

Detection of Hepatitis C Virus (HCV) RNA in Saliva of HCV-Infected Patients by qualitative techniqueHesham H. Radwan¹, Sonya A, El-Gaaly¹, Manal Sabry¹ and Manal Ahmed Shams^{1,2}Department of ¹Internal Medicine and ²Clinical Pathology, Faculty of Medicine, AM Shams University
Hamdyhesham63@yahoo.com

Abstract: The search for hepatitis C virus (HCV) in body fluids other than blood is important when assessing possible non parenteral routes of viral transmission. However, the role of oral fluids in HCV transmission remains controversial. The saliva test maybe a useful alternative in the event of refusal of blood test. These findings have important implications for medical personnel and suggest that epidemiological studies designed to understand the significance of the oral route of transmission of HCV are warranted (**Chen, M., et al., 1995**). **The aim of this study** is to evaluate the possible existence of hepatitis C Virus in the saliva. A cross sectional study was conducted on 40 patients with chronic liver disease who were positive for anti-HCV and serum HCV RNA. Each patient was subjected to a full history, examination, liver functions, quantitative PCR in serum and qualitative saliva PCR for HCV. Here we qualitatively determined HCV RNA in saliva and quantitatively in the blood of antiHCV-positive patients. In this study it was found a significantly high incidence of PCR positivity for HCV RNA in saliva in patients positive for HCV Ab and HCV PCR. The results revealed a highly significant relation between the serum level and the incidence of positive HCV RNA in saliva. Twenty three of HCV positive patients in the blood showed positive PCR in the saliva related to the viral load. It can be concluded from the current study that the presence of HCV-RNA in the saliva provides a possible source of infection. However, our results suggest the need for further investigations.

[Hesham H. Radwan, Sonya A, El-Gaaly, Manal Sabry and Manal Ahmed Shams. **Detection of Hepatitis C Virus (HCV) RNA in Saliva of HCV-Infected Patients by qualitative technique.** *Life Sci J* 2014;11(11):950-955]. (ISSN:1097-8135). <http://www.lifesciencesite.com>. 168

Keywords: Hepatitis C Virus (HCV); RNA; Saliva; Patient; qualitative technique

1. Introduction

Hepatitis C virus (HCV) infection represents a major public health problem in the world today. The infection primarily causes liver disease; however, HCV infection has also been associated with extra hepatic abnormalities, including mixed cryoglobulinemialmalignant lymphoma, Sjogren's syndrome, and oral lichen plans (**Aceti, A., G et al 1992**) and (**Aizaki, H et al., 2004**). Moreover, blood leakage into the Oral cavity is possibly the main source of salivary HCV-RNA (**Pastore et al., Pub Med.**). Lymph tropism of HCV has been observed, and several laboratories have detected the virus in blood mononuclear cells (BMC) (**Muller, H.et al., 1993**) and (**Roque Afonso, et al., 1999**).

Common risk factors for HCV infection include blood transfusion from unscreened donors as well as injection drug use. Although sexual and vertical transmissions have also been reported, there remain a large number of HCV carriers in whom no route of infection has been identified (**Liou TC et al., 1992**).

Epidemiological surveys demonstrate that body fluids other than blood, including saliva, might be potential sources of HCV infection (**Shafique 2009**). Experimental inoculation of saliva obtained from chronic HCV carrier chimpanzees has been reported to transmit hepatitis to recipient animals (**Abe, K., et al., 1991**). Several studies have demonstrated HCV RNA

in the saliva of hepatitis C patients by reverse transcription (RT)-nested PCR. However, the detection rates of viral RNA within saliva have varied widely, and some groups have failed to demonstrate HCV RNA within saliva. However there were two cases of acute hepatitis C occurring after bites from HCV infected individuals have been reported (**Figueiredo et al 1994**). A potential source of HCV RNA within saliva includes gingival crevicular fluid (GCF), which might contain HCV-infected BMC in the setting of periodontal inflammation. Since the efficiency of HCV transmission is likely related to its viral load, it is important to quantitate viral RNA levels within body fluids in order to properly evaluate possible non parenteral routes of HCV infection. Moreover salivary HCV RNA detection was associated with serum HCV RNA load in individuals who were chronically or acutely infected with HCV (**Chia et al 2006**).

