Resistance mutations of NS3 and NS5b in treatment-naïve patients infected with Hepatitis C virus in Santa Catarina and Rio Grande do Sul States, Brazil.

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Abstract

Hepatitis C virus (HCV) infection is a worldwide health problem. Nowadays, direct-acting antiviral agents (DAAs) are the main treatment; however, the high level of variability of HCV lead to development of resistance-associated variants (RAVs). Thus, look into the RAVs among infected patients is an important tool for monitoring the efficacy of the therapy. The aim of our study was to investigate the presence of naturally occurring resistance mutations in HCV NS3 and NS5 regions in treatment-naïve patients. Ninety-six anti-HCV positive serum samples from blood donors at Center of Hematology and Hemotherapy of Santa-Catarina State (HEMOSC) were collected retrospectively in 2013, and evaluated in this study. HCV subtypes 1a (37.9%), 1b (25.3%) and 3a (36.8%) were found. The frequency of patients with RAVs in our study was 6.9%. The HCV NS5b sequencing revealed 1 sample with L320F mutation and 4 samples with the polymorphism C316N/R. The analysis of the NS3 region revealed mutations D168A/G/T (3.45%), S122G (1.15%) and V55A (2.3%). All samples from genotype 3a (36.8%) presented the non-synonymous mutation V170 I/V. In conclusion, we have shown that mutation in NS3 and NS5b genes are present in Brazilian isolates from therapy-naïve patients.

Keywords: Direct-acting antivirals, resistance-associated substitutions, blood donors, NS3, NS5b.

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Hepatitis C virus (HCV) infection is a worldwide health problem, according to The Global Hepatitis Report, from the World Health Organization (WHO), approximately 71 million people have chronic HCV infection, and nearly 399,000 people die each year, mostly due to cirrhosis or hepatocellular carcinoma (WHO, 2017). HCV has a high genetic heterogeneity and is classified into seven genotypes (1-7) and 67 subtypes (Smith et al., 2014). The genotype distribution depend on geographical location or risk groups (Cantaloube et al., 2005). Genotype 1 is the most frequent in Brazil, followed by genotype 3 and 2 (Campiotto et al., 2005; Lampe et al., 2013).

There is no vaccine available for preventing HCV infections. The main antiviral treatment, before 2011, was PEGylated interferon-alfa (αPeg-IFN) monotherapy or in combination with ribavirin, leading to a sustained virological response (SVR) in 50% of treated patients, depending on the virus genotype causing the HCV infection (Peres-da-Silva et al., 2012; Paolucci et al., 2013; Gross et al., 2018). Nowadays, direct-acting antiviral agents (DAAs) have been approved for the HCV infection treatments, with an average SVR above 95%, at least for genotypes 1 and 4 (Leuw and Stephan, 2018). In Brazil, since 2015, DAAs were incorporated, by the Ministry of Health, for the treatment of hepatitis C under the Unified Health System (SUS) (Brasil, 2018). Unfortunately, there is almost no data about the efficacy of DAAs in Brazil, some information can be found in Settle Jr (2017).

The primary targets of DAAs are nonstructural proteins essentials for HCV replication, which include the NS3 protease, NS5B polymerase and NS5A protein. (Paolucci et al., 2013; Lontok et al., 2015). However, a challenge for HCV treatment is the emergence of viral resistance mutations that reduce susceptibility of the virus to DAA therapies (Hoffman et al., 2015; Gededzha et al., 2017). The development of variants associated with resistance (RAVs) is due to the high level of variability of HCV. Result of the combination of high replication rate, low RNA polymerase fidelity rate and selective pressures of drug or immunomediated treatment. (Peres-da-Silva et al., 2012; Paolucci et al., 2013; Gededzha et al., 2017).
The presence of RAVs in patients not yet in treatment have been reported previously worldwide (Peres-da Silva et al., 2010; Gededzha et al., 2017; Paolucci et al., 2013; Zeminian et al., 2013). Even a systematic review, with the information available regarding the HCV resistance associated substitution and their clinical relevance, was published recently (Sorbo et al., 2018). Therefore, look to the RAVs among infected patients is an important tool for monitoring the efficacy of the therapy (Loggi et al., 2017) and the epidemiological monitoring of HCV in Brasil. Thus, the aim of our study was to investigate the presence of naturally occurring resistance mutations in HCV NS3 and NS5 regions in treatment-naïve patients.

