ORIGINAL RESEARCH

Prevalence and Pattern of Resistance in NS5A/NS5B in Hepatitis C Chronic Patients Genotype 3 Examined at a Public Health Laboratory in the State of São Paulo, Brazil

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Purpose: Globally, it is estimated that 71 million people are chronically infected with hepatitis C, and 10–20% of these will develop cirrhosis and hepatocellular carcinoma. The development of new direct-acting antiviral (DAA) drugs has contributed to sustained virological response (SVR), eliminating the infection and achieving cure of chronic hepatitis C. However, treated patients can develop HCV resistance to DAAs, which can contribute to the failure of treatment. Here, we aimed to evaluate the prevalence and specific pattern of NS5A and NS5B resistance-associated substitutions (RAS) in samples from patients chronically infected with HCV genotype 3a at a public health laboratory, Instituto Adolfo Lutz, São Paulo, Brazil.

Patients and Methods: Serum samples from the enrolled individuals were submitted to "in-house" polymerase chain reaction amplification of NS5A and NS5B non-structural protein genes, which were then sequenced by Sanger method.

Results: A total of 170 and 190 samples were amplified and analyzed for NS5A and NS5B, respectively. For NS5A, 20 (12.0%) samples showed some important RAS; 16 (9.0%) showed some type of substitution and 134 (79.0%) showed no polymorphism. No sample showed any RAS for NS5B.

Conclusion: This study found important RAS in samples from naïve chronic HCV patients in some areas from São Paulo. The most prevalent were A62S, A30K, and Y93H, which could indicate an increase in resistance to some DAAs used in HCV treatment.

Keywords: HCV, nonstructural NS5A/NS5B, resistance, polymorphism, RAS

Introduction

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease, with approximately 71 million chronically infected individuals worldwide, many of whom are unaware of their infection. Developments in diagnostic procedures and improvements in therapy, with the discovery of new drugs, and prevention have ensured better clinical care for patients with HCV-related liver disease.[1–3]

HCV is classified into 7 genotypes (GT) and approximately 67 subtypes. GT1 is the most prevalent HCV genotype worldwide followed by GT3 (22%), GT2 (13%), and GT4 (13%). Studies carried out in Brazil have indicated a GT3 prevalence of 30.2%.[4] Recent studies have shown that HCV GT3 is associated with more rapid disease progression and lower rates of response to treatment, in comparison with

other genotypes, especially in patients with cirrhosis and those who have not showed response to previous treatment.[5,6]

The non-structural proteins NS3/NS4, NS5A, and NS5B, together, contribute to the HCV life cycle, including HCV RNA translation, posttranslational processing, HCV replication, and virus assembly and release.[7,8] The goal of therapy of HCV infection is sustained virological response (SVR), and the development of direct antiviral agents (DAAs) has revolutionized the therapeutics for chronic hepatitis C. These include the NS3/4A protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors, which are further subdivided into nucleoside and non-nucleoside polymerase inhibitors, with or without ribavirin, besides the combined therapies.[9,10]

Resistance-associated substitutions (RAS) are generated at a baseline and in chronic hepatitis C patients who fail to respond to DAA treatment. RAS in NS5A may affect the efficacy of re-treatment, as NS5A inhibitor is included in all available DAA regimens.[11] A study by Hezode et al in G1-G6 HCV patients, treated with Sofosbuvir and Velpatasvir, detected 28% of RAS in NS5A at baseline, but irrespective of this, high rates of SVR were observed at 12 weeks. In addition, the study did not report any observed resistance of NS5B.[6]

The current study aimed to evaluate the prevalence and the specific pattern of NS5A and NS5B RAS in samples from DAA naïve patients chronically infected with HCV (GT3), whose samples were sent to a public health laboratory of Viral Hepatitis, Instituto Adolfo Lutz, São Paulo, Brazil.

Methods
Samples
This study included serum samples from DAA naïve patients chronically infected with HCV GT3a, and those

were sent to a public health laboratory in São Paulo State/ Brazil, from January 2015 to February 2016; the serum samples were stored at −20°C until use. The samples were obtained from different regions of São Paulo State: Vale do Paraíba, Vale do Ribeira, and São Paulo Metropolitan area (São Paulo and ABCD).

