Detection of Hepatitis C Virus RNA in Saliva Samples from Patients with Seric Anti-HCV Antibodies

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We examined the frequency of HCV-RNA in saliva samples from anti-HCV positive patients. Both plasma and saliva samples from 39 HCV patients (13 with normal liver enzymes, 19 with abnormal liver enzymes and 13 with cirrhosis) were investigated. Stimulated saliva and fresh plasma were centrifuged (900 x g,10 min) and stored at –70°C, after the addition of guanidine isothiocyanate RNA extraction buffer. HCV-RNA was detected by RT- nested-PCR (amplification of HCV-cDNA for two rounds, using HCV primers 939/209 and 940/211). HCV genotyping was carried out by RFLP (using Mva I and Hinf 1 or Hae III and Rsa I restriction enzymes). Thirty-two out of 39 (82%; 95% CI=70-94%) anti-HCV-positive patients had HCV-RNA in plasma samples. Eight out of 39 (20.5%; 95% CI=6.6-34.4%) had HCV-RNA in the saliva. The HCV genotype in saliva samples from these patients matched the genotype found for plasma HCV-RNA. No significant correlation between the presence of HCV and either age, gender, HCV genotype or any risk factor for HCV infection was found. The observed prevalence (20.5% of anti HCV positive patients or 25% of the patients with HCV-RNA in plasma) was lower than that previously reported from other countries. The low frequency of HCV-RNA in saliva samples observed in our study may be due to the use of cell-free saliva. Other authors reporting higher frequencies of HCV-RNA in saliva used whole saliva, without centrifugation.

Key Words: HCV-RNA in saliva, hepatitis C, saliva.

Significant variability in HVC-RNA detection frequency in saliva samples was found in a careful review of the HCV literature from 1990 to 2003. Data from 38 published papers [1-38] indicated a frequency of HCV-RNA in saliva samples from 0 to 100% (Tables 1 and 2). Five out of 38 publications reported100% HCV-RNA detection in saliva samples from HCV-positive patients [16,17,25,29,38]; however, only a few cases or only patients with sialoadenitis were considered in these studies, which may represent a caveat. When reports with sample sizes larger than 30 HCV-positive patients were considered separately, the broad variability of frequencies was evident, ranging from 0 to 66% [1-16]. This variation in HCV-RNA detection frequencies in saliva samples may be due to the saliva sampling method used. In one study, oropharyngeal washes were used as the sampling method, giving 20% HCV RNA positivity [4]; whereas in another publication, oral sponges (Salivette and Omnisal) were used, but the authors were unable to detect HCV RNA in the saliva of HCV and HIV coinfected patients [11]. On the other hand, when whole saliva samples from anti-HCV positive patients were used, HCV RNA was detected in 35% to 52% of the samples (Tables 1 and 2).

Infection with HCV has been implicated in sialodenitis with sicca syndrome [39]. Considering that the presence of HCV virus in saliva samples may be indirect evidence of salivary gland involvement, it would be useful to determine the frequency of HCV-RNA in

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Table 1. Frequency of HCV RNA in saliva from HCV+ patients (samples with more than 30 patients) in studies published from 1992 through 2002

Author and reference	N	Clinical data	Type of saliva	Blood in saliva	HCV-RNA	
					Plasma N(%)	Saliva N(%)
Liou et al. [1]	31	CLD	Whole	21%(+)	31 (100)	15 (48.3)
Young et al. [2]	50	CLD	Whole	ND	41 (82)	25 (50)
Couzigou et al. [3]	37	CH	NI	(-)	NR	23 (62)
Puchhammer-Stockl et al. [4]	35	Malignancies HCV+	Oral wash	ND	18 (51.4)	7 (20)
Komiyama et al. [5]	32	CH, LC, HCC	NI	24%(+)	NR	21 (66)
Sugimura et al. [6]	76	CH (aged)	Whole	20%(+)	24 (44)	27 (36)
Roy et al. [7]	50	CH , i.v. DA ,27 HIV+	Whole, salivette	ND	33 (66)	19 (57)
Roy et al. [8]	50	CH , i.v. DA ,27 HIV+	Whole, sponges	(-)	33 (66)	25 (50)
Fabris et al. [9]	48	CH	Whole	ND	39 (81.2)	22 (56.4)
Rey et al. [10]	59	CH, HIV (+)	Whole	ND	45 (76.3)	22 (37)
vanDoornum et al. [11]	102	CH,HIV (+)	sponges	ND	76 (75)	0 (0)
Mastromatteo et al. [12]	NI	CH and relatives	NI	ND	ND	44%
Matiac et al. [13]	48	CH	Whole	(+)	48 (100)	17 (35)
Savoldi et al. [14]	32	Dentistry clinic	NI	ND	18 (56)	20 (62.5)
Hermida et al. [15]	61	CH	NI	ND	61 (100)	32 (52)

