Characterization of Primary Direct Acting Antiviral Drugs (DAA) Resistance Mutations in NS5A/NS5B Regions of Hepatitis C Virus with Genotype 1a and 1b from Patients with Chronic Hepatitis

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Abstract

Hepatitis C virus (HCV) infection is a public health problem with an estimated 71 million infected people worldwide. The high level of HCV replication and its lack of post-transcriptional correction mechanisms result in the rapid emergence of viral variants, difficulty in determining polymorphisms, and variants that contain substitutions associated with resistance and/or reduction of susceptibility towards new antivirals. The aim of this study was to map the polymorphisms in NS5A and NS5B and resistance mutations to new antiviral drugs in HCV strains with genotype 1a and 1b derived from patients with chronic hepatitis C infection. Serum samples from patients who underwent routine viral load tests and monitoring at the laboratory of viral hepatitis at the Adolfo Lutz Institute, São Paulo, Brazil were collected. A total of 698 and 853 samples were used for the characterization of NS5A and NS5B regions respectively; comprising HCV genotypes 1a and 1b. The prevalence of resistance mutations in the NS5A region was found to be 6.4%, with Y93H, L31M, Q30R, and Y93N as the main resistance-associated substitutions (RAS). No NS5B-associated RAS was observed for any of the drugs analyzed. This study reveals the presence of significant RAS in the HCV serum samples. These findings support the RAS test should be offered to individuals with poor response to double combination regimens prior to treatment initiation, thereby assisting strain vigilance and selection of effective treatment or retreatment options using DAA regimens.

Introduction

Hepatitis C virus (HCV) is a serious public health problem, affecting approximately 71 million people worldwide [1]. HCV is classified into eight genotypes (GT) with approximately 30% variability, and 86 subtypes that have been described with 20% variability [2]. In Brazil, HCV genotypes 1a and 1b are the most common, prevalent in approximately 64.9% of all the patients infected with HCV, followed by GT 3 (30.2%). On the other hand; the GT 2, 4, and 5 are found less frequently in Brazil with 4.6%, 0.2%, and 0.1% prevalence, respectively [3].

Fortunately, the development of direct-acting antivirals (DAAs) has reached a sustained virological response (SVR) with successful treatment in nearly 95% of patients with HCV and with few adverse events [4]. The high level of HCV replication and its lack of post-transcriptional correction mechanisms result in the rapid emergence of viral variants in quasispecies. These factors are responsible for determining not only the viral polymorphisms, but also the variants that contain substitutions associated with resistance and/or reduction of susceptibility to DAAs [5,6].

The current study highlights NS5B polymerase and NS5A inhibitors, as classes of drugs used in treatment, which are available at Sistema Único de Saúde – SUS in Brazil (Unified National Health System) [7].

Despite the overall success, antiviral treatment for certain groups of patients remains a challenge. For some patients, Ribavirin is still recommended; as it improves the rate of SVR, especially in patients with advanced liver diseases [8].

NS5A inhibitors quickly select resistant variants when administered alone, as they have a low genetic barrier to resistance. Among the main mutations in the NS5A region that impart high levels of resistance to NS5A inhibitors, the most common are Q30E/H/K, Y93N/C, L31M, and Y93H [9]. In contrast, despite having a high genetic barrier; some substitutions in NS5B have been associated with resistance to Sofosbuvir [10]. As described by Jiménez-Pérez et al., some compensatory mutations that can recover the capacity for viral replication in presence of certain mutations have also been described [11].

The real impact of resistance mutations that are relevant in the treatment-naïve patients on the effectiveness of treatment, are issues that still need to be clarified. This study aimed to characterize primary resistance mutations and map the RAS in NS5A and NS5B regions in the HCV-1a and -1b genotypes from naïve patients with chronic hepatitis C DAA in the Brazilian state of São Paulo.

Materials And Methods

Sampling analyzed

Samples from patients belonging to different regions of São Paulo State, including Vale do Paraíba, Vale do Ribeira, and São Paulo Metropolitan area, which included São Paulo and ABCD regions; were included in this study. These serum samples were from DAA-naïve patients chronically infected with HCV GT-1a and GT-1b, and were sent to Adolfo Lutz Institute (IAL), a public health laboratory in São Paulo state of Brazil, from 2012 to 2014. Patient samples were used for HCV diagnosis and stored at -20 °C.

Amplification and sequencing of NS5A and NS5B regions

Nucleic acid extraction was performed using the NucliSENS easyMAG™ kit (BioMérieux, Marcy l'Etoile, France), as per the manufacturer's instructions.