Thus, we examined the presence of HCV RNA in the saliva of anti-HCV antibody-positive and serum HCV PCR positive patients using real-time quantitative RT PCR.

2. Material and Methods

Sample collection: Forty HCV positive patients attending Ain shams university hospital. All of the patients were anti-HCV antibody seropositive on the basis of screening using a fourth-generation enzyme immunoassay (**Cinnagen Diagnostics Company**).

Saliva samples were collected before antiviral treatment. Patients spit into a cup to obtain saliva samples. Whole saliva samples (approximately 2 ml) were then transferred into sterile containers. Also, blood samples were taken from venous blood (2 ml). The samples were stored at -20°C immediately. None of the samples were macroscopically observed to contain blood.

Serum samples were also collected for quantitative HCV PCR assessment.

A -HCV Antibody (Hepatitis Virus C fourth generation) detection in serum

This test system is an indirect enzyme immunoassay kit (**Cinnagen company**) for the detection of antibodies fourth generation of hepatitis C Virus (HCV) in human serum for possible new detection.

B - RNA extraction:

Total RNA was extracted from 200 ul of saliva specimens using guanidinium isothiocyanate and phenol in prechloroform isoamylalcohol, precipitated by isopropanol.

Quantitation of HCV RNA

To determine the quantity of HCV RNA, real-time RT-PCR involving single-tube reactions was performed using RT-PCR Core reagents. Briefly, the reaction mixture contained 1. TaqMan EZ buffer, 500 nM concentrations of each primer from the HCV 5' noncoding region (5'-GAG TGT CGT GCA GCC TCC A-3' and 5'-CAC TCG CAA GCA CCC TAT CA-3'), a 200 nM concentration of fluorogenic probe [5'-(6-carboxyfluorescein) CCC GCA AGA CTG CTA GCC GAG TAG TGT TGG (6-carboxytetramethylrhodamine)-31 200 uM concentrations of each deoxynucleoside Liphosphate, 3 mM Mn(OAc), 5 U of *Thermos thermophilis* DNA polymerase, 0.5 U of AmpErase uracil N-glycosylase, and template RNA. The primers and probe were designed on the basis of the conserved sequences among HCV genotypes. The RT step was started with a 1-mM incubation at 50°C, followed by 50 min at 65°C. Thermal cycling conditions were as follows: a pre-cycling period of 5 min at 95°C followed by 50 cycles of denaturation at 94°C for 15 s and annealing at 55°C for 10 s and extension at 69°C for 1 min. All reactions and analyses of the amplification plots were performed on an Applied Biosystems PRISM 7700 sequence detector (PE Applied Biosystems). Standard curves of the assays were obtained by plotting 10-fold serial dilutions of known concentrations of a synthetic HCV genotype 1b transcript. HCV RNA copy numbers of the synthetic transcript were calculated from the quantity and its molecular weight. Using a standard curve, the Sequence Detector software calculated automatically the concentration of RNA copies in the experimental samples. We found that results obtained

from our in-house real-time RTPCR method were well correlated with those from the COBAS AMPLICOR.

Monitor Test, version 2.0 and that 1 HCV RNA copy/ml in our method corresponded to approximately 1 international unit/ml by the above-mentioned commercial assay (data not shown).

PCR amplification of P-globin DNA

Total DNA was extracted from saliva samples using a QIAamp DNA Mini kit (QIAGEN) according to the manufacturer's instructions. To characterize the degree of cell contamination in saliva, isolated DNA was subsequently used as a template to amplify the human 13-globin gene fragment of 268 bp with the following primers: 5'-GAA GAG CCA AGG ACA GGT AC-3' and 5'-CAA CTT CAT CCA CGT TCA CC-3' (McElhinney, L. M. et al., 1995).

Statistical analysis

The Mann Whitney test was used for evaluating the saliva PCR positivity relation to the viral load in the blood.

