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DETECTION OF HEPATITIS C VIRUS-RNA IN SALIVA FROM CHRONICALLY HCV-INFECTED PATIENTS

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The possibility of the non-parenteral Hepatitis C Virus (HCV) transmission is supported by the demonstration that the actual virus is present in several body fluids, including saliva. From a review of the literature many investigators have found the presence of HCV-RNA in saliva, however, widely contrasting results emerge, with detection rates ranging from 0-100%. To further examine HCV salivary shedding, saliva samples were collected from 46 chronically HCV-infected patients and tested for HCV-RNA and occult blood. Quantification and genotyping of serum HCV-RNA were also carried out for each patient. HCV-RNA was detected in 39.13% of the saliva samples. The viral salivary shedding was significantly related to viraemia levels, serum viral genotype and the presence of salivary occult blood. Our findings indicate that the HCV salivary shedding occurs in about one third of HCV-infected patients, but seem to suggest that it is unlikely when the serum viral genotype is 3a. Moreover, blood leakage into the oral cavity is possibly the main source of the salivary HCV-RNA. Although the occurrence of the viral salivary shedding does not necessarily mean that HCV trasmission occurs by saliva, our results suggest the need for further investigations into the biological factors possibly involved in HCV mucosal transmission related to both the source and the exposed subjects.

According to WHO estimates, more than 170 milion people (i.e. approximately 3% of the world population) are chronically infected with the Hepatitis C Virus (HCV), which results in > 100,000 cases of liver cancer and > 280,000 deaths each year (1). HCV is mainly transmitted through parenteral exposure to infected blood, although non-parenteral transmission is also acknowledged, since many HCV-infected patients relate no recognizable parenteral risk factor (2). It is thought that the virus could also be transmitted by sexual contact, even if the risk of infection across this route is considered lower compared to other sexually transmitted viruses, such as Hepatitis B Virus (HBV) and Human Immunodeficiency Virus (HIV) (3). Mother-to-child transmission may also occur, but infrequently (4). The possibility of the non-parenteral

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HCV transmission is supported by the demonstration of the virus in several body fluids: seminal fluid, vaginal fluids, urine, breast milk, ascites, sweat and saliva (5). The possibility of HCV transmission by saliva was first speculated by Dusheiko et al., who reported the case of a patient infected with HCV due to a human bite (6). Afterwards, Abe and Inchaupse demonstrated the presence of HCV-RNA in the serum of a chimpanzee previously inoculated with the saliva of a chronic HCV carrier chimpanzee (7). Other studies investigating the presence of HCV-RNA in saliva among HCV-infected patients, however, provided widely contrasting results, with detection rates ranging from 0% (8-9) to 100% (10-13).

The aim of this study was to further examine the salivary shedding of HCV in relation to the viraemia levels, serum viral genotype and presence of salivary occult blood. A review of previous publications on this issue was also carried out.

MATERIALS AND METHODS

Patient recruitment

Forty-six patients (35 males, 11 females, mean age 46.9 ± 12.2 years, range 23-66 years) were included in our study. All patients had histologically proven chronic liver disease and detectable HCV-RNA serum levels. Exclusion criteria were: positivity to Hepatitis B surface Antigen (HBsAg) and/or HIV test, history of alcohol abuse or exposure to hepatotoxic drugs, concomitant antiviral treatment and presence of macroscopic oral mucosal lesions. Informed consent was obtained from each patient involved in the study.

Specimen collection and processing

Blood and saliva samples were simultaneously collected from each patient. Whole saliva specimens were collected non-invasively and without stimulation by dribbling into a sterile container. Each patient was requested not to eat, drink, brush his teeth or smoke for at least two hours before salivary collection. No saliva sample showed macroscopic blood contamination. Whole saliva was clarified by centrifugation (560 x g, 10 min) within 1 hour of collection, the supernatant was collected and immediately stored at –80°C. By blood centrifugation (560 x g, 10 min) serum was separated from the clot, then collected and immediately frozen at –80°C.

Occult blood detection

Occult blood presence in saliva specimens was assessed by reactive visual test strips (Aution™ Sticks 10EA, Arkray, Kyoto, Japan), with declared hemoglobin detection limit of 0.06 mg/dL.

