Evaluation of viral load in saliva from patients with chronic hepatitis C infection

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Received 24 December 2014; received in revised form 26 March 2015; accepted 3 April 2015

KEYWORDS
Chronic hepatitis C infection; qPCR; Viral subtype; Ethnicity

Summary Hepatitis C virus can be detected in blood and other bodily fluids, such as saliva. The aim of this study was to detect and quantify the HCV-RNA in saliva and plasma from patients with chronic hepatitis C infections, as well as check the level of viral load in sex groups (age, ethnicity and virus subtypes). Whole saliva and blood from 70 patients with chronic hepatitis C infections attended at the department of gastroenterology from University Hospital. The HCV-RNA load was performed by qRT-PCR using Sybr Green I master mix. HCV-RNA was detected in 80% (56/70) of patients in saliva and 92.85% (65/70) in plasma. The median of the viral load in the plasma was of 4.87 log10, and in saliva, it was 3.32 log10, (p = 0.0005). Female patients and
black patients exhibited a negative correlation between the HCV-RNA load in saliva vs. the HCV-RNA load in plasma ($r = -0.3172$, $CI_{95\%} = 0.6240$ to $-0.03736$, $p = 0.0491$) and ($r = -0.3141$; $IC_{95\%} = -0.6069$ to $-0.05926$; $p = 0.0209$), respectively. HCV-RNA was detected and quantified in saliva samples, and according to the quantification levels, saliva may be a possible transmission source of HCV, particularly in women and people of black ethnicity who develop chronic HCV infections.

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Introduction

Hepatitis C is a public health problem with approximately 170 million people chronically infected [1–3]. Approximately 80% of patients infected with the hepatitis C virus (HCV) develop chronic infections that could lead to cirrhosis and hepatocellular carcinoma [4] because HCV plays an important role not only in diseases of the liver but in a variety of extrahepatic manifestations and immune abnormalities [2].

While the detection of HCV antibodies in serum is a traditional diagnostic method, the use of saliva samples for the diagnosis of HCV infection offers a number of potential advantages, such as minimal invasiveness and skill necessary to collect a sample. These samples are especially suitable for prevalence studies when blood samples are difficult to obtain, as in injecting drug users, hemophiliacs and children. Furthermore, the analysis of saliva may provide a better cost-benefit for screening in large populations [5,6].

Many studies have documented the suitability of saliva samples for the diagnosis of the HCV infection using enzyme immunoassays and polymerase chain reaction in real-time (RT-qPCR) [1,5,7–10]. These studies have shown differences in methodology; a number tested whole saliva for HCV, while others tested centrifuged supernatant [11].

Regarding the detection, the analysis of viral load, the best methodology, the barriers to transmission of HCV in saliva, and the possible mechanisms and infectious ability of a biological fluid, there are still no conclusive answers among researchers. The aim of this study was to detect HCV-RNA and quantify the viral load in saliva and in plasma from patients with chronic hepatitis C infections not receiving any antiviral treatment and to characterize and to analyze the viral loads according to demographics (gender, age, ethnicity and virus subtypes); depending on the viral load level, saliva may be a possible route of HCV transmission for certain risk groups.

Materials and methods

Ethical aspects

This research was approved by the Ethics Committee of Research (Centro de Ciências da Saúde/UFPE), CAAE: 10148913.7.0000.5208. All patients completed an informed consent document about the research.

Patients and samples

We studied 70 patients with chronic hepatitis C infections and positive HCV serum antibody tests, who had not started treatment with antiviral drugs, seen in the gastroenterology outpatient facility of the Clinical Hospital of the Federal University of Pernambuco from April 2013 to September 2013.

Blood collection: 4 mL of peripheral blood were collected in tubes containing EDTA anticoagulant, which was centrifuged (1500 rpm/15 min) and separated in plasma and cells. Two hundred µL of guanidine isothiocyanate solution was added to 1 mL of plasma (4 M) and stored at $-80^\circ$ C.

The patient chewed a piece of sterile soft rubber (diameter of 0.5 cm and length of 1.0 cm), then 5 mL of saliva were collected, which was put into a sterile falcon tube (capacity 50 mL). After the saliva collection, 1 mL of saliva was separated for the addition of 200 µL of guanidine isothiocyanate solution (4 M) and stored at $-80^\circ$ C. The maximum time between the collection and storage of samples was 2 h.
For the HCV-RNA extraction (saliva and plasma), 400 μL of sample was used, according to the method based on phenol acid (pH ± 5.0)/chloroform according to Sambrook and Russell [12]. The sample was resuspended in 20 μL of water with diethylpyrocarbonate-DEPC (0.1%) and stored at −80°C. The cDNA was constructed using random primers and the IMPROM-II™ Reverse Transcription System kit (Promega®; Fitchburg, Wisconsin, USA, 2013). Subsequently, the RNA was quantified using Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, USA, 2012), with an average concentration of 800 ng/μL.

