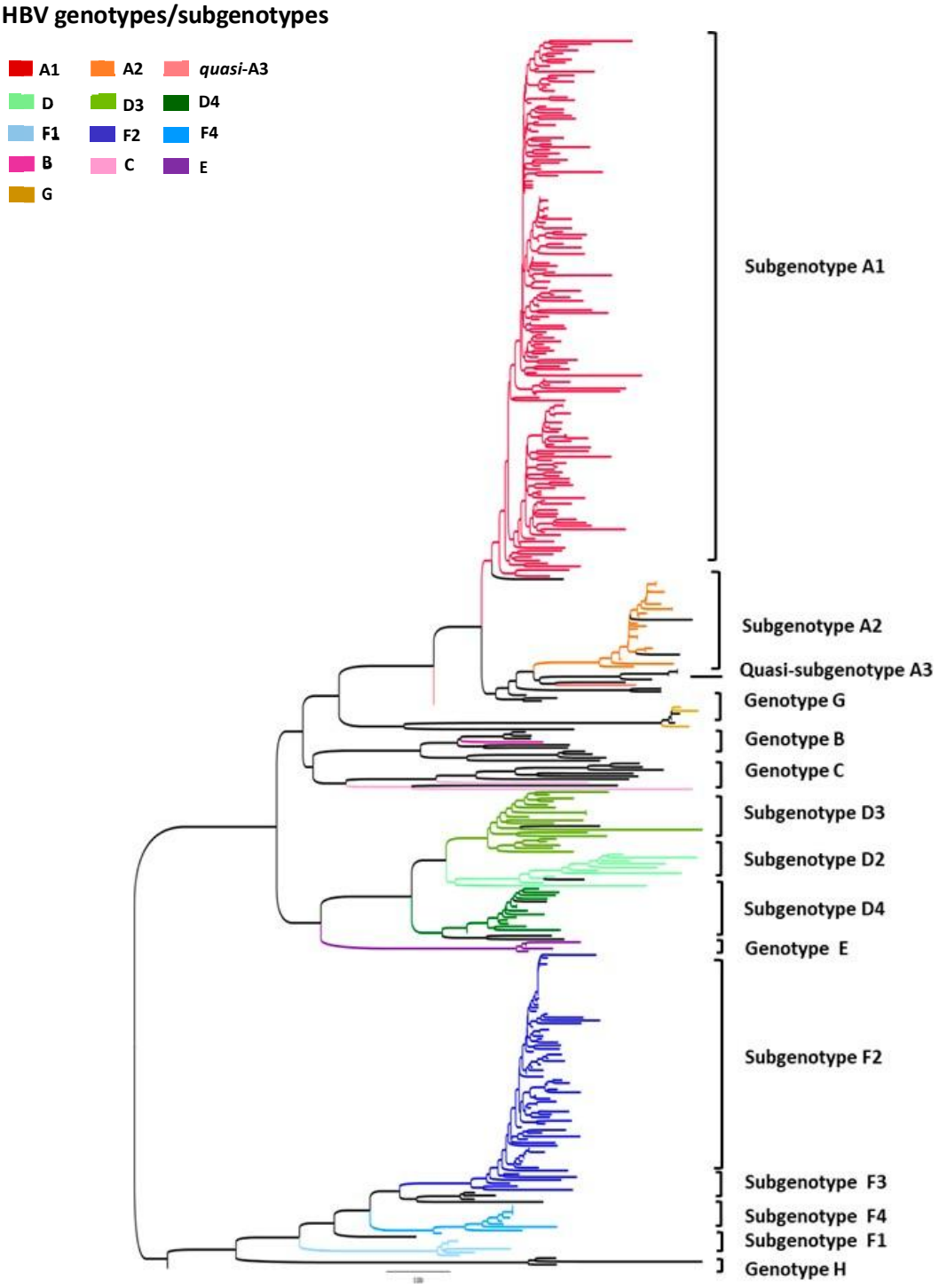


Figure 2. Maximum likelihood phylogenetic tree of all samples enrolled in this study (n = 321). Each genotype/subgenotype clade is represented by colours discriminated in the legend and taxa used as reference sequences are displayed in black.

HBV subgenotype distribution according to region is detailed in Figure 3. HBV/A1 was the most prevalent in all but the Southern region, with all Brazilian isolates clustering in the Asia-American clade (Figure 4A). In the North, A1 accounts for 57.3% of HBV isolates. HBV/A2 was detected in Northeast (5.8%) and Southeast (25.7%). HBV/D2 (47.8%) and D3 (39.1%) were the most prevalent subgenotypes in South, presenting high genetic identity with European samples. HBV/D4 was found in North and Northeast and clustered with strains from Brazil, Cape Verde and India. As identified by

Lampe and colleagues, most of HBV/F samples were observed in Northeast. From all Brazilian HBV/F isolates, HBV/F2 was the most frequent (84.5%), followed by F4 (10.1%) and F1 (5.8%), presenting genetic relatedness with strains from Venezuela, Argentina and Chile, respectively. Genotypes B, C, E and G were found mainly cases in the Southeast region (Figure 3).