3. Results

The clinical and virological characteristics of 40 patients are presented in Table 1. The study group consisted of 22 males (38%) and 18 females (62%) with a mean age of 50 years (range 45 to 61 years). Their average liver enzyme values were as follows: 65 IU/liter for alanine aminotransferase (ALT) and 66 IU/liter for aspartic aminotransferase (AST). HCV RNA levels in the serum of 31 out of 40 patients (77%) were determined by real-time RT-PCR assay, which showed a detection limit of 10² copies/ml and a linear range over 5 logs.

A mean serum HCV RNA level of 5.1 10⁵ copies/ml was observed among samples with viral loads greater than 10² copies/ml. In a number of cases (32 of 40; 80%), HCV RNA was detected in 31% of the saliva samples using real-time RT-PCR.

Significant association was observed between viral RNA levels in the serum and viral RNA positivity in the saliva. However, relatively high serum viral loads (>10⁵ copies/ml) were observed in twenty three out of forty patients with HCV RNA-positive saliva, while serum viral loads were 1.5 × 10³ copies/ml or less in most of the patients whose saliva specimens were negative. Although no visible contamination of the saliva with blood was observed by naked eye, there may be a small amount of cells or lysed cells in the fluids.

Table (2) shows a significant difference between negative values and positive saliva PCR Positivity of saliva for PCR had a higher blood PCR for HCV-RNA.

4. Discussion

Identification of HCV in body fluids other than blood is important in order to evaluate possible non parenteral routes of transmission. The role of oral fluids in HCV transmission remains controversial.

Although the presence of HCV RNA in saliva has been reported by several research groups (M. Martin et al., 1991), (Chen, et al., 1995) and (Sosa-Jurado et al 2014) only one study has attempted to quantify HCV RNA in saliva, in which patients confected with HCV and human immunodeficiency virus were examined

using a branched DNA assay (Rey, D. et al., 2001) and (Eirea et al, 2005). In addition there are several investigators stated that the saliva provides a possible source of hepatitis C virus infection (Couzigou, et al 1993).

Table 1 Clinical and virological characteristics of 40 patients examined in this study

Age	AST Iu/L	ALT Iu/L	Serum quantitative PCR	Saliva qualitative PCR
152	68	47	112,000	Positive
247	103	57	1,200,000	Positive
333	72	33	98,000	Positive
442	124	71	12,000	Negative
536	103	115	36,000	Negative
651	43	53	198,000	Positive
748	18	29	1200	Negative
836	55	45	212,000	Positive
950	48	132	2,600,000	Positive
10_34	24	45	33,000	Negative
11_44	24	32	1300	Negative
12_51	64	58	618,000	Positive
13_48	105	120	218,000	Negative
14_46	68	55	1,100,000	Positive
15_42	44	58	218,000	Negative
16_37	42	45	620,000	Positive
17_32	98	113	213,000	Positive
18_55	47	45	97,000	Positive
19_49	60	65	98,000	Negative
20_28	102	113	42,000	Positive
21_51	42	44	117,000	Positive
22_44	54	62	16,000	Negative
23_52	84	52	225,000	Positive
24_52	90	56	88,000	Negative
25_43	94	112	18,000	Negative
26_46	64	84	38,000	Negative
27_38	54	63	1,200,000	Positive
28_39	74	64	217,000	Negative
29_28	40	45	44,000	Positive
30_29	24	26	14000	Negative
31_41	54	42	118,000	Positive
32_27	32	24	86,000	Negative
33_55	105	102	100,000	Positive
34_48	68	75	2,700,000	Positive
35_47	43	38	14,000	Negative
36_52	112	124	1,200,000	Positive
37_43	64	62	227,000	Positive
38_35	45	43	216,000	Positive
39_47	58	56	198,000	Positive
40_37	146	118	196,000	Positive

Table 2. Saliva PCR results among the studied group.

Saliva PCR	Mean serum	+SD	Z	P
Negative	41742	36096	2.4	<0.05
Positive	496692	70429		

The viral RNA levels in the saliva had no correlation with age, gender, or serum levels of ALT or AST. This was also in agreement with **Rey and Colleagues (2001)** who found no correlation between the positivity of saliva with age and gender in their studied groups. Moreover these were in agreement with the finding that HCV-RNA positivity was unrelated to genotype, duration of disease, hepatitis activity index scores or transaminase levels (**Fabris P, et al., 1999**).

