Characterization of primary direct-acting antiviral (DAA) drugs 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

The Hepatitis C virus (HCV) infection is a public health problem. The high level of HCV replication and its lack of post-transcriptional correction mechanisms results in the emergence of viral variants and the difficulty in determining polymorphisms and variants that contain the substitutions associated with resistance towards new antivirals. The main focus of this study was to map the NS5A and NS5B polymorphisms and resistance mutations to new antiviral drugs in HCV strains genotype 1 from patients with chronic hepatitis C infection. Serum samples were collected from patients who underwent routine viral load tests at the Instituto Adolfo Lutz, Sao Paulo city, Brazil. A total of 698 and 853 samples were used for the characterization of NS5A and NS5B regions, respectively, which comprise the HCV genotypes 1a and 1b. The prevalence of resistance mutations found in the NS5A region was 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 analyzed drugs. These findings support that 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.

KEYWORDS: DAA. Hepatitis C virus. Genotype 1. RAS. NS5B. NS5A.

INTRODUCTION

The Hepatitis C virus (HCV) is a serious public health hazard. In 2020, there were approximately 56.8 (55.2–67.8) million infected people worldwide, an estimate of 7.5 (7.3–8.9) million people having chronic HCV infection, around 8.7 (8.5–10.4) million cured patients, and 5.5 (5.3–6.5) deaths1. The HCV is classified into eight genotypes (GT) with approximately 30% variability and 86 subtypes that have been described with 20% variability2. In Brazil, the HCV genotypes 1a and 1b are the most common and prevalent by approximately 64.9% of all patients infected with HCV, followed by GT 3 (30.2%). On the other hand, GT 2, 4, and 5 are less frequently found in the country with 4.6%, 0.2%, and 0.1% prevalence, respectively3.

Fast replication and lack of post-transcriptional correction mechanisms in HCV result in the rapid emergence of viral variants as quasispecies, in other words, patients
are infected by mixtures of genetically distinct, but closely related viral populations. The resistance-associated substitutions (RAS) are generated in HCV patients who failed direct-acting antiviral (DAA) treatment. RAS in NS5A/NS5B may affect the sustained virological response (SVR) to treatment in patients undergoing re-treatment or at the beginning of treatment.

When a DAA is administered, a positive selection of viral variants with reduced susceptibility to this drug defines the viral resistance, so-called compensatory or fitness-associated substitution. Fortunately, DAAs elicit a SVR with successful treatment and fewer adverse events in nearly 95% of patients with HCV.

These factors are responsible for determining not only the viral polymorphisms but also the variants that contain the substitutions associated with resistance and/or reduction of susceptibility to DAAs.

The current study highlights the NS5B polymerase and NS5A inhibitors as classes of drugs used for treatment, which are distributed by Sistema Unico de Saude – SUS (the Brazilian Unified National Health System).

Despite the overall success, the 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. Although there is effective antiviral therapy for the treatment of HCV infection, other small-molecule drugs – for use in HCV new-drug therapies – have been approved and recommended by the FDA.

The impact of resistance mutations on the effectiveness of treatment for treatment-naïve patients is an issue that still needs to be clarified. Studying naïve patients is a way to avoid RAS prior to treatment/re-treatment initiation and, consequently, avoid treatment failure.

This study aimed to map the polymorphisms in the NS5A region and resistance mutations to antiviral drugs in the HCV strains of genotypes 1a and 1b, derived from treatment-naïve patients with chronic hepatitis C in Sao Paulo State, Brazil.

### MATERIALS AND METHODS

#### Sampling analyzed

The samples were obtained before the availability of NS5A/NS5B inhibitors in Brazil. The patients who have never received NS5A/NS5B DAAs were called “naïve patients”. The samples from patients belonging to different regions of Sao Paulo State – including Vale do Paraiba, Vale do Ribeira, and the Sao Paulo metropolitan area, which included Sao Paulo city and the ABCD region, 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 Instituto Adolfo Lutz (IAL), a public health laboratory in Sao Paulo State, from 2012 to 2014. Patient samples were used for HCV diagnosis and stored at -20 °C.

#### Amplification and sequencing of NS5A and NS5B regions

NS5A (1200bp) and NS5B (400bp) analyzed fragments were previously described. NS5A gene RAS were all covered, NS5A mutations were covered up to amino acid residue 406. The amino acid substitutions in the NS5B region were carried out between residues 159 to 495. The mutations after this residue could not be detected.

Nucleic acid extraction was performed using the NucliSENS easyMAG™ kit (BioMerieux, 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.