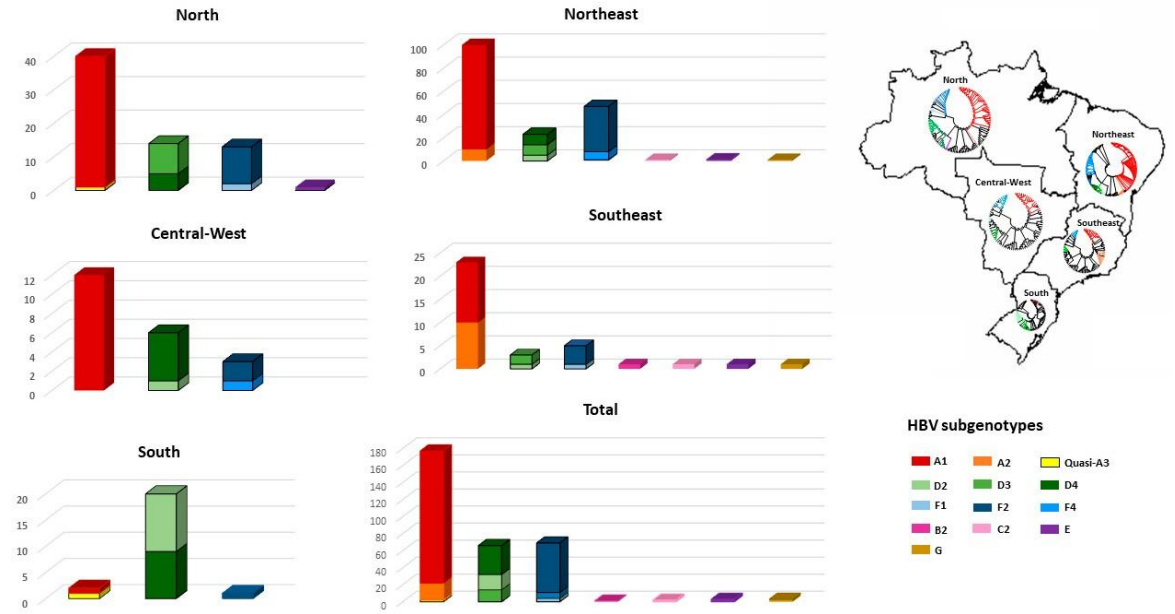
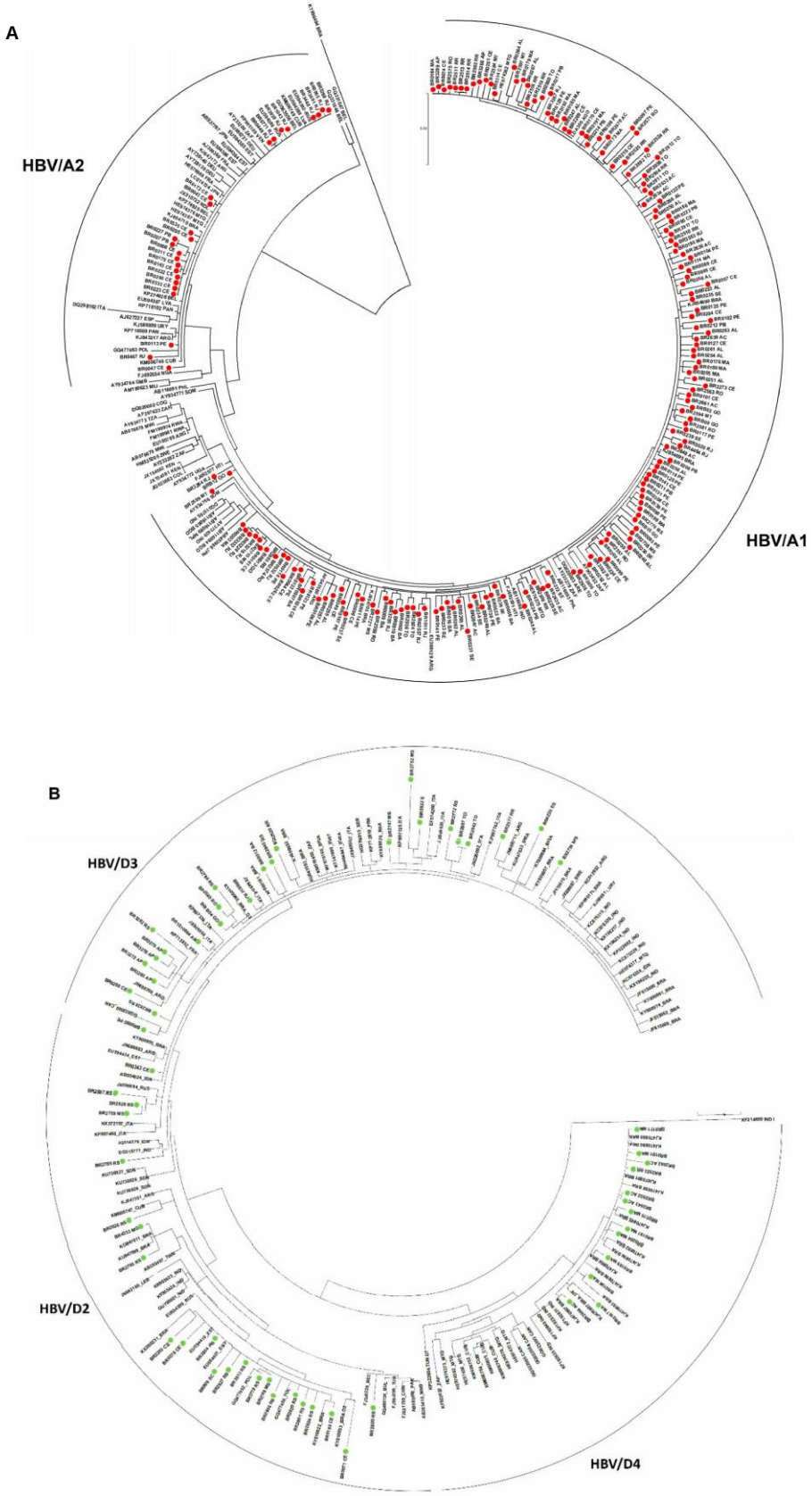