To search for a possible oral route of HCV transmission, whole saliva containing cell fractions (without centrifugation) were used to determine the viral loads in this study.

Goncalves and colleagues in 2005 observed low frequency in saliva samples may be due to the use of cell free saliva, and reported the use of whole saliva without centrifugation for better detection. Although many saliva samples tested were not macroscopically observed to contain blood, cannot rule out the possible effect of a small amount of bleeding as a source of HCV RNA. **Pastore and colleagues (2006)** stated that blood leakage into the oral cavity is possibly the main source of salivary HCV RNA. These were in agreement with **Fabris P and colleagues (1999)** who found HCVRNA in the cell fraction of saliva in highly viremic patients with chronic hepatitis, but its presence does not seem to be associated with increased transmission among sexual partners. Also the detection of HCV in saliva does not correlate with salivary flow or xerostomia in patients with chronic hepatitis C (**de Mattos et al 2010**).

We further found negative saliva PCR results for HCV RNA in face of positive sera in some patients this may be partially due to the presence of PCR inhibitors in saliva. We also found a significant correlation between the degree of serum viremia as detected by serum quantitative PCR for HCV RNA and the higher incidence of positive saliva samples for HCV RNA by Qualitative PCR. This was in agreement with the finding of a trend that patients with HCV RNA-positive saliva showed higher viral loads in sera than patients with HCV RNA-negative saliva (**Taliani, G., 1997**) and (**PuchhammerStockl, E., et al., 1994**) and also might be explained by the findings of **Menezes et al 2012** who detected that salivary HCV viral load was significantly lower than the viral load in the serum which points at the

possibility of negative PCR in saliva in patients with lower degrees of viraemia. In addition **Hermida et al, 2002** stated that HCV RNA is often present in saliva of HCV infected patients with higher plasma viral load as the only predictable factor. A study done in 1992 found the presence of HCV-RNA in the saliva of patients with chronic hepatitis C and the presence was correlated with the degree of HCV viremia (**Wang et al., 1992**). Moreover there was a study in **2001** by **Rey et al**, who found a correlation between qualitative PCR in serum with saliva results.

Although HCV is a hepatotropic virus, convincing evidence of HCV lymphotropism has been demonstrated in tissue culture (**Kato, N. 1999**). HCV has been widely detected in BMC in patients with chronic HCV infection, and differences in quasi species identification within serum and BMC suggest that viral replication occurs within BMC (**Lerat, H., S 1998**) and (**Young, K. C., et al., 1993**). There also might be transudation of HCV containing serum into the mouth. Generally, periodontal inflammation increases the excretion of BMC-rich GCF (Gingival crevicular fluid). There is also a possibility that HCV exists within mucosal epithelial cells. HCV has been identified in the mucosal tissue, as well as salivary glands, of anti-HCV positive patients with oral lichen planus using various techniques, including in situ hybridization, strand-specific RT-PCR, and immunohistochemistry (**Arrieta, J. J., 2001**). Multiple factors contribute to the infectivity of body fluids, including the presence of intact and infectious viral particles, the viral titer, and the presence of appropriate target cells in the exposed area of the uninfected individual (**Chia et al 2006**). Thus, it is likely that several possible sources discussed above are involved in HCV penetration into the saliva and GCF. Whatever the sources or mechanisms are, the findings obtained provide important implications for medical personnel regarding HCV transmission in health care settings as well as for HCV epidemiology, as it was stated that the origin of the viral infection remains unclear in up to 40% of case (**Takamatsu, k., et al., 1992**).

In this study, although the numbers of specimens were limited, we qualitatively determined HCV RNA in oral fluids from patients, including incidentally some patients with oral diseases, and demonstrated frequent detection of HCV in the saliva. Further large-

scale epidemiological studies employing quantitative real-time RT-PCR assays are required to clarify the clinical significance of HCV in the saliva, including the potential for viral transmission through exposure to these fluids may be through experimental animal inoculation.