The amplification of HCV NS5A region was performed in a single step using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen®, Thermo Fisher, Carlsbad, USA), and a set of primers described in Table 1 [12]. PCR products were obtained using the following thermal cycling conditions: 50 °C for 30 min and 94 °C for 3 min, followed by 45 cycles of 94 °C for 15 s, 48 °C + 0.3 °C/cycle for 30 s, 68 °C for 1 min and 68 °C for 5 min.

The amplification of HCV NS5B region was performed in two steps using the protocol described by Santos et al [13], and a set of primers described in Table 1 [14]. For both regions, amplified fragments were visualized by electrophoresis, using 2% agarose gel and SYBR Safe DNA gel stain (Invitrogen™, Carlsbad, CA, USA).

Samples were sequenced by Sanger method [15], using commercial Kits, according to the protocol described by Santos et al. [13]. To characterize genotypes and identify polymorphisms, the sequences were submitted to Geno2Pheno site (<https://hcv.geno2pheno.org>).

Results

Study population

For the characterization of NS5A region, a total of 698 samples were analyzed; of which 456 patients (65.3%) were from the São Paulo metropolitan area and 242 (34.7%) from Vale do Paraíba or Vale do Ribeira, Brazil. For NS5B characterization, a total of 853 samples were analyzed; of which 541 patients (63.4%) were from the São Paulo metropolitan area and 312 (36.6%) from Vale do Paraíba or Vale do Ribeira.

Of the 698 samples used for NS5A region characterization, 305 (43.7%) belonged to GT-1a and 393 (56.3%) to GT-1b. Of the total, 371 (53.2%) and 327 (46.8%) were men and women respectively. Of the 853 samples used for NS5B region characterization, 456 (53.5%) belonged to GT-1a, and 397 (46.5%) belonged to GT-1b. Of the total, 458 (53.7%) and 395 (46.3%) were men and women respectively.

Characterization of polymorphisms for NS5A and NS5B regions

A total of 698 samples of HCV GT-1a and GT-1b were used for characterizing the NS5A region. The results revealed absence of polymorphism in 531 (76.1%) samples, natural polymorphism in 116 (16.6%) samples, 6 (0.9%) samples with reduced susceptibility, and 45 (6.4%) samples exhibiting RAS at baseline. The most frequent age group that showed RAS was in the range of 40–59 years (Table 2).

Some relevant RAS were observed in 45 (6.4%) out of the 698 samples analyzed for NS5A region characterization. The most prevalent polymorphisms observed were Y93H, L31M, Q30R, and Y93N; in isolation or association with other polymorphisms. Of the 45 samples demonstrating the presence of RAS, 13 and 32 belonged to GT-1a and GT-1b respectively. The most frequent RAS for GT-1a was L31M, followed by Q30R and Y93N. For GT-1b, the most frequent RAS was Y93H, followed by L31M.

Among the mutations in the NS5A region detected for Daclatasvir resistance, the most frequent RAS was Y93H (62.5%); followed by L31M (12.5%) and Q30R (9.4%). For Elbasvir resistance, Y93H was also the most frequent RAS (52.4%), followed by L31M (28.6%). Ledipasvir resistance presented RAS Y93H in 43.9% and L31M in 26.9% of patients. For Velpatasvir resistance, RAS Y93H was observed as an isolated polymorphism in 88% of the samples and as an associated polymorphism with Q30H in 4.0%. Y93N polymorphism was detected at a concentration of 8.0%. For Pibrentasvir resistance, only two samples showed RAS with the Y93N polymorphism. Ombitasvir resistance demonstrated Y93H in 70% of the samples and Q30R in 10.0%. However, other polymorphisms were observed at a lower rate. These data are presented in Table 3. Table 4 shows the frequency of RAS for each DAA in the NS5A region.

In this study, none of the 853 samples analyzed for polymorphisms in NS5B region of HCV GT-1a and GT-1b presented RAS that confer resistance to Sofosbuvir.

Discussion

Since 2014, DAAs have been used for the treatment of chronic Hepatitis C in Brazil. They are dispensable freely for all patients but drugs are controlled by Ministry of Health (Brazil). To ensure that the patients have reached the SVR, the absence of HCV-RNA must be observed six months after the treatment has already finished [7].

Adolfo Lutz Institute (IAL) in São Paulo City, Brazil has played an important role in diagnosing viral load and monitoring patients from public health services, starting from diagnosis to analysis of virological sustained response. In addition, IAL monitors the appearance of resistance mutations in these patients.

There is no gold standard available to detect HCV drug resistance so far. In this study, the Sanger sequencing method was employed for the detection of mutations associated with antiviral drug resistance associated with HCV [15], as a tool with great potential for application in a public health diagnostic laboratory. Many studies on HCV RAS have used this methodology to detect important polymorphisms, as it has a low cost and is easier to perform [13,16,17].