### Table 1 - Sequence of primers used for GT1a and 1b for regions NS5A and NS5B. Adapted from Santos et al.14

| Region | Direction | Sequence (5’ – 3’) | Position | Reference |
|--------|-----------|--------------------|----------|-----------|
| **NS5A:** | | | | |
| 2F     | Sense     | 5’ACTGTAAGACGGCAGTCAGTGGAAGGCCGACTGCTGGATGAA3’ | 6066-6091 | 13 |
| R      | Antisense | 5’ACCAGGAAAACAGCTATGAGCTRTGrGAIgGRTGCTGTAARCATTGA3’ | 6882-6858 | |
| 3R     | Antisense | 5’ACCAGGAAAACAGCTATGAGCTRTGrGAIgGRTGCTGTAARCATTGA3’ | 6882-6858 | |
| **NS5B:** | | | | |
| PR1    | Sense     | 5’TGGGGAATCTCCGATATGATAAGGCGCTGGCTTGGTGGATGAA3’ | 8245-8275 | 15 |
| PR2    | Antisense | 5’GGGCGGATTCTCCTGCTGCTAGGCACTGCTCCGCTGGATGAA3’ | 8616-8645 | |
| PR3    | Sense     | 5’TATGAYACCCCTGTTGGACTG3’ | 8256-8278 | |
| PR5    | Antisense | 5’GCTAGTCATAGCCTCCTCGGT G3’ | 8619-8636 | |
The amplification of HCV NS5B region was performed in two steps using the protocol described by Santos et al.\textsuperscript{14}, and a set of primers described in Table 1\textsuperscript{15}. For both regions, the amplified fragments were visualized by electrophoresis, using 2% agarose gel and SYBR Safe DNA gel stain (Invitrogen\textsuperscript{™}, Carlsbad, CA, USA).

The samples were sequenced by the Sanger method\textsuperscript{16}, using commercial kits, according to the protocol described by Santos et al.\textsuperscript{14} In order to characterize genotypes and identify polymorphisms, the sequences were submitted to Geno2Pheno website\textsuperscript{17}.

Ethical standards

All the proceedings of this study were initiated only after obtaining ethical approvals from the relevant ethics committees of the participating institutions. This study was approved by the Ethical Committee in Research at the Instituto Adolfo Lutz, Brazil (CEPIAL) Nº 1040338.

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 Sao Paulo metropolitan area and 242 (34.7%) from Vale do Paraiba or Vale do Ribeira, Sao Paulo State. For NS5B characterization, a total of 853 samples were analyzed; of which 541 patients (63.4%) were from the Sao Paulo metropolitan area and 312 (36.6%) from Vale do Paraiba or Vale do Ribeira.

Of the 698 samples used for the 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 the 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 the 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 old (Table 2).

Some relevant RAS were observed in 45 (6.4%) out of the 698 samples analyzed for the 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 the 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 provided free of charge for all patients and are controlled by the Brazilian Ministry of Health. To ensure that the patients have reached the SVR, the absence of HCV-RNA must be observed six months after the end of treatment\textsuperscript{7}.

The Instituto Adolfo Lutz in Sao Paulo city has played an important role in diagnosing viral load and monitoring patients from public health services, starting from diagnosis to analysis of the virological sustained response. In addition, IAL monitors the emergence of resistance mutations in these patients.

There is no gold standard method available to detect HCV drug resistance so far. In this study, the Sanger sequencing method\textsuperscript{16} was employed for the detection of mutations associated with antiviral drug resistance associated with HCV 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 it is easier to perform.\textsuperscript{14,18,19}

All patients enrolled in this study were treatment-naive until the samples were collected to process for the molecular tests. The population examined represents the patients with the most advanced chronic HCV disease in Brazil awaiting treatment. The population was heterogeneous, composed mainly of male individuals who had a high viral load, were approximately 50 years old and from different regions of Sao Paulo State.\textsuperscript{14,20,21}

The distribution of HCV GTs varies according to the geographic location, and monitoring this distribution is important to define the epidemiological trends of infection regarding the introduction of new GTs and for the determination of the transmission routes.\textsuperscript{22,23} In this study, the 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 that the frequency of RAS in the NS5A region of HCV, determined by conventional and next-generation sequencing assays, was between 6.0% and 16.0%.\textsuperscript{24,25} The prevalence of NS5A RAS was 18.0 to 21.0% in Asia, 15.0% in North America, and 19.0% in Europe. The prevalence of NS5B RASs was 1.0% to 5.0%, 4.0% and 20.0% in Asia, North America and Europe, respectively.\textsuperscript{26}

Some studies in Latin America have also published data of RAS in NS5A, NS3 and NS5B. Esposito et al.\textsuperscript{27} studied patients in Buenos Aires and observed that the prevalence of NS3 RASs (39.2%) was higher than NS5A RASs (25.0%) and NS5B RASs (8.9%). In the three regions, the frequencies of RASs were significantly higher in HCV-1b than in HCV-1a.