Figure 3. Distribution of HBV genotypes according to geographic regions. The phylogenetic trees in Brazilian map reflects the HBV subgenotypic diversity in each region. Taxa represented in black are reference sequences retrieved from Genbank.

Bayesian evolutionary analyses were conducted for the subgenotypes rarely found in Brazil as B2, C2 and E. Maximum clade credibility trees displayed in Figure 5 shows the most probable country of origin and dissemination routes of these genotypes to Brazil. According to Bayesian Inference, HBV/B2 and HBV/C2 were likely introduced in Brazil from China. Our results confirmed that both HBV/B2 and C2 would have originated in China, as previously proposed [3,25]. HBV/E clustered with samples from Liberia, Ghana and Guinea and it was probably introduced in Brazil from Guinea.


A
HBV/A2
HBV/A1
B
HBV/D3
HBV/D2
HBV/D4

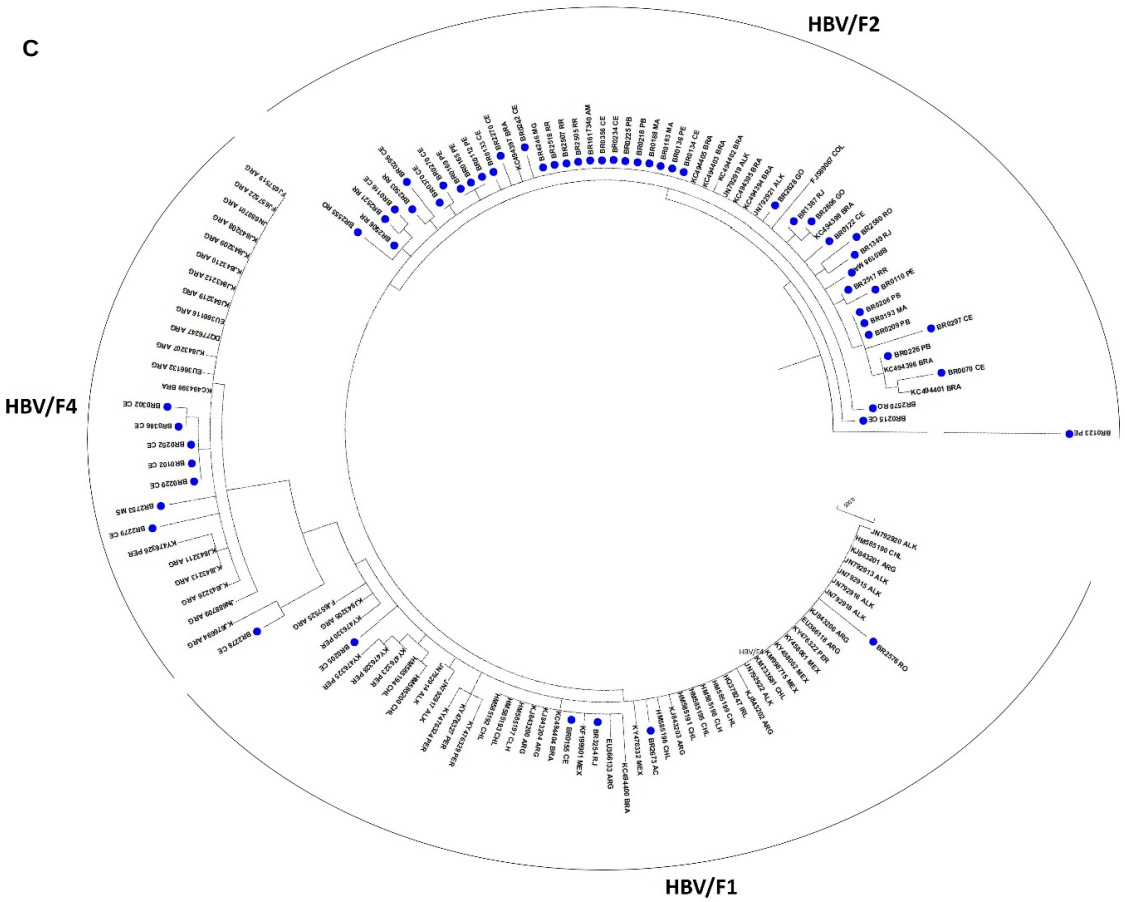
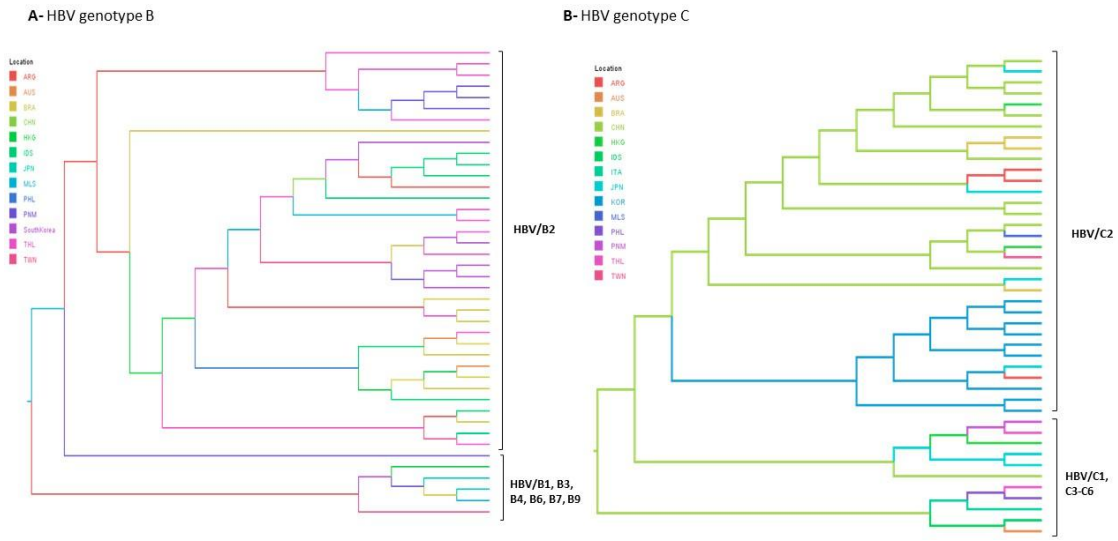


Figure 4. Phylogenetic analysis based on HBV partial S/Pol nucleotide sequences. Maximum likelihood phylogenetic trees for each genotype were constructed using (**A**) 177 HBV/A; (**B**) 66 HBV/D, and (**C**) 69 HBV/F sequences determined in this study and reference sequences representing all subgenotypes.