All the patients enrolled in this study were treatment-naïve until the samples were collected to process for the molecular tests. The population examined represents the patients with most chronic HCV in Brazil who were awaiting treatment. The population was heterogeneous, composed mainly of male individuals who had a high viral load, were approximately 50 years of age and from different regions of the state of São Paulo, Brazil [13,18,19].

The distribution of HCV GTs varies according to geographic location, and monitoring this distribution is important to define epidemiological trends of infection regarding the introduction of new GTs and determining transmission routes [20, 21]. In this study, samples belonging to GT-1 were evaluated to determine the frequency of DAA-resistant variants. The available drugs for the treatment of HCV GT-1 include Daclatasvir, Elbasvir, Ledipasvir, Ombitasvir, Pibrentasvir, Velpatasvir, Sofosbuvir, and Dasabuvir. For the GT-1 NS5A region, our study revealed the presence of RAS in 6.4% out of a total of 698 samples analyzed. Previous studies have indicated the frequency of RAS in the NS5A region of HCV, determined by conventional and next-generation sequencing assays, to be between 6.0% and 16.0% [22, 23]. The prevalence of NS5A RAS is 18.0 to 21.0% in Asian countries, 15.0% in North America, and 19.0% in Europe. The prevalence of NS5B RASs is 1.0% to 5.0%, 4.0% and 20.0% in Asian countries, North America, and Europe respectively [24].

This study shows that patients with HCV GT-1 already have some RAS at baseline. The most prevalent polymorphisms observed in GT-1 are Y93H, L31M, Q30R, and Y93N, existing in isolation or in association with another polymorphism. These polymorphisms were also reported by Rao et al. in China [24]. In samples that presented RAS, a significant association was observed with GT-1b (71.1%), as opposed to GT-1a (28.9%). Previous studies suggest that GT-1b may accumulate more resistance mutations than the GT-1a strain to overcome the action of DAAs [25]. The most frequent RAS found for GT-1a was L31M, followed by Q30R and Y93N whereas for GT-1b; it was Y93H followed by L31M. The single mutation of Y93H or Q30R confers high levels of Ledipasvir resistance to both HCV strains with GT-1a and GT-1b. For GT-1b, this resistance was observed after a short period of treatment; whereas for GT-1a, it was only observed in patients who received high doses [26].

According to Cuypers et al., the most important RAS that interfere with the effectiveness of NS5A inhibitors are M/L28T/V, Q/L30E/H/R/S, L31M/V, H58D, and Y93C/H/N, which reduce susceptibility to NS5A inhibitors and induce conformational changes [27]. In addition, Gottwein et al. have demonstrated that the Y93H polymorphism shows clinical results on the effectiveness of Pibrentasvir [28]. As a result, the mutation Y93H may well be responsible for selection of resistant strains in the NS5A region, which may interfere with treatment based on DAAs [16, 28].

The most frequent mutations in GT-1 were Y93H and L31M. Of all the samples analyzed, the resistance to Daclatasvir and Ledipasvir with L31M was observed in 4 and 11 samples respectively. The Y93H mutation was observed in 20 and 18 samples that presented resistance to Daclatasvir and Ledipasvir respectively. A study carried out *in vitro* by Wyles and Luetkemeyer, demonstrated that the substitution of the amino acid L31M is more likely confers a clinical impact due to the high fold change observed *in vitro* towards Daclatasvir and Ledipasvir sensitivity [29].

However, an *in vivo* study by Costa et al. has indicated that the basal presence of RAS L31M and Y93H does not influence the treatment results, since all patients with this mutation have already reached SVR [30].

In our study, two samples that showed the polymorphism Y93N also presented resistance to Pibrentasvir. However, a study performed by Wyles & Luetkemeyer has shown that this mutation does not seem to have a significant clinical impact with respect to this drug, due to the low fold-change (<10X for GT-1a), but, due to the high fold-change, this polymorphism is more likely to confer a clinical impact for other drugs like Elbasvir, Velpatasvir (>1000X for GT-1a), Daclatasvir, Ledipasvir and Ombitasvir (>10,000X for GT-1a) [29]. The absence of RAS in the NS5B region was observed in all the analyzed sequences. Polymerase inhibitors have a high genetic barrier; therefore, only a few patients experience therapeutic failure with the Sofosbuvir regimen [31]. The persistence of NS5A RAS has been well described and can be selected by long-term immune pressure or other factors that are still undetermined [32, 33].