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

### Table 2 - Characteristics of the patients who participated in this study according to the sample sequence results for NS5A region (n = 698).

| Sample sequence for NS5A region | Samples without RAS\textsuperscript{*} | 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)**                 |                                        |                             |                                   |                 |       |
| 1–9                             | 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 Sao Paulo State**  |                                        |                             |                                   |                 |       |
| Sao Paulo metropolitan area     | 343                                    | 80                          | 4                                 | 29              | 456   |
| Vale do Paraiba/Vale do Ribeira | 188                                    | 36                          | 2                                 | 16              | 242   |
| **Genotype**                    |                                        |                             |                                   |                 |       |
| 1a                              | 224                                    | 64                          | 4                                 | 13              | 305   |
| 1b                              | 307                                    | 52                          | 2                                 | 32              | 393   |

\textsuperscript{*}Resistance-associated substitutions.
Table 3 - Distribution of polymorphisms found for the HCV NS5A region, according to the DAA (n=45).

| Sample | Genotype | Daclatasvir | Elbasvir | Ledipasvir | Omibasvir | Pibrentasvir | Velpatasvir |
|--------|----------|-------------|----------|------------|------------|--------------|-------------|
| 73     | 1a       | L31M        | R        | L31M       | R31M       | S            | R31M        |
| 95     | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 108    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 142    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 140    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 254    | 1a       | Q30R        | R        | Q30R       | Q30R       | S            |            |
| 255    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 279    | 1b       | L31M        | RS       | L31M       | L31M       | SB           |            |
| 285    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 462    | 1b       | L31M        | RS       | L31M       | L31M       | SB           |            |
| 491    | 1a       | Y93S        | SB       | Y93S       | Y93S       | S            |            |
| 497    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 577    | 1a       | Y93N        | R        | Y93N       | Y93N       | -            | S           |
| 602    | 1b       | Y93N        | R        | Y93N       | Y93N       | -            | S           |
| 611    | 1b       | L31F        | SB       | L31F       | L31F       | S            |            |
| 641    | 1b       | L31M        | RS       | L31M       | L31M       | SB           |            |
| 652    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 660    | 1b       | L31M        | RS       | L31M       | L31M       | SB           |            |
| 685    | 1a       | Q30H,Y93H   | R        | Q30H,Y93H  | Q30H,Y93H  | S            |            |
| 762    | 1a       | Q30R        | R        | Q30R       | Q30R       | S            |            |
| 783    | 1b       | L31M        | RS       | L31M       | L31M       | S            |            |
| 787    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 821    | 1b       | Y93H        | R        | Y93H       | P58,Y93H   | S            |            |
| 830    | 1b       | L31F        | SB       | L31F       | L31F       | S            |            |
| 861    | 1a       | H58P,Y93S   | SB       | H58P,Y93S  | H58P,Y93S  | S            |            |
| 886    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 940    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 960    | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |
| 964    | 1a       | L31M        | R        | L31M       | L31M       | S            |            |
| 967    | 1b       | Y93N        | R        | Y93N       | Y93N       | -            | S           |
| 972    | 1a       | L31M        | R        | L31M       | L31M       | S            |            |
| 977    | 1a       | H58P        | SB       | P32L,H58P  | P32L,H58P  | S            |            |
| 987    | 1b       | L31M        | RS       | L31M       | L31M       | S            |            |
| 1040   | 1b       | Y93N        | R        | Y93N       | Y93N       | -            | S           |
| 1065   | 1a       | Q30R        | R        | Q30R       | Q30R       | S            |            |
| 1073   | 1b       | Y93H        | R        | Y93H       | Y93H       | -            | S           |

R = Resistance; RS = Reduced Susceptibility; S = Susceptible; SB = Substitution.
both HCV strains with GT-1a and GT1b. 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.28

According to Cuypers et al. 29, 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 the susceptibility to NS5A inhibitors and induce conformational changes. In addition, Gottwein et al. 30 have demonstrated that the Y93H polymorphism shows clinical results on the effectiveness of Pibrentasvir. As a result, the mutation Y93H may well be responsible for the selection of resistant strains in the NS5A region, which may interfere with treatment based on DAAs.18,30

The most frequent mutations in GT-1 were Y93H and L31M. Out 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 11 samples that presented resistance to Daclatasvir and Ledipasvir, respectively. An in vitro study carried out by Wyles and Luetkemeyer31 demonstrated that the substitution of the amino acid L31M is more likely to confer a clinical impact due to the high fold-change observed in vitro towards the Daclatasvir and Ledipasvir sensitivity.