HCV RNA Extraction
HCV RNA was extracted from 200 µL of plasma using the NucliSENS easyMAG™ kit (BioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions.

Amplification of the NS5A Region
The HCV NS5A region was amplified by PCR in two overlapping parts using the same sets of primers (Table 1). Complementary DNA (cDNA) and the first round were performed using the SuperScript III One-Step RT-PCR System with the Platinum Taq DNA Polymerase kit (Invitrogen™, ThermoFisher Brand, Carlsbad, USA). The reverse transcription was performed under the following conditions: an initial denaturation step of 50°C for 30 min and 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 62°C for 30 s, and 68°C for 90 s, and a final extension at 68° C for 5 min. The second round was performed using Platinum Taq kit (Invitrogen™, ThermoFisher Brand, Carlsbad, USA) with a genotype-specific primer for GT3a, as described previously.[12] After the amplification cycles, a final extension at 72°C for 7 min was added for all reactions. The amplified fragments were visualized by 2% agarose gel electrophoresis and gel red staining.

Amplification of the NS5B Region
For the NS5B region, the cDNA was generated using M-MLV reverse transcriptase and random hexamers (Invitrogen™, Carlsbad, CA, USA). After incubation at 70°C for 10 min, reverse transcription was carried out at

Table 1 Oligonucleotides for NS5A and NS5B Gene Amplification of HCV

Region	Direction	Sequence (5′ - 3′)	Position	Reference
NS5A				[12]
F1S	Sense	5′TAT GTT CCC GAG AGC GAT GC-3′	6157–6176	
F1AS	Antisense	5′CGG CAC TTG GCA CGG ACA CTT GAG3′	6685–6708	
NS5B				[13]
PR1	Sense	5′TGGGGATCCCGTATGATACCCGCTGCTTTGA3′	8245–8275	
PR2	Antisense	5′GGCGGAATTCCTGGTCATAGCCTCCGTGAA3′	8616–8645	
PR3	Sense	5′TATGAYACCCCCTGYTTTGACTC3′	8256–8278	
PR5	Antisense	5′GCTAGTCATAGCCTCCGT 3′	8619–8636	

37°C for 60 min, followed by heat inactivation at 95°C for 15 min. The HCV NS5B region was amplified by nested PCR in two overlapping parts (primers are described in Table 1).[13] The first round of PCR was performed under the following conditions: 49 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The second round was performed under the following conditions: an initial denaturation step of 95°C for 5 min, 34 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The amplified fragments were visualized by 2% agarose gel electrophoresis and gel red staining.

Sequence Analysis

After quantification of the amplified product, each sample was sequenced using the commercial BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc., USA). For the NS5A region, an initial purification step was performed using the ExoSAP-IT PCR Clean-up Kit (GE Healthcare, Chalfont St. Giles, UK), following the manufacturer's instructions. The final fragments were purified using the Ethanol/EDTA method, following the instructions of the BigDye® Terminator kit. Thereafter, the sequencing reaction was analyzed on an ABI3500 automated sequencer (Applied Biosystems, Thermo Fisher Brand, Foster City, CA, USA).

Chromatograms obtained from the sense and antisense sequences were edited to obtain consensus sequences using Sequencher™ 4.7 software (Gene Codes Corporation, Michigan, USA). The file generated in the "fasta" format was submitted to Geno2Pheno site (https://hcv.geno2pheno.org/) for characterization of genotypes and identification of polymorphisms and mutations related to drug resistance.

Results
Study Population

All samples were from naïve DAA patients. For NS5A, 87 patients were from São Paulo Metropolitan area and 83 from Vale do Paraiba or Vale do Ribeira. For NS5B, 108 patients were from São Paulo Metropolitan area and 90 from Vale do Paraiba or Vale do Ribeira. Patients were waiting for new drugs, and all were being monitored to receive DAAs from public health service. RAS was analyzed in sequences obtained from chronic HCV-infected patients naïve to DAA - NS5A (N = 170; 110 males and 60 females) and NS5B-inhibitors (N = 198; 126 males and

72 females). Viral load of these patients ranged from log 2.78 – log 7.24 (median log 5.74). Patients were aged 17 to 79 years (median 54 years).