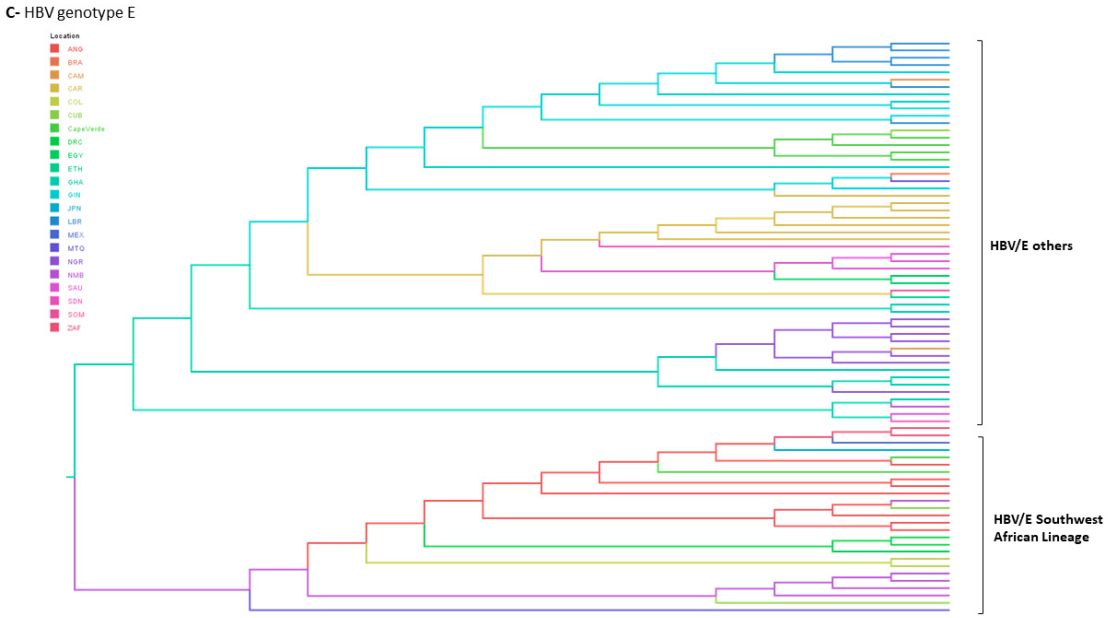


Figure 5. Bayesian maximum clade credibility tree of (**A**) HBV/B, (**B**) HBV/C and (**C**) HBV/E full length genome sequences. B1–B9 and C2–C6 represent HBV subgenotypes within genotypes B and C respectively. HBV/E tree is composed by two branches corresponding to the Southweast African lineage countries (Angola, Namibia and Democratic Republic of the Congo) and the other HBV/E sequences found in other African countries. Branches are coloured according to probable country of origin represented by the following abbreviations: ANG: Angola; ARG: Argentina; BRA: Brazil; CAM: Cameroon; CAR: Central African Republic; CHN: China; CAN: Canada; COL: Colombia; CUB: Cuba; CPV: Cape Verde; DRC: Democratic Republic of the Congo; EGY: Egypt, ETH: Ethiopia; GHA: Ghana; GIN: Guinea; HKG: Hong Kong; IDN: Indonesia; JPN: Japan; LBR: Liberia; MEX: Mexico; MTQ: Martinique; NGR: Nigeria; NMB: Namibia; PNM: Panama; SAU: Saudi Arabia; SDN: Sudan; SOM: Somalia; THL: Thailand; VTN: Viet Nam; ZAF: South Africa.

4. Discussion

Recently, a nationwide, large-scale survey on the prevalence and geographic distribution of HBV genotypes in Brazil was published by our group [18]. The present study is a complementation of the previous data, aiming to elucidate in detail the genetic variability of HBV subgenotypes across Brazil, as well as to investigate the introduction and dispersion patterns of foreign viral variants. Phylogenetic analysis of 321 HBV isolates revealed the presence of three HBV/A (A1, A2, quasi-A3), three HBV/D (D2-D4) and three HBV/F (F1, F2 and F4) subgenotypes circulating endemically in Brazil. Genotypes B, C, E and G were mostly linked to travelers or immigrants.

As genotype A has been found worldwide, it has been suggested that it has emerged in Africa [4,26,27] and after a long evolutionary process and di**ff**erentiation events, it has accumulated enough genetic variability to permit the classification in di**ff**erent subgenotypes before and after spreading to other continents [28]. Previous studies proposed that HBV/A1 was first introduced in Brazil by the slave trade from 16th to 19th centuries [15,17]. In this study, HBV/A1 was by far the most prevalent subgenotype, accounting for 87.6% of HBV/A samples, while A2 was detected in 11.3%. These findings corroborated with previous observations in which HBV/A1 has been found at a frequency about ten times higher than A2 in Brazil [15,16,29,30]. Although not presenting a monophyletic origin — which indicate multiple introduction events over time — all Brazilian HBV/A1 isolates clustered in the 'Asia-American' instead of the 'African' clade. This observation reinforces the suggestion that HBV/A1 isolates were carried to Brazil by an alternative route, possibly being imported from East Africa or Asia by merchants in the middle of the 19th century [30]. Further studies enrolling samples from East

African countries as Mozambique would shed a light on HBV/A1 dispersion patterns between Africa and Brazil.

Assuming an intermediate mutation rate of 2×10^{-5} s/s/y [1,26], it was suggested that HBV/A1 and A2 have diverged more than 2000 years ago, with HBV/A2 isolates being endemic in Europe and countries with European colonization [8,26]. It is known that the main subgenotypes circulating in Europe are HBV/A2 in the North-western countries and HBV/D1, D2 and D3 in the South-eastern European and Mediterranean countries [31,32]. In this study, HBV/A2 isolates clustered with sequences from distinct European countries such as Germany, Belgium, Russia and Poland and are phylogenetically divided into several branches, with no evidence of monophyly (intragroup and overall mean genetic distance 0.007 ± 0.001).

Two HBV/A sequences from this study (one from North and the other from South region) clustered close to the recently described quasi-subgenotype A3, that comprises sequences from the former A3, A4 and A5 subgenotypes [12]. Unfortunately, some ambiguities found in these sequences could not be resolved due to volume limitation for resequencing, so more sequences are needed to confirm the circulation of quasi-subgenotype A3 in Brazil.