The Center of Hematology and Hemotherapy of Santa-Catarina State (HEMOSC) is currently responsible for the nucleic acid testing for HIV, HCV and HBV in samples from blood donor of the Santa Catarina and Rio Grande do Sul States. Annually, HEMOSC receives around 300 thousand donations. In 2013, a total of 96 samples from blood donor were positive to HCV and they were all collected to this study, retrospectively.

HCV RNA was extracted from plasma previously conserved at -80°C, using a molecular biology workstation (BioRobot MDx, Qiagen), with Qiamp one-for-all nucleic acid kit (Qiagen), according to the manufacturer’s instructions. Plasma HCV RNA viral load were quantified using COBAS/Taqman HCV Test v2.0 (Roche).

Genotyping/subtyping was performed by amplifying and sequencing a 339-bp amplicon of the NS5b region, according to Cantaloube et al., 2005. The nucleotide sequences obtained were analyzed in the Geno2pheno [HCV] (Kalaghatgi et al. 2016), for geno- and subtypes, and possible resistance against licensed DAAs.

The amplification of the entire NS3 region of HCV genome, followed by a second PCR was performed as described previously (Peres-da-Silva et al., 2010), using primers specific to subtypes 1a, 1b and 3a. The nucleotide sequences obtained from each subtype were analyzed in the Geno2pheno [HCV] (Kalaghatgi et al. 2016), with respect to drug resistance.
The statistical program SPSS (IBM SPSS Statistics Base 22.0) was used. Multivariate analysis (ANOVA) was used to compare means of continuous variables with normal or significant distribution ($p < 0.05$).

From 96 samples HCV positive collected to this study, nine of them had not enough material to perform the assays and, for this reason, they were excluded. Eighty-seven samples were used for genotyping and analysis of NS3 and NS5b regions.

From 87 samples evaluated by our study, 33 (37.9%) were genotype 1a, 22 (25.3%) were genotype 1b and 32 (36.8%) were genotype 3a. Genotype 1 (1a plus 1b) was the most frequent, followed by genotype 3, a result that is in agreement with what was previously reported in Brazil (Campioto et al., 2005; Nishyia et al., 2014; Lampe et al., 2013). We did not found the genotypes 2, 4 and 5, described to be less frequent genotypes in Brazil. Sixty-three samples were successfully geno- and subtyped by the NS3 and NS5b region, and there were no discordance between the HCV genotype in both region sequenced. Twenty-four samples had no amplification of NS5b region, not even with an alternative protocol (Sandres-Saune, 2003). Those 24 samples were genotyped according to Peres-da-Silva (2010) protocol (NS3 region). This amplification divergence has already been discussed in Larrat et al., 2013, when was reported the failure of some quantitative RT-PCR assays to detect or to amplify correctly the NS5b region in some strains of HCV, even when were used three sets of primers covering two different regions. This could be explained by the greater variety of the viruses, the use of primers not suitable for these peculiar strains, or even by a mixed infection in the plasma sample.

The mean viral loads to each genotype found were, 5.31 log IU/mL for genotype 1a, 5.18 log IU/mL for 1b and 5.38 log IU/mL for 3a. There was no difference in viral load between the genotypes ($p=0.6$). The detected HCV genotypes and viral loads are both important predictors for therapeutic outcomes. It has been reported that patients infected with genotype 1 were more likely to have higher viral loads than those infected with genotype 2 and 3 (Scott et al., 2007, Soriano et al., 2008, Nishiyama et al, 2014). We did not observe a significant difference between the mean viral loads of the genotypes.
in this study. In contrast, in a study carried out with blood donors from São Paulo, the viral load from genotype 3a (5.22 log10 IU/mL) had a lower log mean than genotype 1a (5.99 log10 IU/mL) (3a vs 1a, p = 0.0002) and genotype 1b (6.35 log10 IU/mL) (Nishiya et al, 2014), in agreement with the previous reported.

The total frequency of patients with variants associated with resistance in our study showed an intermediate result (6.9%) when compared with other Brazilian studies among HCV chronic carriers not treated with protease inhibitors (3.2% - 18.9%) (Hoffmann et al., 2013; Nishiya et al., 2014).