NS5A Region

A total of 170 HCV GT3a samples were amplified, sequenced, and analyzed for NS5A RAS and 134 (79.0%) samples showed no polymorphism; 16 (9.0%) samples showed natural polymorphism and 20 (12.0%) samples showed RAS at baseline. As the majority of samples were from male patients (64.7%) and aged from 40 to 59 years old, these were the groups with the greatest number of detected polymorphisms for NS5A. These data are shown in Table 2.

Some relevant polymorphisms were found in our analysis. From 170 samples analyzed for NS5A, we observed RAS in 20 samples (12.0%) (Table 3). Among mutations in the NS5A detected for Daclatasvir, the most frequent RAS was A30K+A62S (70.0%), followed by A30T/E/K/S (10.0%) and polymorphisms A30K+A62P, A30S+Y93H +A62M, Y93H+A62L, and A30K (with the frequency of 5.0% each). For Elbasvir and Ledispavir the most frequent RAS was A30K (80.0%), followed by A30T/E/K (10.0%), A30S+Y93H (5.0%), and Y93H (5.0%). For Velpatasvir, only 2 samples showed RAS, 1 sample with polymorphism A30S+Y93H and 1 sample with Y93H. For Pibrentasvir, no samples showed RAS. These polymorphisms are described in Table 4.

NS5B Region

None of the 198 samples of HCV GT3a analyzed for NS5B region showed RAS for sofosbuvir.

Discussion

Assessment of RAS in baseline in a candidate for therapy seems to be an option to define therapeutic strategies and to predict resistance to sustained response to DAAs. In Brazil, DAAs are freely dispensed in the entire country for the treatment of chronic HCV patients. Instituto Adolfo Lutz in São Paulo, besides performing diagnostic test by viral load, also monitors the emergence of resistance strains in this population. Samples are not sent to the laboratory with medical records or any clinical information. Hence, it was not possible to follow up with the patients after drug administration; it was only possible to detect RAS in a pre-administration phase of the drugs.

Sequencing was performed by Sanger method,[14] which is useful in a diagnostic laboratory and for public health.

Table 2 Demographic Characterization of the Patients According to NS5A Region Results

	Samples Sequence for NS5A Region			
	Samples without RAS*	Samples with Polymorphisms	Samples with RAS	Total
Gender				
Female	47	7	6	60
Male	87	9	14	110
Age (years)				
17	1	0	0	1
20–29	2	0	0	2
30–39	12	1	2	15
40–49	33	3	5	41
50–59	55	8	7	70
60–69	27	1	6	34
70–79	3	3	0	6
No information	1	0	0	1
Regions of São Paulo State				
São Paulo Metropolitan area	65	10	12	87
Vale Paraíba/Ribeira	69	6	8	83

Note: *Resistance associated substitutions.

Although deep sequence is more sensitive than other methods, Sanger sequencing is easier to perform and most of the studies on HCV RAS have used this methodology to detect polymorphisms that are clinically important.[15,16] Pawlotsky (2016) highlighted the importance of resistance in baseline and how Sanger method was useful for detecting RAS in diagnostic laboratory and detect strains with clinical importance.[9]

The population examined in the current study was heterogeneous, composed mainly by men above 50 years old, from different regions of São Paulo state and with high viral load. These characteristics represent most of the chronic HCV patients waiting for DAA treatment in Brazil and maybe these results could help other patients in the country. Besides, GT3a is the second most prevalent HCV genotype in Brazil and is associated with faster disease progression and lower rates of response to treatment, especially in patients with cirrhosis.[4,17]