Genotype D has been found in all five Brazilian geographic regions, being the second most prevalent genotype [16,18]. In this study, we observed a marked prevalence of HBV/D2 and D3 in South region (87.0%). These findings were in agreement with other studies conducted in South Brazil, where these subgenotypes accounted for 57–100% of viral isolates [33–37]. Phylogenetic analysis revealed that HBV/D2 and D3 isolates were genetically related to sequences from East Europe and Italy, respectively. HBV/D3 is highly prevalent in Italy [31,38] and have been described in populations of Italian ancestry in Brazil [20,36,39–41] and Argentina [42]. In our study, all Brazilian D3 clustered together, suggesting closer ancestral relationships (intragroup divergence of 0.005 ± 0.001, versus 0.007 ± 0.001 when compared to international sequences). However, a study enrolling full-genome analysis of HBV/D3 from Brazilian Amazon Region published by Spitz and colleagues [43] did not find a close relatedness between the Brazilian and European D3 sequences. However, this might be due to the limited number of HBV/D3 full-length genomes assigned to Italy in Genbank that hindered more robust phylogenetic analyses. Despite this, combined evidence as historical background, partial HBV/D3 sequencing, and Y-chromosome heritage pointed out Italy as the most plausible source of HBV/D3 circulating in Brazil [36,39,41,44].

While HBV/D3 may be related to Italian settlement in South region in the early 1900s, the detection of HBV/D4 in Brazilian North and Northeast region seems to be linked to the forced migration of Africans to Brazil. Despite virtually confined to Africa, HBV/D4 has been found in the islands of Cuba, Haiti and Martinique, where, as HBV/A1, its introduction has been linked to slave trade [28,45]. Here, HBV/D4 was detected in 21.2% of HBV/D samples, all of them from North and Northeast Brazil. This finding is in agreement with previous studies conducted in rural populations of the same Brazilian regions, where HBV/D4 have been found in a variable frequency, from 2.5 to 24% [29,46,47]. The relatedness of the Brazilian strains thus suggests single/few introductions of HBV/D4 in North/Northeast Brazil. Phylogenetic analyses showed that the Brazilian HBV/D4 formed a distinct clade, with few sequences from East Africa, Cape Verde and from the region of Tripura, India. As Brazil, India also faced successive waves of colonization, and multiple episodes of human migration [48]. It is possible that, due to the geographical proximity of Tripura with the Portuguese colony of Hughli in India, HBV/D4 isolates would have been introduced in Brazil from Asia or East Africa by the Transatlantic trade, as stated for the Asian-American clade of subgenotype A1.

Latin America is the most plausible origin of HBV/F, where it circulates since the pre-Columbian times [49]. HBV/F subgenotypes are highly dispersed in South, Central America and in the native population of Alaska, being rarely found in other parts of the globe [33,50–54].

Although South American countries present a marked predominance of HBV/F, several historical processes shaped Brazil as an exception [18,36,54]. HBV/F is the third most prevalent genotype circulating in Brazil, with a remarkably prevalence in the Northeast, region with a high interchange

with the indigenous population from Amazon region [16,18]. In this study, the most frequent HBV/F subgenotype was F2 (84.1%), followed by F4 (10.1%) and F1 (5.8%). A very similar prevalence was found by Mello and colleagues [54] in a survey on the phylogeography of HBV/F in Brazil, in which HBV/F2 was suggested as being the oldest HBV/F subgenotype, thus representing the original native HBV of Brazil [54]. HBV/F2 have been described in variable proportions across Brazil [16,39,55,56]. Here, HBV/F2 was mostly found in North and Northeast displaying high similarity with other Brazilian sequences and with sequences from Venezuela. HBV/F4 and F1 were related to strains from Argentina and Chile, respectively.

Although Brazilian ethnical background seems to explain the genetic variability of HBV strains, viral variants of unusual/rare subgenotypes were mostly associated to immigrants or travelers. In this study, one HBV/B2 sample was identified in an Asian patient living in Southeast Brazil, and clustered with HBV/B2 sequences from China and Panama. To our knowledge, a complete genome of subgenotype B2 has not been characterized in Brazil before. Although HBV/B has been previously reported in a Chinese female who had moved to Northeast Brazil [57] and in patients from an Asian community in South region [58], no information regarding subgenotype variability was provided.

Phylogeographic analyses revealed that HBV/B2 would have originated in China and have been introduced in Brazil and other Latin America countries by China/Hong Kong. These findings are in agreement with the fact that Brazil, Argentina and Panama were the main destinations for Chinese immigrants from the 1960s onwards. Moreover, Brazil was the fourth country with the largest number of Chinese migrants in the XXI century, of whom 80.7% settled in Southeast region. This fact may be related to the expansion in commercial relations between Latin America and China, with an active Brazilian participation [59].