CLD= chronic liver disease; CH=chronic hepatitis; LC= liver cirrhosis; HCC = hepatocellular carcinoma; i.v. DA=intravenous drug abusers; NI = not informed; ND= not determined.

saliva samples from HCV-infected patients. Since geographic variations, changes in the predominant genotypes, and their association with environmental factors, may affect extrahepatic manifestations of HCV infection, such as sialodenitis [40], we decided to investigate the frequency of HCV RNA in saliva samples from anti-HCV positive patients who had no evident clinical features of sialoadenitis, in Vitoria, ES, Brazil.

Material and Methods

Patients

Thirty-nine anti-HCV positive patients (13 with normal liver enzymes, 19 with abnormal liver enzymes and 13 with cirrhosis), attended at the Gastroenterology Outpatient Unit of the Hospital Universitario Cassiano Antonio Moraes, in Vitória, Espírito Santo, Brasil, were enrolled. All the patients received a complete clinical and physical examination, and laboratory tests were performed to evaluate liver function and to detect HBV infection (HbsAg, anti-HBc, anti-HBs). Liver biopsy, ultrassonography and upper gastrointestinal endoscopy were performed, when necessary, to confirm diagnosis of advanced liver disease.

Sampling of plasma and saliva for HCV RNA detection

Fresh plasma samples, processed within two hours of collection by venous puncture, were stored at –70ºC, after addition of guanidine isothiocyanate RNA

Table 2. Frequency of HCV RNA in saliva from HCV patients (samples smaller than 30 patients) reported from1990 through 2002

Author and reference	N	Clinical data	Type of saliva	Blood in saliva	HCV-RNA	
					Plasma N(%)	Saliva N(%)
Takamatsu et al. [16]	5	CH, LC, HCC	Whole,S	ND	ND	5 (100)
Wang et al. [17]	3	CH	NI	(-)	3 (100)	3 (100)
Komiyama et al. [18]	13	CH	NI	ND	13 (100)	2 (15.3)
Hsu et al. [19]	19	CH	Whole	(-)	19 (100)	0 (0)
Nakano et al. [20]	10	CH	NI	ND	10 (100)	6 (60)
Fried et al. [21]	14	CH	Whole	(-)	14 (100)	0 (0)
Wang et al. [22]	14	CH	NI	+	10 (71.4)	7 (50)
Numata et al. [23]	23	CH	NI	(-)	23 (100)	8 (34.8)
Ogasawara et al. [24]	10	Pregnant HCV(+)	NI	ND	10 (100)	5 (50)
Harle et al. [25]*	9	-	-	-	0 (0)	9 (100)
Chen et al. [26]	26	CH (11 HIV+)	Whole	ND	23 (88)	4/23 (17)
Mariette et al. [27]	28	CH (13 HIV+)	Whole	ND	28 (100)	17 (61)
Roy et al. [28]	14	CH	Whole; salivette	ND	14 (100)	9 (64)
Biasi et al. [29]	1	CH; sialoadenitis	Whole	ND	0 (0)	1 (100)
Roy et al. [30]	21	Hemophilic (6HIV+)	Whole; salivette	ND	21 (100)	10 (47)
Jorjensen et al. [31]	16	CH and SS	NI	ND	15 (93)	13 (83)
Caldwell et al. [32]*	21	CH	-	-	21 (100)	5 (21)
Ustundag et al. [33]	10	CH, hemodialysis	NI	ND	10 (100)	3 (30)
Taliani et al. [34]	20	CH,LC	Whole	(-)	20 (100)	3 (15)
Kage et al. [35]*	11	CH (pregnant)	-	-	11 (100)	4 (36)
Becheur et al. [36]*	15	CH	NI	-	15 (100)	4 (26.6)
Nagao et al. [37]*	6	CH	-	-	6 (100)	3 (50)
Arrieta et al. [38]	4	CH, sialoadenitis	NI	ND	4 (100)	4 (100)