RT-qPCR reaction

Approximately 2 μL of cDNA was used for a mix containing: 1 × Sybr Green I (Promega®; Fitchburg, Wisconsin, USA, 2013) and 0.5 pmol/μL of primers 940 (5′-TTC ACG CAG AAA GCG TCT AG-3′) and 211 (5′-CAC TCT CGA GCA CCC TAT CAG GCA GT-3′) (adapted of Chang et al.) [13]. The cycling conditions were as follows: 1°C heating 50°C/2 min; 2°C heating 95°C/12 min/45 times (95°C/20 s; 50°C/60 s; 72°C/35 s). Then, the mixture was gradually heated (60–95°C, with increases of 0.7°C per 7 s). The melting (78 ± 1.5°C) was analyzed to verify the specificity of the amplicons. For the quantification of HCV-RNA, a standard curve ranging from 10^1 to 10^9 copies/mL was used from positive HCV-RNA control samples, confirmed in a reference laboratory from state of Pernambuco. The results were analyzed in copies/mL and log_{10}.

Statistical analysis

The GraphPad Prism 5.0 software was used for the association of viral load according to two continuous variables (the Mann–Whitney test). We used the Pearson correlation test for to measure the degree of linear relation (positive or negative) between two continuous variables. All results with p values < 0.05 were indicative of statistical significance.

Results

HCV-RNA was detected in 80% (56/70) of saliva samples and 92.85% (65/70) of plasma samples.

The viral load in plasma was higher (75,699 copies/mL or 4.879 log_{10}; CI_{95%} 4.29 to 5.46), than in saliva (2118 copies/mL or 3.32 log_{10}; CI_{95%} 2.58–4.05) (p = 0.0005, Fig. 1).

Only three patients showed HCV-RNA quantification in saliva (5,293,423 copies/mL or 6.724 log_{10}) and an absence of plasma viral load.

Of the patients, 39 (55.71%) were women, and 31 (44.28%) were men, presenting a range age of 26–77 years (mean of 55 years).

The genotype 1b was most frequent in the HCV patients (32/70). The genotype 3a (5/70) was found only in female patients, and the median viral load was higher in plasma (6.88 log_{10}).

Genotype 1b (32/70) and genotype 1a (5/70) also showed high viral loads in the plasma, with medians of 5.14 log_{10} and 5.30 log_{10}, respectively. Comparing the viral load in plasma vs. saliva, there was a negative correlation; four female patients (r^2 = 10% of female patients) who presented an increased viral load in the plasma also showed a decreased viral load in the saliva (r = −0.3172, CI_{95%} −0.6240 to −0.03736, p = 0.0491, Fig. 2a). Male patients showed no statistically significant correlation (r = −0.0739, CI_{95%} −0.2794 to 0.1896, p = 0.6926, Fig. 2b).

There was no difference in the viral load between men and women, according to age, race, and virus subtype (Table 1). However, in relation black ethnicity, there was a negative correlation (r = −0.3141; IC_{95%} −0.6069 to −0.05926; p = 0.0209), as 13.2% (r^2) of black patients had a high viral load in the saliva, and simultaneously, a low viral load in the plasma (Fig. 3).

Discussion

It is estimated that 3% of the world population are infected with HCV and at risk of developing disease complications. According to WHO, Brazil is considered a country of intermediate endemicity for hepatitis C, ranging from 1% to 2% [4].
Detection of HCV-RNA by real-time qPCR was investigated in a greater sample size in this study than in others [2,10,11,14–17]. The detection frequency of HCV-RNA in serum was 92.85% of the positive samples compared with 82% in another study [15]. The percentages presented by other authors are as follows: 20.5%, 31%, 42.5%, 52.4%, 52.4%, 62%, and 72% [2,10,11,14–17].

The results of this research showed a higher viral load in plasma (median 4.879 log_{10}) than in saliva samples (median 3.32 log_{10}). The results were similar to a study by Menezes et al. [6] with HCV-RNA levels of 5.78 log_{10} copies in serum and 3.32 log_{10} copies in saliva.