However, an in vivo study by Costa et al.32 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.

In our study, two samples that showed the polymorphism Y93N also presented resistance to Pibrentasvir. However, a study performed by Wyles and Luetkemeyer31 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 (>1,000X for GT-1a), Daclatasvir, Ledipasvir and Ombitasvir (>10,000X for GT-1a). The absence of RAS in the NS5B region was observed in all the analyzed sequences. The polymerase inhibitors have a high genetic barrier; therefore, only a few patients experience therapeutic failure with the Sofosbuvir regimen.33 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.34

Aldunate et al.35, in a study from Uruguay, found that naturally occurring substitutions conferring resistance to NS5A and NS5B inhibitors were present in 8.0% and 19.2%, respectively, of treatment-naïve HCV genotype 1 infected patients.

Di Stefano et al.36, in a study to evaluate real-life DAA regimens, observed a strong correlation between the presence of RAS and therapeutic regimen. The study detected that 93.0% of patients treated with regimens containing NS5A inhibitors had RAS associated with therapeutic failure. In contrast, RAS associated with NS5B does not seem to be significant.

Di Maio et al.37, in a multicenter real-world study retrospective (VIRONET-C), comparing RAS analyses and clinical data, observed a significant correlation between the presence of RAS and fibrosis/cirrhosis in HCV GT3

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Table 4 - Frequency and percentage of RAS in the NS5A region, according to each drug.

| RAS*       | Daclatasvir n (%) | Elbasvir n (%) | Ledipasvir n (%) | Velpatasvir n (%) | Pibrentasvir n (%) | Ombitasvir 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.
infected patients. In this study, Y93H was the most frequent RAS observed, in concordance with our results. Furthermore, the VIRONET-C study has also suggested aiding RAS detection in the choice for an optimal second line of treatment.

A limitation of this study was the impossibility to report the patient's clinical information. The IAL receives only serum samples without information about drug adherence, liver damage, or disease outcome. Therefore, it was not possible to follow up on the effectiveness of the administered treatments.

In addition, due to a lack of complete data and clinical follow-up of the patients, it was not possible to evaluate whether these patients reached 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.

CONCLUSION

The prevalence of a baseline RAS determined in this study aligns with that reported in other countries. The NS5A region, as expected, is more susceptible to RAS than the NS5B region, especially in GT1-b, corroborating the conclusions of other studies highlighting that the NS5B region of the HCV genome represents a high barrier to mutations.

Overall, 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 Sao 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 re-treatment 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 the treatment initiation.

AUTHORS’ CONTRIBUTIONS

RCM: wrote the first draft, coordinated the completion of the paper, studied protocol, was head of the project, was in charge of project design, and coordinated the study; APTS: was responsible for the methodology, contributed to the first draft and all revisions, conducted the analysis, and reviewed the paper; MCMC: was in charge of the project conceptualization, helped developing the protocol, wrote the first draft, was responsible for clinical and laboratory interpretations results, contributed to the revisions, and reviewed the final draft; FMM: was in charge of the standardization of molecular methods and interpretation of molecular results, and contributed to the final draft; RAHS, GTFD and VFDC: were the Hospital Albert Einstein team responsible for the molecular methodology and contributed to the final draft; MFL and VCMS: developed the laboratory algorithm, were responsible for the development, analysis and results interpretation of molecular tests, and contributed to the final draft; JRRP: was responsible for the project conceptualization and validation, contributed to the final draft, and reviewed the final paper.

CONFLICT OF INTERESTS

All authors declare that they have no conflict of interests or disclosures to the manuscript.