Unfortunately, the samples used for this study were not sent with clinical information. Hence, it was not possible to carry out a comparative study between RAS found in this study and those that caused effective post-treatment resistance. In addition, due to a lack of complete data and clinical follow-up of patients, it was not possible to evaluate whether these patients had SVR. A study with the clinical follow-up of patients would be important to understand whether the mutations found in the laboratory tests cause effective resistance to drugs, as an inappropriate choice of drugs together with a short duration or poor adherence to treatment are important factors that can influence treatment failure in real life. A study performed by Chen et al. has identified the lack of treatment adherence as the main failure factor in SVR. The negative impact of this factor can be underestimated due to a lack of pre-treatment counseling that reinforces the importance of adherence for successful treatment in practice [34].

The prevalence of a baseline RAS determined in this study aligns with that reported in other countries [22-24]. The NS5A region, as expected, is more susceptible to RAS than the NS5B region, corroborating the conclusions of other studies highlighting that the NS5B region of the HCV genome represents a high barrier to mutations. However, these findings should be supported by further studies including the clinical follow-up of patients and the comparison between the mutations found in laboratory tests and those that cause effective drug resistance. Such additional information is essential to allow the choice of drug alternatives for the treatment or retreatment of patients with chronic hepatitis GT-1a and GT-1b.

Overall, here, we reveal that the prevalence of RAS in the NS5A region of HCV is 6.4 %, in the context of chronic Hepatitis C patients from important regions of the São Paulo State. Importantly, this prevalence may be behind the failure to achieve virological sustained response in some cases. Although pangenotypic drugs are highly efficacious options for the retreatment of chronic HCV patients, our findings support the notion that the RAS test should be offered to individuals with poor response to double combination regimens prior to treatment initiation.

Declarations

Conflict of interest: All authors declare that they have no conflicts of interest or disclosures to the manuscript.

Ethical standards: All the proceedings of this study were only initiated post obtaining ethical approvals from relevant ethics committees of the participating institutions. This study was approved by the Ethical Committee in Research at the Adolfo Lutz Institute, Brazil (CEPIAL) # 1.040.338.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request, but clinical data or other information from patients enrolled in this study, are not available.

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Tables

Table 1: Sequence of primers used for GT1a and 1b for regions NS5A and NS5B. Adapted from Santos et al. [13].

Region	Direction	Sequence (5' - 3')	Position	Reference
NS5A:				
2F	Sense	5'ACTGTAAAACGACGGCCAGTGGIGARGGIGCIGTICARTGGATGAA3'	6066-6091	[12]
R	Antisense	5'ACCAGGAAACAGCTATGACCTRTGRGAIGGRTCIGTIARCATIGA3'	6882-6858	
3R	Antisense	5'ACCAGGAAACAGCTATGACCTRTGRGAIGGRTCICTIARCATIGA3'	6882-6858	
NS5B:				
PR1	Sense	5'TGGGGATCCCGTATGATACCCGCTGCTTTGA3'	8245-8275	[14]
PR2	Antisense	5'GGCGGAATTCTGGTCATAGCCTCCGTGAA3'	8616-8645	
PR3	Sense	5'TATGAYACCCCTGYTTTGACTC3'	8256-8278	
PR5	Antisense	5'GCTAGTCATAGCCTCCGT 3'	8619-8636	

Table 2: Characteristics of the patients who participated in this study according to the samples sequence results for NS5A region (n=698). Adapted from Santos et al. [13].

Samples sequence for NS5A region					
	Samples without RAS*	Samples with Polymorphisms	Samples with reduced susceptibility	Samples with RAS	Total
Gender					
Female	253	53	3	18	327
Male	278	63	3	27	371
Age (years)					
1-09	2	0	0	0	2
10-19	3	1	0	0	4
20-29	26	4	0	0	30
30-39	97	23	0	11	131
40-49	160	41	1	16	218
50-59	125	26	3	9	163
60-69	94	13	2	8	117
70-79	21	8	0	1	30
>80	3	0	0	0	3
Regions of São Paulo State					
São Paulo Metropolitan area	343	80	4	29	456
Vale Paraíba/Ribeira	188	36	2	16	242
Genotype					
1a	224	64	4	13	305
1b	307	52	2	32	393

* Resistance associated substitutions

Table 3: Distribution of polymorphisms found for the HCV NS5A region, according to the DAA (n=45). Adapted from Santos et al. [13].