Three studies, in particular, showed that the HCV-RNA load in serum is higher than in saliva from patients with chronic hepatitis C infections. In the first study, the mean was 2.52 \times 10^7 copies/mL in serum and 1.15 \times 10^6 copies/mL in saliva [18]. In the second, the mean HCV-RNA in serum was 5.1 \times 10^5 copies/mL and 1.9 \times 10^4 copies/mL in the saliva [16]. In the third study, the viral load ranged from 1.1 \times 10^4 to 1.9 \times 10^6 IU/mL in the plasma and 1.1 \times 10^3 to 1.3 \times 10^5 IU/mL in the saliva [2]. These results were similar to ours; the plasma viral load was higher (75,699 copies/mL) than the saliva load.

There was a negative correlation between viral load in plasma and viral load in saliva in female patients (r = −0.3172, p = 0.0491), whereas in male patients there was no significant correlation. Our results conflict with some studies that claim to have found no statistical correlation between the presence of HCV-RNA in the saliva and gender [2,14,15]. Hermida et al. [14] found a statistically significant positive correlation between the HCV-RNA load in plasma and HCV-RNA detection in the saliva. Wang et al. [10] reported that HCV-RNA is frequently found in saliva from patients with high serum HCV-RNA loads.

HCV is classified in six major genotypes (designated of 1–6), and several dozen subtypes and strains based on the heterogeneity of the genomic sequence. Genotypes 1, 2, and 3 are distributed worldwide; among them, genotypes 1a and 1b are more prevalent, representing 60% of the infection genotypes [19]. As described in other studies [6,15,19,20], genotype 1b was present in 64% of our cases (32/50 of patients with genotype 1).

However, epidemiological studies suggest that the infectivity of HCV in the saliva is low, but it was not possible to determine the infectious potential. Furthermore, HCV-specific receptors have been defined in buccal epithelial cell pellets, and the role of host defense mechanisms has been determined [21].

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**Figure 2** Correlation of viral load levels in the plasma vs. viral load in the saliva in female patients, n = 39 (a) and viral load in saliva, male patients, n = 31 (b), by the Pearson correlation test.

**Figure 3** Correlation of HCV load levels in the plasma vs. HCV load in the saliva in black patients (n = 40), by the Pearson correlation test.
Several hypotheses are evaluated in scientific circles; blood mononuclear cells infected with HCV may allow the virus to infiltrate the crevicular and saliva fluids [22]. In recent research, the authors concluded that CD3+ cells are the dominant site for extrahepatic replication of HCV, although other subpopulations of peripheral blood can also support virus replication [23]. Saliva containing HCV can penetrate the gingival sulcus [24]; however, saliva may not be the only route for HCV-RNA to gain systemic access in edentulous patients [25]. There may be transudation of serum containing HCV into the mouth. There is also the possibility that HCV exists within the mucosal epithelial cell pellet because HCV has been identified in the mucosal tissue and salivary glands [6,16,20]. This mechanism would explain the detection of different viral genotypes in the serum and saliva of the same patient [25] and patients who are HCV-RNA seronegative but saliva-positive [26].

The use of viral RNA quantification as an evolutionary determinant or a predictor of hepatitis C disease severity is of relative value. The correct interpretation of viral load should be made considering multiple factors, more complex than the simple value obtained in the quantification [27].

Wang et al. [10] demonstrated that the levels of serum and saliva viral loads in HCV-RNA patients change during the course of infection. In the study by Goncalves et al. [15], all patients with detectable HCV-RNA in saliva samples were also positive for HCV-RNA in the plasma. However, some authors reported that they were able to detect HCV in the saliva, but failed to detect it in the blood, as occurred in 5 patients in the studied samples [25,28–30].

We observed a higher viral load in plasma than in saliva. Some patients presented a high viral load in the saliva and low viral load in the serum, suggesting a possible transmission route of the HCV. This information is a relevant knowledge to health professionals, such as dentists with a high exposure risk. This study has useful biological material for use in epidemiological studies; however, further cohort studies are warranted to monitor the plasma from patients showing variable viral load and identify receptors that favor the presence of HCV in the oral mucosa.

**Funding**

No funding sources.
Conflict of interest

None declared.

Ethical approval

This research has the approval of the Ethics Committee of Research (Centro de Ciências da Saúde/UFPE), CAAE: 1014913.7.0000.5208.

Acknowledgment

The authors gratefully acknowledge the Hospital das Clínicas of Federal University of Pernambuco, Brazil and the financial support of CAPES.

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