HCV-RNA detection

Quantification of serum HCV-RNA was performed by a quantitative RT-PCR assay (Cobas Amplicor™ HCV Monitor Test v2.0, Roche Diagnostics, Basel, Switzerland) with a declared detection limit of 600 IU/mL, according to the manufacturer's specifications. For saliva samples, HCV-RNA extraction was performed from 700 µl of each specimen by a fully automated extraction system (Cobas AmpliPrep™, Roche Diagnostics, Basel, Switzerland). Reverse transcription and amplification were carried out by a qualitative RT-PCR assay (Cobas Amplicor™ HCV Test v2.0, Roche Diagnostics, Basel, Switzerland) with a declared detection limit (on serum) of 50 IU/mL. To confirm the detection sensibility on the saliva samples, a quality control was performed by diluting 30 µl of HCV-RNA positive control (Accurun 305 s100, BBI Diagnostics; declared average HCV-RNA concentration of 1.3×10^3 IU/mL) in 750 µl of HCV-RNA-negative saliva specimen, thus obtaining a control specimen with HCV-RNA concentration of approximately 50 IU/mL. When tested, the control sample resulted positive.

Serum HCV genotypes

Serum HCV genotypes were determined by the INNO-LiPA HCV II assay (Innogenetics, Gent, Belgium), which discriminates among HCV genotypes according to the classification system of Simmonds et al. (14).

Statistical analysis

Viral loads were expressed as International Units per milliliter (IU/mL). Moreover, a quantitative assessment of viraemia was performed and scored as follows: low (less than 3×10^5 IU/mL), medium (from 3×10^5 to 7×10^5 IU/mL) and high (more than 7×10^5 IU/mL). Mean serum viral loads from patients providing HCV-RNA positive and negative saliva were compared by using the two-tailed Student's t-test. To compare the chosen parameters between the two groups, the Chi-squared test was applied; if not applicable, the Fisher's exact test was used. Statistical significance was considered for P values equal to or less than 0.05.

RESULTS

Laboratory data and statistical analysis are summarized in Table I. HCV-RNA was detected in 18 out of 46 saliva samples (39.13%). The average serum viral load among all patients was 9.6×10^5

Table I. *Virological data and occult blood presence from patients with HCV-RNA positive and negative saliva.*

	HCV-RNA positive saliva (No = 18)	HCV-RNA negative saliva (No. = 28)	P value
Mean serum load (IU/ml ± SD)	$9.8 \times 10^5 \pm 6.6 \times 10^5$	$10 \times 10^5 \pm 7.9 \times 10^5$	P > 0.05 (Student's t-test)
Serum load*			
low	0	9	P = 0.019 (Chi-squared test)
middle	7	5	
high	11	14	
Genotype			
1a	1	3	P = 0.038 (Chi-squared test)
1b	13	8	
2a/2c	4	8	
3a	0	8	
4c/4d	0	1	
Occult blood			
yes	16 (88.89%)	17 (60.71%)	P = 0.013 (Fisher's exact test)
no	2 (11.11%)	11 (39.29%)	

* *serum load: low: < 3×10^5 IU/ml; middle: 3×10^5 IU/ml – 7×10^5 IU/ml; high: > 7×10^5 IU/ml.*

IU/mL (range 1.4×10^5 – 29.2×10^5; standard deviation [SD] 7.1×10^5). No significant difference (P > 0.05) was found between the average serum viral loads from patients providing HCV-RNA-positive (9.8×10^5 IU/mL; range 3.4×10^5 – 27.9×10^5; SD 6.6×10^5) and negative (10×10^5 IU/mL; range 1.4×10^5 – 29.2×10^5; SD 7.9×10^5) saliva. However, when the viraemia scores were considered, a skewed distribution in HCV salivary shedding was observed. In fact, among the 9 patients with low serum viral load, none had HCV-RNA-positive saliva; on the contrary, the viral genome was detected in 7 out of the 12 patients with middle serum viral load, and in 11 out of the 25 patients with high viraemia (P = 0.019).

The genotype distribution showed a significant correlation related to the occurrence of the virus in saliva (P = 0.038). In fact, out of the 18 HCV-RNA-positive saliva patients, 13 had genotype 1b, 4 had genotype 2a/2c and 1 had genotype 1a. Out of the 28 patients with HCV-RNA-negative saliva, genotypes 1b, 2a/2c and 3a were each found in 8 subjects; 3 patients had genotype 1a, and 1 had genotype 4c/4d. Thirty-three saliva specimens (71.74%) were contaminated with occult blood. Among the 18 saliva samples positive for the HCV genome detection, 16 (88.89%) were positive for the microscopic blood contamination detection, compared to the 60.71% out of the HCV-RNA-negative saliva specimens which showed a positive occult blood test (17 out of 28). Hence, the presence of occult blood in saliva was significantly related to viral salivary shedding (P = 0.013).