The two HBV/C isolates from this study were classified as quasi-subgenotype C2, one from Southeast and one from Northeast regions. According to phylogeographic analyses, China was the most plausible source of this subgenotype. HBV/C2 had been previously described in people of Japanese and Chinese ancestry from South and Southeast Brazil [20,37,58]. Despite HBV/B and C are widely found in the Asian region with an expressive number of HBV chronic carriers (revised in [8,60]), these genotypes are rarely detected out of this continent. In Brazil, most reports of genotypes B and C have been linked to Asian communities and probably have been restricted to these groups due to cultural habits and/or intrafamilial transmission routes [58,61].

It has been established that genotype C has a higher propensity to fix mutations compared to genotype B, and for this reason have been linked to severe liver disease and hepatocarcinogenesis [8]. These findings highlight the need to manage the dispersion of HBV/C in Brazil, since its spread may seriously impact the public health.

HBV/E is largely spread in West Africa but its low genetic variability pointed out to a recent evolutionary history in humans [28,62–64]. Despite the three centuries of slave trade from Africa to Americas and the evidences of the contribution of forced migration in the spread of other HBV genotypes, HBV/E has not been reported in the Americas, except in people who maintained relations with Africa [17,19,37]. In this study, HBV/E was characterized in samples from North, Northeast and Southeast, in individuals whose names indicate African ancestry. In Brazil, HBV/E was previously found in a patient from Guinea-Bissau living in South region [37], in an Angolan individual living in an Afro community from Central Brazil [17] and in our multicentric previous study, where HBV/E was detected in sporadic cases in all Brazilian regions, except the South [18].

Phylogenetic analysis of HBV/E partial sequences indicates independent introductions of this genotype in Brazil. From all three HBV/E sequences from this study, two clustered in the Southwest African Lineage (SWA), that comprises samples from the Democratic Republic of Congo, Namibia and Angola, countries located in the West African coast, that historically were suppliers of slave labor to Brazil [62].

Phylogeographic analyses were performed with one HBV/E complete genome that was successfully amplified. To our knowledge, no complete genome of HBV/E has been characterized in Brazil before.

This isolate did not fit in the SWA and may not represent the consensus of HBV/E strains circulating in Brazil. This analysis revealed that the most recent common ancestor of this strain was possibly introduced in Brazil through Guinea, probably linked to the recent episodes of human migration from Africa. However, due to the remarkably differences in the HBV/E sampled in Brazil, these results cannot be extrapolated to other HBV/E isolates.

Genotype G could not be successfully amplified in its full length. Nevertheless, phylogenetic analysis of the three HBV/G partial sequences revealed multiple introductions through distinct origins. HBV/G is a cosmopolitan genotype that have been linked to sexual transmission by men who have sex with men worldwide [8,60]. HBV/G is commonly found in coinfection with other genotypes, however, few cases of mono-infection have been described [65,66], as the chronic carriers enrolled in this study.

Brazil is a continental country with expressive ethnic diversity shaped by several historical events. The genetic variability of HBV expressed in the different subgenotypes reflects the Brazilian ethnic background that seems to be allied with the distinct waves of migration to and inside Brazil. In addition, recent episodes of Arab and Asian migration have contributed to ethno-cultural enrichment of the Brazilian population as well as to the dispersion of pathogens.

In conclusion, a comprehensive overview of the current situation of HBV subgenotypes circulation among chronic carriers in all Brazilian regions is addressed in this study. These data may contribute to epidemiological surveillance of HBV isolates circulating in Brazil, introduction of new strains as subgenotypes B2, quasi-C2 and E and its dispersion patterns, mostly linked to the recent events of human migration. Combined efforts of research groups and the Brazilian Ministry of Health are needed to better understand the dynamics of viral variants, to establish a permanent molecular surveillance to monitor the introduction of new strains and, thus, to interrupt the chain of HBV dissemination in Brazil.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/11/9/860/s1. Table S1: Sequences used in HBV complete genome phylogenetic analyses

Author Contributions: B.V.L., F.C.A.M. and E.L. prepared and revised the manuscript. B.V.L., F.C.A.M. and M.P.d.E.-S. sequenced and performed the phylogenetics analysis. L.L.L.-X. contributed to recruiting and medical care of patients. V.A.M., V.D.C., L.M.V. and M.P.d.E.-S. worked on data curation, performed the molecular assays, also providing the interpretation of the molecular results obtained. E.L. supervised/managed the project and worked on funding acquisition. All authors revised the final version of this manuscript.

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LETTER TOEDITOR

USEFULNESS OF SALIVA SAMPLES FOR DETECTING SARS–CoV–2 RNA AMONG LIVER DISEASE PATIENTS

Dear Editor,

In a recent article in the *Journal*, Azzi and colleagues (1) evaluated saliva samples of 25 COVID-19 patients by real time RT-PCR. In this study, all individuals had severe or very severe infection. All of them had SARS CoV-2 detected in saliva samples and there is no information regarding the presence of liver diseases.