The main polymorphisms observed in this study were A62S, A30K, and Y93H, alone or associated with another polymorphism, which confers high levels of resistance to the NS5A inhibitors Daclatasvir, Ledipasvir, Elbasvir, and Velpatasvir, but not to Pibrentasvir. The presence of NS5A polymorphisms at position 62 can reduce susceptibility to some NS5A inhibitors in other genotypes, but for GT3, their impact is unclear.[18] A previous study suggested that the variations at position 62 did not have substantial impact on daclatasvir potency.[19] Hernandez et al observed that A30K and Y93H mutations in NS5A reduced viral susceptibility to Daclatasvir and Ledispavir, respectively. The real impact of these resistances depends on some factors such as drug regimen, treatment adherence, and cirrhosis.[20]

A previous study showed that RAS Y93H and Q30K confer resistance to daclatasvir, ledipasvir, ombitasvir, elbasvir, and velpatasvir, but has low impact on the potency of pibrentasvir. Pibrentasvir is a next-generation pan-genotypic drug that remains active even with common polymorphisms that can occur for NS5A genotype 3.[21] In contrast to the above study, another study demonstrated that Y93H polymorphism has clinical relevance to pibrentasvir.[22]

In our study, several combinations of RAS were observed, as shown in Tables 3 and 4. Some substitutions when occurring alone have been associated with weak resistance to NS5A inhibitors, but when combined with other substitutions, can decrease the viral sensitivity to DAAs.[9,12] In addition, we observed some sequences that contained a polymorphism associated with resistance, although it has not yet been determined to be an important mutation associated with therapeutic failure for some drugs, such as pibrentasvir. The characterization (substitution on scored position) determined by geno2pheno software occurs based on its role in resistance to DAAs, which for pibrentasvir is possible, but not immediate.[23] In G3a

Table 3 Distribution of the Amino Acid Mutations in the HCV NS5A Region in DAA-Naïve Patients Infected with HCV Genotype 3a (n=20)

Sample	Polymorphism	Daclatasvir	Polymorphism	Elbasvir	Polymorphism	Ledipasvir	Polymorphism	Pibrentasvir	Polymorphism	Velpatasvir
12	A30K	R	A30K	R	A30K	R	A30K	RS	A30K	RS
31	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
37	A30S, Y93H, A62M	R	A30S, Y93H	R	A30S, Y93H	R	A30S, Y93H	SB	A30S, Y93H	R
56	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
62	A30T, A30E, A30K, A62S	R	A30T, A30E, A30K	R	A30T, A30E, A30K	R	A30T, A30E, A30K	RS	A30T, A30E, A30K	RS
75	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
93	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
104	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
110	A30T, A30E, A30K, A62S	R	A30T, A30E, A30K	R	A30T, A30E, A30K	R	A30T, A30E, A30K	RS	A30T, A30E, A30K	RS
119	Y93H, A62L	R	Y93H	R	Y93H	R	Y93H	S	Y93H	R
138	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
162	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
165	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
183	A30K, A62P	R	A30K	R	A30K	R	A30K	RS	A30K	RS
186	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
193	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
206	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
221	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
222	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS
225	A30K, A62S	R	A30K	R	A30K	R	A30K	RS	A30K	RS

Abbreviations: R, resistance; RS, reduced susceptibility; S, susceptible; SB, substitution.

Table 4 Frequency of Mutations Associated with Resistance to DAAs in the NS5A Region of Genotype 3a

Polymorphism	Drugs			
	Daclatasvir	Elbasvir	Ledipasvir	Velpatasvir
A30K	1	16	16	0
A30K, A62P	1	0	0	0
A30K, A62S	14	0	0	0
A30S, Y93H, A62M	1	0	0	0
A30T, A30E, A30K, A62S	2	0	0	0
Y93H, A62L	1	0	0	0
A30S, Y93H	0	1	1	1
A30T, A30E, A30K	0	2	2	0
Y93H	0	1	1	1

patients, the A30K substitution has never appeared in combination with Y93H, which could suggest a restriction for co-selection of these RAS in the same genome.[24]

Variations in amino acid residues can occur in all sites of action of antiviral drugs. According to Cuypers et al, the most important RASs to NS5A inhibitors are M/L28T/V, Q/L30E/H/R/S, L31M/V, H58D, and Y93C/H/N, which are capable of reducing susceptibility to NS5A inhibitors by conformational changes.[25]