DISCUSSION

The necessity to detect HCV transmisson routes other than parenteral exposure to infected blood has led several researchers to assess the presence of HCV-RNA in saliva in order to evaluate the possible role of saliva in the transmission of the virus. As shown in Table II, however, previous studies have reported widely diverging results. Therefore, whereas some authors demonstrated the presence of HCV-RNA in all tested saliva samples (10-13), others failed to detect the viral genome in saliva of any examined HCV-infected patient (8-9). Several factors could account for this extreme variability. First, the patient recruitment criteria used in previous studies were not the same. In our series, all enrolled patients were positive for serum HCV-RNA and had histologically proven chronic liver disease; furthermore, possible bias factors (such as HBV and/or HIV coinfection, alcohol abuse, exposure to hepatotoxic drugs, concomitant antiviral treatment, presence of macroscopic oral mucosal lesions) were excluded for each patient. Also, the collection methods have to be considered. Various techniques were used in previous studies to collect saliva: dribbling, expectoration, washing and specific commercial devices. Besides, while in the majority of the studies saliva samples were collected without stimulation, in some reports patients were requested to chew a paraffin stick before providing saliva. In our study saliva was collected without stimulation by simple dribbling into a sterile container.

In addition, handling and storage protocols could likely affect the detectability of HCV-RNA in saliva, as previously shown by Roy et al. (24). To minimize the risk of alteration of the viral genome stability, we performed centrifugation of the saliva specimens within 1 hour of collection, then the supernatants were immediately stored at –80°C. Furthermore, the subsequent thawing was carried out in an ice bath. Also RNA extraction and RT-PCR methods are obviously of crucial importance. We performed RNA extraction by a fully automated extraction system and RT-PCR by a commercial qualitative assay with a declared detection limit of 50 IU/mL

Table II. *Literature review of the studies investigating the presence of HCV-RNA in saliva.*

Author, year	No. of patients	Diagnosis	anti-HCV Ab positive serum	HCV-RNA positive serum	HCV-RNA positive saliva
Present series	46	CHC	NR	46	18
Diz Dios *et al.*, 2005 (15)	44	HCV-infected before treatment	NR	44	26
Bélec *et al.*, 2003 (16)	28	CHC, cirrhosis	28	28	12
Chernetsova *et al.*, 2003 (17)	38	CHC with Sjogren's syndrome	NR	38	23
Toussirot *et al.*, 2002 (10)	5	HCV-infected with Sjogren's syndrome	NR	5	5
	1	HCV-infected	NR	1	1
Hermida *et al.*, 2002 (18)	61	HCV-infected	NR	61	32
Savoldi *et al.*, 2001 (19)	32	HCV-infected dental patients	32	18	20
Mastromatteo *et al.*, 2001 (20)	45	CHC	45	23	13
	11	relatives, cohabitants	8	7	5
Matičič *et al.*, 2001 (21)	48	CHC	NR	48	17
Rey *et al.*, 2001 (22)	59	HCV/HIV-coinfected	59	45	22
Arrieta *et al.*, 2001 (11)	4	HCV Ab-positive with xerostomia	4	4	4
Nagao *et al.*, 2000 (23)	6	CHC	6	6	3
Roy *et al.*, 1999 (24, 25)	23	IVDUs HCV Ab-positive	23	14	13
	27	IVDUs HCV Ab/HIV Ab-positive	27	19	12
Fabris *et al.*, 1999 (26)	45	CHC	45	39	22
	3	healthy HCV Ab-positive	3	0	0
Üstündağ *et al.*, 1997 (27)	10	HCV/HGBVC-coinfected haemodialysed	NR	10	3
Kage *et al.*, 1997 (28)	11	HCV-infected pregnant women	11	11	4
Taliani *et al.*, 1997 (29)	20	CHC, cirrhosis	NR	20	3
Jorgensen *et al.*, 1996 (30)	12	HCV Ab-positive with sicca syndrome	12	11	10
Caldwell *et al.*, 1996 (31)	21	liver transplantated with recurrent CHC	NR	21	4
	12	CHC	NR	12	0
Roy *et al.*, 1996 (32)	15	HCV-infected haemophiliacs	15	15	8
	6	HCV/HIV-coinfected haemophiliacs	6	6	2
Tang *et al.*, 1996 (33)	16	CHC	16	16	5
Sugimura *et al.*, 1995 (34)	76	HCV Ab-positive	76	24	27
Mariette *et al.*, 1995 (35)	15	HCV-infected	NR	15	10
	13	HCV/HIV-coinfected	NR	13	7
Chen *et al.*, 1995 (36)	15	HCV Ab-positive	15	14	2
	11	HCV Ab/HIV Ab-positive	11	9	2
Komiyama *et al.*, 1995 (37)	32	CHC, cirrhosis, HCC	NR	32	21
Roy *et al.*, 1995 (38)	14	CHC	14	13	9
Numata *et al.*, 1993 (39)	23	CHC, cirrhosis, HCC	23	23	8
Young *et al.*, 1993 (40)	50	CHC	50	41	25
Ogasawara *et al.*, 1993 (41)	10	HCV-infected pregnant women	10	10	5
Couzigou *et al.*, 1993 (42)	37	CHC	37	NR	23
Nakano *et al.*, 1992 (43)	10	CHC	10	10	6
Liou *et al.*, 1992 (44)	36	CHC	36	31	15
Fried *et al.*, 1992 (8)	12	CHC	12	12	0
Wang *et al.*, 1992 (45)	14	CHC, AHC	13	10	7
Hsu *et al.*, 1991 (9)	16	CHC	NR	16	0
Komiyama *et al.*, 1991 (46)	6	HCV-infected not treated	6	NR	2
	7	CHC under treatment	NR	7	0
Abe *et al.*, 1991 (7)	4	HCV-infected chimpanzees	NR	4	2
Wang *et al.*, 1991 (12)	3	CHC	3	3	3
Takamatsu *et al.*, 1990 (13)	5	CHC, cirrhosis, HCC	NR	5	5