The HCV NS5b sequencing from 63 samples were analyzed. The mutation L320F was present in only one sample (1.59%), genotype 1a. L320F is known to confer low resistance to Sofosbuvir and Sofosbuvir associated with Mericitabine (Paolucci et al., 2013) and are associated to treatment failure in clinical trials (Constantino et al., 2015). In a previous study, L320F single mutation had no significant impact on the 50% effective concentration (EC50) and EC90 values for mericitabine (≤2.7fold) (Tong et al., 2013). To our knowledge, this is the first time that mutation L320F is reported as naturally occurring in DAA treatment-naïve patients and it should be monitored due to the treatment failure reported previously in clinical trials. The polymorphism C316N/R was present in 4 samples (6.35%), genotype 1b. C316N is reported to confer low level of resistance to Sofosbuvir (Paolucci et al., 2013, Lontok et al., 2015). C316N mutation has been associated with a 10-fold increase in EC50 to a new experimental non-nucleoside drug, HCV796 (Castilho et al., 2011). Previous studies have found variable prevalence of C316N in Brazil, from 3.85% (Peres-da-Silva et al., 2017) to 11.6% (Castilho et al., 2011),16.3% (Noble et al., 2017) and 24% (Castilho et al., 2011), and higher prevalence in North America (16.81%), Europe (7.47%) and Asia (49.71%) (Peres-da-Silva et al., 2017). The higher prevalence of mutations in genotype 1b has been reported previously and it was due to the presence of C316N (Paolucci et al., 2013; Peres-da-Silva et al., 2017).

Although it was not a goal of our study, evaluating the NS5b region from the 63 samples, we also observed the presence of D244N, Q309R and A333E, mutations conferring resistance to
Ribavirin and interferon in 42 samples (57.14%). Twenty samples (26.98%) presented Q309R, and three (1.58%) A333E. Seventeen were Q309R and D244N, two were Q309R and A333E, and two were triple positive. In a previous work, the most frequent mutation observed in Brazil was Q309R, present in all HCV subtypes (Castilho et al., 2011), present in 38 samples in our study. No double mutations in NS5b region conferring resistance to DAAs were observed in our samples. The emergence of double or triple-sites RAVs in the clinic is threatening the effectiveness of anti-HCV therapies, as published previously (Gane et al., 2016).

The analysis of the NS3 region revealed mutations D168A/G/T (3.45% - 3/87), S122G (1.15% - 1/87) and V55A (2.3%- 2/87) that confer resistance to Asunaprevir, Boceprevir, Grazoprevir, Simeprevir and Paritaprevir (Zeminian et al., 2013; Lontok et al., 2015; Sorbo et al., 2018). V55A was observed at a higher frequency, 4.1% (Moreira et al., 2018) and 6% (Nishyia et al., 2014), in previous works with DAA naïve patients and blood donor, respectively, in São Paulo, and in Europe (6%) (Bartel et al., 2013). The V55A variant have been shown to confer 6.9-fold increase in EC50 to boceprevir (Vermehren et al., 2012). Regarding S122G, it was found in a higher frequency in Spain (6.23%) and China (85.48%) (Li et al., 2017). One in vitro study have shown that S122G did not reduce susceptibility to simeprevir (Izquierdo et al., 2014). However, another study have shown that S122G reduced the susceptibility in 0.5 fold (Lenz et al., 2010). In São Paulo, mutation D168G was found previously in one sample from 125 HCV infected blood donors (Nishyia et al., 2014). In a transient susceptibility assay, D168G conferred low- to moderate-level asunaprevir resistance (5- to 21-fold) for HCV genotype 1a. For genotype 1b a higher level of asunaprevir-associated resistance was observed ranging from 170- to 400-fold relative to wild-type control. (McPhee et al., 2012).

No mutations were found to confer resistance to Glecaprevir and Voxilaprevir, drugs known to present a high barrier to resistance (Sorbo et al., 2018). However, this study have found samples with mutations that decrease the susceptibility of HCV to these drugs, this corroborate with the importance of monitoring the HCV RAVs.
Samples from genotype 3a, presented no mutations that confers or diminishes resistance to Glecaprevir and Voxilaprevir, drugs recommended for treatment of patients infected with this genotype. This means that the standard protocol for treatment of patients with genotype 3 will be effective in Santa Catarina and Rio Grande do Sul. However, we found the non-synonymous mutation V170 I/V in all 32 samples of this genotype. In agreement with our results, Peres-da-Silva (2010) found that 100% (32/32) of the sequences of HCV genotype 3a contained the V170I substitution. Few data are available on effects of V170I substitution. The conservative substitution at this site was detected up to 45% of patients infected with HCV genotype 1 (López-Labrador et al., 2008).