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REFERENCES

1. Polaris Observatory HCV Collaborators. Global change in hepatitis C virus prevalence and cascade of care between 2015 and 2020: a modelling study. Lancet Gastroenterol Hepatol. 2022;7:396-415.
2. Borgia SM, Hedskog C, Parhy B, Hyland RH, Stamm LM, Brainard DM, et al. Identification of a novel hepatitis C virus genotype from Punjab, India: expanding classification of hepatitis C virus into 8 genotypes. J Infect Dis. 2018;218:1722-9.
3. Campiotto S, Pinho JR, Carrilho FJ, Silva LC, Souto FJ, Spinelli V, et al. Geographic distribution of hepatitis C virus genotypes in Brazil. Braz J Med Biol Res. 2005;38:41-9.
4. Bhattacharjee C, Singh M, Das D, Chaudhuri S, Makhopadhyay A. Current therapeutics against HCV. Virusdisease. 2021;32:228-43.
5. Liu Z, Mao X, Yu K, Suo C, Jin L, Zhang T, et al. Prevalence of HCV resistance-associated substitutions among treatment-failure patients receiving direct-acting antiviral agents. J Viral Hepat. 2020;27:585-92.
6. Jiménez-Pérez M, González-Grande R, Contreras PE, Martínez IP, Lombardo JC, Martín RO. Treatment of chronic hepatitis
C with direct-acting antivirals: the role of resistance. World J Gastroenterol. 2016;22:6573-81.

7. Brasil. Ministério da Saúde. Departamento de Vigilância, Prevenção e Controle das Infecções Sexuamente Transmissíveis, do HIV/ Aids e das Hepatites Virais. Protocolo clínico e diretrizes terapêuticas para hepatite C e coinfecções. Brasília: Ministério da Saúde; 2015. [cited 2022 Aug 16]. Available from: http://bvsms.saude.gov.br/bvs/publicacoes/protocolo_clinico_diretrizes_hepatite_co coinfeccoes.pdf

8. Foster GR, Irving WL, Cheung MC, Walker AJ, Hudson BE, Verma S, et al. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. J Hepatol. 2016;64:1224-31.

9. Meshram RJ, Kathwate GH, Gacche RN. Progress, evolving therapeutic/diagnostic approaches, and challenges in the management of hepatitis C virus infections. Arch Virol. 2022;167:717-36.

10. Sayan M, Yildirim FS, Akhan S, Yildirim AA, Sirin G, Cabalak M, et al. NS5A resistance-associated substitutions in chronic hepatitis C patients with direct acting antiviral treatment failure in Turkey. Int J Infect Dis. 2020;95:84-9.

11. Noble CF, Malta F, Lisboa-Neto G, Gomes-Gouvêa MS, Leite AG, Castro VF, et al. Natural occurrence of NS5B inhibitor resistance-associated variants in Brazilian patients infected with HCV or HCV and HIV. Arch Virol. 2017;162:165-9.

12. Malta F, Gaspareto KV, Lisboa-Neto G, Carrilho FJ, Mendes-Correia MC, Pinho JR. Prevalence of naturally occurring NS5A resistance-associated substitutions in patients infected with hepatitis C virus subtype 1a, 1b, and 3a, co-infected or not with HIV in Brazil. BMC Infect Dis. 2017;17:716.

13. Andre-Garnier E, Besse B, Rodallec A, Ribeyrol O, Ferre V, Luco C, et al. An NS5A single optimized method to determine genotype, subtype and resistance profiles of Hepatitis C strains. PLoS One. 2017;12:e0179562.

14. Santos AP, Silva VC, Mendes-Correa MC, Lemos MF, Malta FM, Santana RA, et al. 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. Infect Drug Resist. 2021;14:723-30.

15. Sandres-Sauné K, Deny P, Pasquier C, Thibault V, Duverlie G, Izopet J. Determining hepatitis C genotype by analyzing the sequence of the NS5b region. J Virol Methods. 2003;109:187-93.

16. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977;74:5463-7.

17. Kalaghagi P, Sikorski AM, Knops E, Rupp D, Sierra S, Heger E, et al. Geno2pheno[HCV]: a web-based interpretation system to support hepatitis C treatment decisions in the era of direct-acting antiviral agents. PLoS One. 2016;11:e0155869.

18. Sharafi H, Alavian SM. Hepatitis C resistance to NS5A inhibitors: is it going to be a problem? World J Hepatol. 2018;10:543-8.

19. Sarrazin C. The importance of resistance to direct antiviral drugs in HCV infection in clinical practice. J Hepatol. 2016;64:486-504.

20. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol. 2014;61 Suppl 1:S45–57.

21. Sorbo MC, Cento V, Di Maio VC, Howe AY, Garcia F, Perno CF, et al. Hepatitis C virus drug resistance associated substitutions and their clinical relevance. Drug Resist Updat. 2018;37:17-39.

22. Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology. 2005;42:962-73.

23. Lavanchy D. Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect. 2011;17:107-15.

24. Suzuki F, Sezaki H, Akuta N, Suzuki Y, Seko Y, Kawamura Y, et al. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J Clin Virol. 2012;54:352-4.

25. Sarrazin C, Berg T, Buggisch P, Dollinger M, Hinrichsen H, Huppe D, et al. Current recommendations for the treatment of chronic hepatitis C. Z Gastroenterol. 2014;52:1185-97.

26. Rao H, Yang X, Tan Y, Ning Q, Yang D, Wang J, et al. Efficacy and safety of all-oral Emitasvir and Sofosbuvir in patients with genotype 1b HCV infections without cirrhosis. J Clin Transl Hepatol. 2020;8:255-61.

27. Esposito I, Marciano S, Haddad L, Galdame O, Franco A, Gadano A, et al. Prevalence and factors related to natural resistance-associated substitutions to direct-acting antivirals in patients with genotype 1 hepatitis C virus infection. Viruses. 2018;11:3.

28. Cheng G, Tian Y, Doehle B, Peng B, Corsa A, Lee YJ, et al. In vitro antiviral activity and resistance profile characterization of the hepatitis C virus NS5A inhibitor ledipasvir. Antimicrob Agents Chemother. 2016;60:1847-53.

29. Cuypers L, Ceccherini-Silberstein F, Van Laethem K, Li G, Vandamme AM, Rockstroh JK. Impact of HCV genotype on treatment regimens and drug resistance: a snapshot in time. Rev Med Virol. 2016;26:408-34.

30. Gottwein JM, Pham LV, Mikkelson LS, Ghanem L, Ramirez S, Scheel TK, et al. Efficacy of NS5A inhibitors against hepatitis C virus genotypes 1-7 and escape variants. Gastroenterology. 2018;154:1435-48.

31. Wyles DL, Luettekemeier AF. Understanding hepatitis C virus drug resistance: clinical implications for current and future regimens. Top Antivir Med. 2017;25:103-9.

32. Costa VD, Brandão-Mello CE, Nunes EP, Silva PG, Rodrigues LL, Lamp E, et al. 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;14:e0216327.
33. Sagnelli E, Starace M, Minichini C, Pisaturo M, Macera M, Sagnelli C, et al. Resistance detection and re-treatment options in hepatitis C virus-related chronic liver diseases after DAA-treatment failure. Infection. 2018;46:761-83.

34. Eltahla AA, Leung P, Pirozyn MR, Rodrigo C, Grebely J, Applegate T, et al. Dynamic evolution of hepatitis C virus resistance-associated substitutions in the absence of antiviral treatment. Sci Rep. 2017;7:41719.

35. Aldunate F, Echeverría N, Chiodi D, López P, Sánchez-Cicerón A, Fajardo A, et al. Pretreatment hepatitis C virus NS5A/NS5B resistance-associated substitutions in genotype 1 Uruguayan infected patients. Dis Markers. 2018,2018:2514901.

36. Di Stefano M, Faleo G, Farhan Mohamed AM, Morella S, Bruno SR, Tundo P, et al. Resistance associated mutations in HCV patients failing DAA treatment. New Microbiol. 2021;44:12-8.

37. Di Maio VC, Barbaliscia S, Tetti E, Fiorentino G, Milana M, Paolucci S, et al. Resistance analysis and treatment outcomes in hepatitis C virus genotype 3-infected patients within the Italian network VIRONET-C. Liver Int. 2021;41:1802-14.

38. Chen CY, Huang CF, Cheng PN, Tseng KC, Lo CC, Kuo HT, et al. Factors associated with treatment failure of direct-acting antivirals for chronic hepatitis C: a real-world nationwide hepatitis C virus registry programme in Taiwan. Liver Int. 2021;41:1265-77.

39. Peres-da-Silva A, Almeida AJ, Lampe E. NS5A inhibitor resistance-associated polymorphisms in Brazilian treatment-naive patients infected with genotype 1 hepatitis C virus. J Antimicrob Chemother. 2015;70:726-30.

40. Aguiar BF, Campos GR, Rodrigues JP, Marques NN, Molina BF, Bittar C, et al. Baseline resistance associated substitutions in HCV genotype 1 infected cohort treated with Simeprevir, Daclatasvir and Sofosbuvir in Brazil. Clin Res Hepatol Gastroenterol. 2020;44:329-39.