AHC = acute hepatitis C; CHC = chronic hepatitis C; HCC = hepatocellular carcinoma; HCV = hepatitis C virus; HCV Ab = anti-HCV antibodies; HGBVC = hepatitis GB virus type C; HIV = human immunodeficiency virus; HIV Ab = anti-HIV antibodies; IVDU = intravenus drug user; NR = not reported.

and with an internal control that monitors the possible inhibition of the PCR. Furthermore, to definitively avoid false-negative results, we used quality control by testing an HCV-RNA-positive control saliva specimen. Finally, the examined saliva fraction has to be taken into consideration. The majority of the studies tested whole saliva, though some researchers tested cell fractions or supernatants. In the present study HCV-RNA detection was performed on supernatants.

In our series, HCV-RNA was detected in 39.13% of the saliva samples. This result is consistent with that obtained by other Authors (16, 21, 28, 39). Hence, we confirm that HCV salivary shedding occurs in a considerable portion of HCV-infected patients. Our findings derived from the examination of supernatants, in contrast to previous studies of which all but two (13, 38) reported very low or non-detectable HCV-RNA (8, 16, 26, 29, 36). This discrepancy may be due to handling and storage factors, since cell-free viral genome is more easily susceptible to stability alterations than cell-associated ones.

The presence of salivary occult blood correlated significantly with the HCV salivary shedding, in agreement with Matičič et al. (21). These findings seem to suggest that blood leakage into the oral cavity is possibly the main source of the salivary HCV-RNA. Consistent with this hypothesis, we found a statistical correlation between the detection of salivary HCV-RNA and the serum viral load scoring. In fact, when viraemia scores were considered, the viral genome was detected in saliva specimens from none of the 9 patients with low serum viral loads. This result is in accordance with those previously obtained by others (21, 26, 29, 39) and could mean that HCV salivary shedding is unlikely to occur when viraemia is low, or occurs at levels lower than the PCR detection limit. HCV-containing peripheral blood mononuclear cells (PBMCs) may also contribute to viral detection in saliva (26, 36, 44, 47). Gingival crevicular fluid could be another source of salivary HCV-RNA (21), while the occurrence of virus replication in salivary gland tissue is still controversial (10, 29, 48). Surprisingly, we found a statistical correlation between the serum viral genotype distribution and the likelihood of virus salivary shedding, while previous studies failed to do so (15, 21, 26). However, the number of subjects enrolled in our study was too low and does not allow to draw any definitive conclusion.

With regard to the risk of HCV infection by saliva, it is valid to point out that the occurrence of viral salivary shedding does not necessarily mean that viral trasmission via saliva occurs. As Fabris et al. remarked, the detectability of HCV-RNA in saliva represents a necessary, but not sufficient, condition for HCV transmission (26). Factors related to both the source (viral load, size of inoculum, biological factors involved in viral mucosal transmission) and the exposed (integrity of skin or mucosae, innate or acquired defence factors) subjects obviously affect the risk of HCV transmission by saliva (49). Recent findings by Bélec et al., who investigated biological factors possibly involved in mucosal transmission of HCV, seem to suggest a poor infectivity of saliva (16).

In summary, we confirm that the HCV salivary shedding occurs in a considerable proportion of HCV-infected patients, mostly in the presence of salivary occult blood and only in saliva samples from patients with middle or high viraemia. Further investigations on the biological factors possibly involved in mucosal transmission of HCV are required for definitive conclusions.

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