References

1. Abe, .K., and G. Inchauspe.(1991) Transmission of hepatitis C by saliva. *Lancet* 337:248, 1991.
2. Aceti, A., G. Taliani, M. Sorice, and M. A. Amendolea. (1992). HCV and Sjogren's syndrome. *Lancet* 339:1425-1426.1992.
3. Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.(2004) Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 324:450-461, 2004.
4. Aizaki, H., A. Saito, I. Kusakawa, Y. Ashiura, S. Nagamori, G. Toda, T. Suzuki, K. Ishii, Y. Matsuura, and T. Miyamura. (1996). Mother-to-child transmission of a hepatitis C virus variant with an insertional mutation in its hypervariable region. *J. Hepatol.* 25: 608-613.
5. Arrieta, J. J., E. Rodriguez-Inigo, N. Ortiz-Movilla, J. Bartolome, M. Pardo, F. Manzarbeitia, H. Oliva, D. M. Macias, and V. Carreno.(2001) In situ detection of hepatitis C virus RNA in salivary glands. *Am. J. Pathol.* 158:259- 264, 2001.
6. Chen, M., Z. B. Yun, M. Sallberg, R. Schvarcz, I. Bergquist, H. B. Berglund, and A. Sonnerborg(1995). Detection of hepatitis C virus RNA in the cell fraction of saliva before and after oral surgery. *J. Med. Virol.* 45:223-226, 1995.
7. Chia C. Wang, Chihiro Morishima, Minjun Chung, Rebecca Engelberg, Elizabeth Krantz, Meighan Krows, Daniel G. Sullivan, David R. Gretch, and Lawrence Corey(2006). High Serum Hepatitis C Virus (HCV) RNA Load Predicts the Presence of HCV RNA in Saliva from Individuals with Chronic and Acute HCV Infection. *The Journal of Infectious Diseases* 2006; 193:672-6.
8. Couzigou, P., L. Richard, F. Dumas, L. Schouler, and H. Fleury. (1993). Detection of HCV-RNA in saliva of patients with chronic hepatitis C. *Gut* 34:S59-S60, 1993.
9. de Mattos Camargo Grossmann S, Teixeira R, de Oliveira GC, do Carmo MA (2010). Detection of HCV RNA in saliva does not correlate with salivary flow or xerostomia in patients with chronic hepatitis C. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* Jun;109(6):851-6. 2010.
10. Eirea M. Dios PD. Hermida M. Rodriguez I. Castro A. Ocampo A (2005) Detection of HCV-RNA in saliva of HI V-H CV coinfectd patients. *AIDS Res Hum Retroviruses.* Dec: 21(12):1011-5, 2005.
11. Fabris, P., D. Infantolino, M. R. Nasin, G. Marchelle, E. Venza, V. Terribile Wiel Marin, P. Benedetti, G. Tositti, V. Manfrin, and F. de Lalla. (1999). High prevalence of HCV-RNA in the saliva cell fraction of patients with chronic hepatitis C but no evidence of HCV transmission among sexual partners. *Infection* 27:86-91, 1999.
12. Figueiredo JF, Borges AS, Martinez R, et al.(1994) Transmission of hepatitis C virus but not human immunodeficiency virus type 1 by a human bite. *Clin Infect Dis* 1994; 19:546-7.
13. Goncalves PL, Cunha CB, Bused SC, Oliveira GC, RibeirO-Rodrigues R, Pereira FE. (2005) Detection of hepatitis C virus RNA in saliva samples from patients with serum Anti-HCV antibodies. *Braz J Infect Dis.* Feb; 9(1) 28-34.2005.
14. Hermida, M., M. C. Ferreira, S. Barra!, R. Laredo, A. Castro, and P. Diz Dios.(2002) Detection of HCV RNA in saliva of patients with hepatitis C virus infection by using a highly sensitive test *J. Virol. Methods* 101:29-35, 2002.
15. M. Martin, Hsu, H. H., T. L. Wright, D. Luba, S. M. Feinstone, G. Garcia, and H. B. Greenberg (1991). Failure to detect hepatitis C virus genome in human secretions with the polymerase chain reaction. *Hepatology* 14:763-767. 1991.
16. Kato, N. Systems to culture hepatitis C virus, p. 261-278. In C. H. Hagedorn and C. M. Rice (ed.), (1999) *The hepatitis C virus.* Springer-Verlag, Berlin, Germany. 1999.
17. Lerat, H., S. Rumin, F. Habersetzer, F. Berby, M. A. Trabaud, C. Trepo, and G. Inchauspe(1998) In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* 91:3841-3849. 1998.
18. Liou, T. C., T. T. Chang, K. C. Young, X. Z. Lin, C. Y. Lin, and H. L. Wu(1992). Detection of HCV RNA in saliva, urine, seminal fluid, and ascites. *J. Med. Virol.* 37:197-202. 1992.
19. McElhinney, L. M., R. J. Cooper, and D. J. Morris. (1995). Multiplex polymerase chain reaction for human herpesvirus-6, human cytomegalovirus, and human beta-globin DNA. *J. Virol. Methods* 53:223-233 ,1995.
20. Menezes GB, Pereira FA, Duarte CA, Carmo TM, Silva Filho HP, Zarife MA, Krieger MA, Reis EA, Reis MG. (2012) Hepatitis C virus quantification in serum and saliva of HCV-