Diagnostic testing for COVID-19 is made through nasopharyngeal (NP) and oropharyngeal (OP) swabs. Saliva samples could be useful specimens since collection is less invasive, safer and allows the option of self-collection. Saliva samples have been evaluated for detecting viral hepatitis markers (2,3), however there is a lack of studies about usefulness of these samples for detecting SARS CoV-2 in hepatitis infected individuals and non-severe cases of COVID-19. The saliva collection can be safer than NPS samples, especially for those patients that presenting decompensated cirrhosis or other severe sequels, like hepatocarcinoma. This study aims to evaluate the usefulness of saliva for detecting SARS-CoV-2 RNA according the presence of liver disease patients.

Nowadays, Brazil has the second number of confirmed cases of COVID-19 in the world and no information is available regarding the number of cases in liver disease patients. The study protocol was approved by the Brazilian National research ethics committee under the number n° 4.014.273 and complied with the clinical research guidelines of the Declaration of Helsinki.

First, we evaluated extraction method and limit of detection of artificially spiked SARS-CoV-2 saliva samples (estimated viral load: 10^3, 10^2, 10^1, 10^0 copies/mL). Saliva were collected using Salivette Device as previous described (3). These samples were tested in triplicate using two extraction methods (M1: PureLink RNA Mini Kit, Thermo Fisher Scientific, Waltham, USA and M2: QIAamp Viral RNA Mini Kit, QIAGEN, Germany) following manufacturer's recommendations with some modifications (low elution volume) along to real time PCR that amplifies N1 and N2 regions (2019-nCoV CDC EUA Kit, Integrated DNA Technologies, Coralville, USA) (4). M1 used 200μL of samples to extraction and RNA was eluted in 100 μL, M2 used 140 μL of sample volumes and was eluted in 50 μL.

Both methods were feasible to extract SARS-CoV-2 RNA saliva, however using M1 the detection limit was 10 copies/mL and M2 the limit of detection was 1 copy/mL. M2 was applied to extract RNA from saliva and NPS from 13 volunteers (5 hepatitis cases and 8 non hepatitis cases).

Volunteers gave saliva samples using Salivette device after signing informed consent. A total of four individuals (two hepatitis cases and two without liver disease) were negative to SARS CoV-2 in NPS and saliva (100% of specificity). The overall positivity was 9/13 (69.2%) lower than observed in saliva from ambulatory patients without liver disease (84.6%) (5). A total of 11/13 (84.6%) had concordant results in saliva and NPS samples what is lower than observed by Azzi and coleagues (1) and probably is the reflex of severity of disease among both studies. Positive concordant results in NPS and saliva were observed in seven individuals (two hepatitis cases and 5 without liver disease) until 7 days after onset of symptoms (100% of sensitivity). After 7 days of onset of symptoms, RNA was detected in NPS but it was not observed in paired saliva samples.

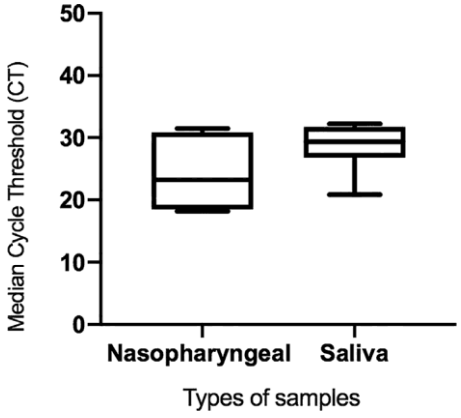

Figure 1. Box Plot Graph of cycle threshold (Ct) values in nasopharyngeal swabs and saliva specimens of positive samples for SARS CoV-2. Vertical lines indicate range of values, and the median Ct value is represented as black horizontal line within the box plot. The box indicates the 25th and 75th percentiles.
Abbreviations: NPS, nasopharyngeal swab.

Figure 1 shows the comparison of median, maximum and minimum of cycle threshold (CT) values. Positive NPS and saliva samples presented median CT of 23.2 and 29.3, respectively.

This is the first report of SARS CoV-2 detection in saliva samples among liver disease patients showing best results until 7 days of beginning of symptoms. There is an urgency for alternative methods for SARS-CoV-2 RNA detection to overcome swab availability and increase the access of diagnosis. Saliva samples have been evaluated for SARS CoV-2 RNA detection in severe cases or hospitalized patients, but there is a lack of data about theses samples in mild cases or a standard protocol for sample collection and viral detection. In addition, there is no information regarding the usefulness of saliva for detecting SARS CoV-2 RNA in individuals presenting comorbidities, such as liver disease. The present study gives new information regarding the presence of SARS CoV-2 in saliva of liver disease patients. Since saliva can be collected easily, SARS CoV-2 RNA detection in saliva can be useful strategy to increase the access of sample collection for the diagnosis of COVID-19 in patients with liver disease.

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