CH= chronic hepatitis; LC= liver cirrhosis; HCC=hepatocellular carcinoma; SS = Sjögren syndrome; NI= not informed; ND= not determined. *Data obtained from abstract.

extraction buffer. Production of whole saliva was stimulated by chewing a sterile rubber cylinder for two minutes. Patients were asked to spit into a funnel placed over a sterile 15-mL Falcon centrifuge tube until a 2-3 mL sample of whole saliva was obtained. After centrifugation of the whole saliva samples (900 x g, 10 min), 1mL of supernatant was collected, added to 1mL of extraction buffer, homogenized, and stored at –70°C. The elapsed time between sample collection and cryopreservation was always less than two hours.

RNA Extraction and Nested RT-PCR for the Identification of HCV RNA

RNA extraction was performed using a commercial viral RNA isolation kit (Qiaamp, Qiagem, Hildem,

Table 3. Gender, age, clinical presentation, main risk factors for HCV infection, results of investigation and genotyping of HCV-RNA in serum and saliva of 39 anti-HCV positive patients diagnosed in Vitoria, ES, Brazil

Variables	HCV-RNA in plasma			HCV-RNA in saliva		
	Positive N=32	Negative N=7	P	Positive N=8	Negative N=31	p
Age (years, mean ± sd)	45.7±11.3	49.4±20.2	0.0456	46.2±12.6	45.9±12.5	0.534
Gender						
Male	23	4		6	21	
Female	9	3	0.364	2	10	0.526
Clinical presentation						
Asymptomatic carrier	3	2	0.213	6	14	0.133
Chronic hepatitis	19	1	0.038	0	5	0.295
Liver cirrhosis	10	4	0.193	2	12	0.388
Risk factors						
Blood transfusion	11	3	0.493	3	10	0.296
i.v drug abuse	11	0	0.159	3	8	0.400
Inhaled cocaine	1	0	0.820	0	1	0.797
Surgery	3	1	0.562	0	4	0.382
Tattoo	1	1	0.330	0	2	0.627
Multiple sexual partners	1	0	0.820	0	1	0.794
HCV+ sexual partner	1	0	0.820	0	1	0.794
Undetected	3	2	0.213	2	4	0.357
HCV genotypes						
Genotype 1	22	na	5	na		
Genotype 2	1	na	0	na		
Genotype 3	4	na	2	na		
Undetermined	5	na	1	na		

na = not applicable.

Germany) following manufacturer's instructions. A nested RT-PCR was carried out, as described by Chan et al. [41], with minor modifications introduced by Oliveira et al. [42]. Briefly, single-strand cDNA was synthesized from 18 µL of the RNA sample at 42°C for 50 minutes with 200 U of reverse transcriptase (Superscript II, GIBCO, BRL, Rockville, MD, USA) in 30 µL of manufacturer-supplied buffer containing 10 pmole of primer 209 (ATACTCGAGGTGCACGGGTCTACGAGACCT), 10 mM of each dNTP, and 10nM of dithiothreitol. For the first-round PCR, 2µL of cDNA was added to a mixture containing 2 µL of 10x supplied PCR buffer, 5mM $MgCl_2$, 10 mM of each dNTP, 10 pmole of primers 939 (CTGTGAGGAACTACTGTCTT) and 209, and 5U of Taq DNA polymerase (GIBCO/BRL). The mixture was covered with 25 µL of mineral oil (Sigma, St Louis, MO, USA) and cycled 30x in a thermocycler (Perkin Elmer Gene Amp PCR System 2400) at 95°C for 5 minutes, 95°C for 1 minute, 50° C for 1 minute, 72°C for 1 minute, and 1x 72° C for 1 minute. The second round PCR was carried out as above, but with primers 940 (TTCACGCAGAAAGGGTCTAG) and 211

(CACTCTCGGAGCACCCTATCAGGCAGT) and 1.5U of Taq polymerase. The PCR products were electrophoresed in a 6% polyacrylamide gel, stained with ethidium bromide, and recorded on a digital imager (Eagle Eye II, Stratagene, LaJolla, CA).