- infected patients Aug;107(5):680-3. Mem Inst Oswaldo Cruz. 2012.
21. Muller, H. M., E. Pfaff, T. Goeser, B. Kallinowski, C. Solbach, and L. Theilmann. (1993) Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. *J. Gen. Virol.* 74:669-676, 1993.
 22. Pastore L. Fiore JR. Tateo M. Benediteis M. Petruzzi M. Casalino C. Genchi C. Lo Muzi L. Angarano G. Serpico R.(2006) Detection of hepatitis C virus -RNA in saliva from chronically HCV- infected patients. *J Med Virol.*63: 117-9,2006.
 23. Puchhammer-Stockl, E., W. Mor, M. Kundi, F. X. Heinz, H. Hofmann, and C. Kunz (1994). Prevalence of hepatitis-C virus RNA in serum and throat washings of children with chronic hepatitis. *J. Med. Virol.* 43:143-147, 1994.
 24. Rey, D., S. Fritsch, C. Schmitt, P. Meyer, J. M. Lang, and F. Stoll-Keller.(2001) Quantitation of hepatitis C virus RNA in saliva and serum of patients coinfectd with HCV and human immunodeficiency virus. *J. Med. Virol.* 63:117-119, 2001.
 25. Roque Afonso, A. M., J. Jiang, F. Penin, C. Tareau, D. Samuel, M. A. Petit, H. Bismuth, E. Dussaix, and C. Feray. Nonrandom (1999) distribution of hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cell subsets. *J. Virol.* 73:9213-9221. 1999.
 26. Sosa-Jurado F, Hernández-Galindo VL, Meléndez-Mena D, Mendoza-Torres MA, Martínez-Arroniz FJ, Vallejo-Ruiz V, Reyes-Leyva J, Santos-López G. (2014) Detection of hepatitis C virus RNA in saliva of patients with active infection not associated with periodontal or liver disease severity. *BMC Infect Dis.* Feb 10;14:72 2014.
 27. Shafique M, Ahmad N, Awan FR, Mustafa T, Ullah M, Qureshi JA. (2009) Investigating the concurrent presence of HCV in serum, oral fluid and urine samples from chronic HCV patients in Faisalabad, Pakistan. *Arch Virol.* ;154(9):1523-7 2009.
 28. Takamatsu, K., I. Okayasu, Y. Koyanagi, and N. Yamamoto (1992). Hepatitis C virus propagates in salivary glands. *J. Infect. Dis.* 165:973-974.1992.
 29. Taliani, G., D. Celestino, M. C. Badolato, A. Pennica, A. Bozza, G. Poliandri, V. Ricciari, G. Benfari, A. Sebastiani, C. De Bac, G. Quaranta, and A. Aceti.(1997) Hepatitis C virus infection of salivary gland epithelial cells. Lack of evidence. *J. Hepatol.* 26:1200-1206, 1997.
 30. Wang, J. T., J. C. Sheu, J. T. Lin, T. H. Wang, and D. S. Chen. (1992). Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. *J. Infect. Dis.* 166:1167-1169, 1992.
 31. Young, K. C., T. T. Chang, T. C. Liou, and H. L. Wu. (1993). Detection of hepatitis C ^yin is RNA in peripheral blood mononuclear cells and in saliva. *.1. Med. Virol.* 41:55-60, 1993.

11/15/2014