Drugs											
Sample	Genotype	Polymorphism	Daclatasvir	Polymorphism	Elbasvir	Polymorphism	Ledipasvir	Polymorphism	Ombitasvir	Polymorphism	Polymorphism
73	1a	L31M	R	L31M	R	L31M	R	L31M	SB	L31M	L31M
95	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
108	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
142	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
160	1a	Y93N	R	Y93N	R	Y93N	R	Y93N	R		Y93N
195	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
254	1a	Q30R	R	Q30R	R	Q30R	R	Q30R	R		Q30R
255	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
279	1b	L31M	RS	L31M	R	L31M	R	L31M	SB		/
285	1a	L31M	R	L31M	R	L31M	R	L31M	SB		L31M
289	1b	A92T,Y93H	R	Y93H	R	A92T,Y93H	R	Y93H	R		/
302	1b	A92T,Y93H	R	Y93H	R	A92T,Y93H	R	Y93H	R		/
308	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
355	1b	L31M	RS	L31M	R	L31M	R	L31M	SB		/
410	1b	Y93H	R	Y93H	R	P58?,Y93H	R	Y93H	R		/
455	1b	L31M	RS	L31M	R	L31M	R	L31M	SB		/
458	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
462	1b	L31M	RS	L31M	R	L31M,P58S	R	L31M	SB		/
491	1a	Y93S	SB	Y93S	SB	Y93S	SB	Y93S	R		Y93S
497	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
577	1a	Y93N	R	Y93N	R	Y93N	R	Y93N	R		Y93N
602	1b	Y93N	R	Y93N	R	Y93N	R	Y93N	R		/
611	1b	L31F	SB	L31F	R	L31F	SB	L31F	RS		/
641	1b	L31M	RS	L31M	R	L31M	R	L31M	SB		/
645	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
652	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
660	1b	L31M	RS	L31M	R	L31M	R	L31M	SB		/
685	1a	Q30H e Y93H	R	Q30H e Y93H	R	Q30H e Y93H	R	Q30H e Y93H	R		Q30H
762	1a	Q30R	R	Q30R	R	Q30R	R	Q30R	R		Q30H
783	1b	L31M	RS	L31M	R	L31M	R	L31M	SB		/
787	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
821	1b	Y93H	R	Y93H	R	P58R e Y93H	R	P58R e Y93H	R		/
830	1b	L31F	SB	L31F	R	L31F	SB	L31F	RS		/
861	1a	H58P, Y93S	SB	H58P, Y93S	SB	H58P, Y93S	SB	H58P, Y93S	R		H58P,
866	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
940	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
960	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R		/
964	1a	L31M	R	L31M	R	L31M	R	L31M	SB		L31M
967	1b	Y93N	R	Y93N	R	Y93N	R	Y93N	R		/
972	1a	L31M	R	L31M	R	L31M	R	L31M	SB		L31M
977	1a	H58P	SB	H58P	SB	P32L,H58P	R	P32L,H58P	SB		P32L
987	1b	L31M	RS	L31M	R	L31M	R	L31M	SB		/

1040	1b	Y93N	R	Y93N	R	Y93N	R	Y93N	R	/
1065	1a	Q30R	R	Q30R	R	Q30R	R	Q30R	R	Q30R
1073	1b	Y93H	R	Y93H	R	Y93H	R	Y93H	R	/

R: Resistance; **RS**: Reduced Susceptibility; **S**: Susceptible; **SB**: Substitution

Table 4: Frequency and percentage of RAS in the NS5A region, according to each drug. Adapted from Santos et al. [13].

	Drugs					
	Daclatasvir	Elbasvir	Ledipasvir	Velpatasvir	Pibrentasvir	Ombitasvir
RAS*	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
L31M	4 (12.5)	12 (28.6)	11 (26.9)	0 (0.0)	0 (0.0)	0 (0.0)
Y93N	2 (6.2)	2 (4.8)	2 (4.9)	2 (8.0)	2 (100.0)	2 (6.8)
Q30R	3 (9.4)	3 (7.1)	3 (7.3)	0 (0.0)	0 (0.0)	3 (10.0)
Y93S	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)
H58P + Y93S	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)
Q30H + Y93H	1 (3.2)	1 (2.3)	1 (2.4)	1 (4.0)	0 (0.0)	1 (3.3)
P32L + H58P	0 (0.0)	0 (0.0)	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)
A92T + Y93H	2 (6.2)	0 (0.0)	2 (4.9)	0 (0.0)	0 (0.0)	0 (0.0)
Y93H	20 (62.5)	22 (52.4)	18 (43.9)	22 (88.0)	0 (0.0)	21 (70.0)
L31F	0 (0.0)	2 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
L31M + P58S	0 (0.0)	0 (0.0)	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)
P58R + Y93H	0 (0.0)	0 (0.0)	2 (4.9)	0 (0.0)	0(0.0)	1 (3.3)

*resistance associated substitutions