A different pattern of resistance associate with NS3 protease domain in therapy-naïve patients was previously reported in Brazil. V36L mutation was found in genotype 1a at a frequency of 5.6%, 100% in genotype 1b (Peres-da-Silva et al., 2010) and in genotype 2, 3, 4 and 5 were found as genetic signatures with frequency of 99% (Vidal et al., 2016), in another work, V36L was found at frequency of 4% in genotype 1a (Nishyia et al., 2014). T54S mutations were found in 4.1% of genotype 1a (Peres-da-Silva et al., 2010) and 100% in genotype 2 (Vidal et al., 2016). The samples investigated by our study presented none of these mutations. The Q80K, a common mutation in the USA (40%) (Bartels et al., 2013) that confers resistance to simeprevir, was not found in our study, and previously have been reported at diferent prevalence ranging from 0.4% to 2.7% in Brazil (Moreira et al., 2018; Vidal et al., 2015; Nishiya et al., 2014). There is a strong geographic correlation regarding the frequency on the Q80K substitution (Moreira et al., 2018), and for this reason, studies from different geographic origins are of great importance, especially in a big country as Brazil.

Of all the samples evaluated, only one sample of genotype 1b showed mutation in the genes NS3 and NS5b, conferring resistance to sofosbuvir (C316N), and decreased susceptibility to Gazoprevir (Y56F). This shows the importance of study both NS3 and NS5 proteins when evaluating or choosing the therapy strategy of HCV positive patient. Patients carrying combinations of resistance mutations are of particular interest, since they may increase the possibility of failure in the treatment of DAAs.
When evaluating the frequency of resistance mutation and genotype, we have found that it was twice as high among patients with subtype 1a compared to those with subtype 1b. A similar result was found in blood donor’s samples from São Paulo (Nishyia et al., 2014). In addition, a higher frequency of virological failure for subtype 1a than 1b has been reported (Pawlotsky et al., 2011)

At last, we observed in our samples several polymorphisms not associated with resistance to DAAs, detailed in table 1, and previously reported by others (Costantino et al., 2015). The polymorphism, prior to therapy, is part of the quasispecies population in infected individuals, and may not alter viral fitness (Peres-da-Silva et al., 2012; Paolucci et al., 2013; Nishyia et al., 2014).

In conclusion, we have shown that mutations in NS3 and NS5b domains are present in Brazilian isolates from therapy-naïve patients, in this case, blood donors with unknown HCV infection. Monitoring the presence of the RAVs are an important tool for predicting response to antiviral therapy, and regional discernment can help determine local policies for treatment. The results presented here will help to ensure a more successful therapy strategies for HCV infected patients in Santa Catarina and Rio Grande do Sul states in Brazil.

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**Figure 1:** Frequency of specific NS3 and NS5b resistance-associated variants, found in this study by HCV subtype.
Table 1: HCV polymorphic sites distribution according to genotype.

| Gene | Position | Polymorphisms* | 1a | 1b | 3a |
|------|----------|----------------|----|----|----|
| NS3  | 62       | R62K           | 7  | 3  | 5  |
|      | 64       | I64L/M         | 7  | 5  | 2  |
|      | 86       | P86Q/H         | 5  | 2  | 7  |
|      | 89       | Q89A/H/P       | 8  | 3  | 3  |
|      | 91       | S91A/T         | 18 | 11 | 10 |
|      | 102      | S102A/F        | 3  | 6  | 8  |
|      | 140      | T140A          |    |    |    |
|      | 147      | A/F147G/M/S/T  | 9  | 2  | 8  |
|      | 153      | L153I          | 24 | 9  | 9  |
|      | 166      | A/S166 A/T/R   | 4  | 5  | 9  |
|      | 170      | I/V170I/V/H    | 3  | 2  | 32 |
|      | 176      | E/S176K/N      | 8  | 9  | 5  |
| NS5  | 244      | D244N          |    |    | 17 |
|      | 254      | K254R/S        | 5  | 13 | 22 |
|      | 300      | R300Q/S/T      | 17 | 17 | 23 |
|      | 309      | Q309R/H        | 15 | 1  | 22 |
|      | 312      | T312D/E/S/R    | 2  | 2  | 23 |
|      | 329      | V329E/F/G/R/T  | 14 | 17 | 7  |
|      | 332      | D332G/N/R      | 5  | 12 | 13 |
|      | 333      | A333E/G/P/Q    | 11 | 6  | 23 |
|      | 334      | A334G/H/V/Q/W  | 12 | 10 | 17 |
|      | 335      | S335E/N/G/Q/T  | 15 | 13 | 23 |
|      | 336      | L336A/P        | 14 | 12 | 20 |
|      | 337      | R337N/T/I      | 11 | 12 | 9  |

* some samples have more than one variant.