The presence of Y93H in NS5A could facilitate the selection of resistant strains under treatment. Therefore, it is necessary to avoid the fitness of resistant variants as the predominant viral population and prevent DAA failure due to baseline resistant variants. In addition, failing DAA-based therapy should be discontinued as soon as possible to avoid an increase in the frequency of RASs, to preserve HCV re-treatment options.[15,24]

Daclastavir proved to be superior against resistant variant Y93H, which has a natural prevalence of >10% and displays high level of resistance to both Ledipasvir and Daclatasvir in GT1b replicon cells. Furthermore, for HCV GTs 2–4, Daclatasvir has significantly higher potency in vitro compared to Ledipasvir, with the highest fold resistance values for variant Y93H in GT3.[26]

Efficacy of the DAAs, depending on baseline polymorphisms, may be associated with the reduction of treatment or re-treatment. Sometimes SVR is not reached or 12 weeks of treatment is not enough. RAS is generated in chronic HCV patients and it could affect re-treatment with other available drugs.[27] A recent study showed that a longer duration of treatment with Sofosbuvir +Ledipasvir and the addition of Ribavirin can reduce or even eliminate the impact of baseline NS5A RAVs. Only 50% of the patients that had two or more baseline resistance-related variants cleared the virus, with the lowest SVR rates observed with variant Y93H/N.[25]

Velpatasvir is a potent pan-genotypic HCV NS5A inhibitor with activity against HCV GT1 to GT6. It has better in vitro activity against GT2 and GT3 than the NS5A inhibitors Ledipasvir and Daclatasvir. A study performed by Lawitz et al observed emergent RAS at three positions in GT3 (Y93, L31, E92) and, although pre-treatment RAS was associated with a slightly low response in patients with GT3, current HCV treatment strategies are based on combined use of DAAs and should overcome any minor impact of pretreatment RASs. Combination treatment with Velpatasvir and Sofosbuvir may provide an effective treatment option for patients infected with HCV GT1 to GT6.[28]

No polymorphism was found for the NS5B region in the 198 sequences analyzed. Polymerase inhibitors have a high genetic barrier, so few patients show therapeutic failure in regimen containing sofosbuvir. Costa et al, in a study at Rio de Janeiro, G1/G3 HCV patients treated with daclatasvir and sofosbuvir showed high SVR. These authors also observed no NS5A RAS in 28% of HCV/HIV coinfected and 16.8% in monoinfected patients. No RAS was observed for NS5B.[29] Sofosbuvir is a nucleoside NS5B polymerase inhibitor with pan-genotypic activity, and whose most frequent resistance mutations are L159F and C316N in GT1a-infected patients, S282G/T and C316N/H/F in the GT1b-infected, L159F and S282T in those with GT2 infection, L159F and V321A in the GT3-infected, and E237G, S282T and V321A, in those with GT4 infection.[5] Chen et al also observed that there was a notable absence of RAS in NS5B, except in 1 patient

who carried the high-resistance S282T alone in 100% of the viral genomes sequenced.[24]

In conclusion, this study found important RAS in samples from naïve chronic GT 3a HCV patients in some areas from São Paulo. The most prevalent were A62S, A30K, and Y93H, which could indicate an increase in resistance to some DAAs offered to treat HCV.

Ethics Statement

The procedures described in this study were initiated only after obtaining approval from the ethics committees of the participating institutions. Following the Ministry of Health guidelines[30], informed consent was obtained from all patients who had detectable viral load, allowing the collection and storage of their blood samples for studying Protease Inhibitor (IP) resistance and other drugs for HCV treatment. Further, in this study, we ensured that all sensitive information remained confidential, guaranteeing patient anonymity. Therefore, the requirement for additional consent to conduct this study was waived. This study was approved by the Ethical Committee in Research at the Instituto Adolfo Lutz (CEPIAL) # 1.040.338.

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Disclosure

All the authors who took part in this study declare that they have no conflicts of interest or disclosures with respect to the manuscript.

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