RFLP analysis

Nested RT-PCR products were digested at 37°C overnight in 15μL of the supplied buffer containing 5μL of PCR products and 0.5μL of each enzyme: Mva I and Hinf I (MH), or Hae III and Rsa I (HR) purchased from Boehringer Mannheim, Germany. The digestion products were separated by electrophoresis in a 12% polyacrylamide gel using a vertical electrophoresis apparatus (Model S2 Life Technologies Inc.) and stained with silver nitrate. The banding pattern was visualized and analyzed as described by McOmish et al. [43].

Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 10.0) and P values lower than 0.05 were considered significant. The Student's t test was used to compare continuous variables and Chi-square or Fisher's exact tests were used to compare proportions.

Results

Patient population descriptive data on age, gender, clinical presentation, presence of HCV-RNA and its genotypes in both serum and saliva samples were recorded (Table 3). HBV markers were detected in 13 (33%) patients (nine were anti-HBc and anti-HBs positive, four were only anti-HBc positive and none had HBsAg). HCV-RNA was detected in plasma samples from 32 out of 39 patients (82.5%). When HCV genotyping was performed using the RNA isolated from plasma samples, genotype 1 was the most prevalent, found in 22 out of 32 (69%) patients, genotype 3 in four out of 32 (12.5%) and genotype 2 in only one patient (3.1%). The genotyping method used was not able to determine the HCV genotype of five patients.

When saliva samples were used, HCV-RNA was detected in saliva samples from 8 out of 32 (25%) patients with detectable plasmatic HCV-RNA. The genotype found in the saliva samples was the same as that observed in the plasma samples. HCV-RNA was not detected in saliva samples from seven patients who were also negative for viral RNA in the serum. No correlation was found between the presence of HCV-RNA in the saliva, and gender, risk factors or clinical presentation of HCV infection (Table 3).

Discussion

In our study, the HCV-RNA frequency in plasma samples from anti-HCV-positive patients (82.5%) was similar to that reported in other studies in Brazil [42, 44]. The predominance of genotype 1 (68.75% of all HCV-RNA positive cases) also concurs with published data from other studies conducted in Brazil [42,44-46].

Although the frequency of HCV-RNA that we found in saliva samples was 25%, lower than that reported by other authors who analyzed samples larger than 30 patients (Table 1), we could not find an explanation for the discrepancies in the observed HCV-RNA frequency in saliva samples from anti-HCV positive patients. It is possible that the sampling, centrifugation and freezing methods used, as well as the presence of blood may have contributed to these differences. Though HCV-RNA has been found in leukocytes [47] and in oral epithelial cells [29,38,47] from HCV patients, the saliva samples in our study were free of such cells. Blood in the saliva samples may contribute to these discrepant results. We did not perform specific tests to investigate the possibility of occult blood in the saliva samples, Liou et al. [1] and Komiyama et al. [5] demonstrated that the presence of blood in saliva samples did not correlate with the presence of HCV-RNA in the same samples.

In our study, all patients with detectable HCV-RNA in saliva samples were also positive for plasmatic HCV-

RNA. However, some authors have reported that they were able to detect HCV in saliva samples from patients, but could not detect HCV in their blood [6,8, 12,14,25]. A possible explanation for such findings is the fact that HCV can replicate in oral epithelial cells in patients without HCV in the blood, as demonstrated by Arrieta et al. [49] in HCV positive patients with and without oral lichen planus.

We also observed total concordance between the HCV genotype detected in serum and saliva samples from the same patient. Discrepancy between blood and saliva HCV genotypes was reported by Roy et al. [7], in drug abusers; he found that genotype 2 was more prevalent in saliva samples from these patients.

The various studies that have compared viral load with the presence of HCV-RNA in the saliva have shown both positive [9,15,23,27] and negative correlations [10,28]. We did not evaluate this association.

The frequency (25%) of HCV-RNA that we found in saliva samples was lower than that reported from other countries, probably due to the fact that we used cell-free saliva samples.

Since it has been demonstrated that HCV can replicate in oral epithelial cells in patients without manifestation of oral diseases [48,49], a follow-up study is being carried out to investigate the prevalence of HCV-RNA in the cell pellet and in the supernatant obtained after centrifugation of whole saliva from HCV-positive patients.

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