Detection of hepatitis C virus RNA in the saliva using real-time PCR with emphasis on oral lichen planus

El-Zarka M. S,[1] El-Nouaem M. I. [1], Metwally D E. [2] and Essawy M. M.[1]

[1] Oral Pathology Department, Faculty of Dentistry, University of Alexandria.
[2] Microbiology Department, Medical Research Institute University of Alexandria.
dr.dalia.ragab@hotmail.com.

Abstract: HCV plays an important role not only in liver diseases but also in the establishment of extrahepatic manifestations and immune abnormalities. Oral lichen planus (OLP) that appears in the oral cavity has been reported as an extrahepatic lesion induced by HCV. HCV RNA has been detected in the saliva of HCV positive patients. If sterilization and disinfection techniques are inadequate, there is an increased risk of HCV transmission to exposed individuals. he current study included a group of 40 HCV RNA positive patients. Paired blood and saliva samples were tested by real time PCR for HCV viral. Dental examination was performed for all patients. HCV RNA was found in 17 out of the 40 saliva specimens (42.5 %), obtained from the patients. No statistical significant relation was found between the detection of HCV RNA in the saliva and the different dental treatments as risk factors. There was no correlation between viral load in the serum and viral load in saliva. Also, there was no statistically significant relationship between serum HCV RNA viral load and the delectability of HCV RNA in the saliva. Three patients out of 40 (7.5%) had OLP.

[El-Zarka M. S, El-Nouaem M. I., Metwally D E. and Essawy M. M. **Detection of hepatitis C virus RNA in the saliva using real-time PCR with emphasis on oral lichen planus.** Journal of American Science 2011;7(4):854-859]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: HCV; saliva; oral lichen planus; real time PCR.

1. Introduction

Hepatitis C virus (HCV) is estimated to have infected 170 million people worldwide [(1)]. The published prevalence rates of HCV infection vary considerably in different countries and even in different parts of one country. Prevalence ranges from as low as 0.3% in Sweden to more than 14% in Egypt. Approximately one-quarter of patients with chronic HCV hepatitis will ultimately develop cirrhosis and a significant proportion will go on to hepatocellular carcinoma (HCC) [(2)].

HCV also plays an important role not only in liver diseases but also in the establishment of extrahepatic manifestations and immune abnormalities [(3)] .A wide variety of extrahepatic disease manifestations have been reported to be associated with HCV infection [(4)] .Infection with HCV has been implicated in sialodenitis with sicca syndrome. The presence of HCV virus in saliva samples may be an indirect evidence of salivary gland involvement. Geographic variations, changes in the predominant genotypes, and their association with environmental factors, may affect extrahepatic manifestations of HCV infection, such as sialodenitis [(5)].

Oral lichen planus (OLP) that appears in the oral cavity has been reported as an extrahepatic lesion induced by HCV [(3)]. Numerous cases of lichen planus in patients with hepatitis C virus (HCV) infection have been published and an association of chronic hepatitis with lichen planus has been established. However, an association between HCV infection and lichen planus is uncertain because the prevalence of HCV infection in patients with lichen planus varies considerably from one geographic area to another, ranging from 4% in northern France to 62% in Japan[(6)].

In developing and transitional economy countries, nosocomial transmission of HCV through the re-use of contaminated or inadequately sterilized instruments used in medical, paramedical and dental procedures remains a major source of HCV infection and puts the public in these areas at high-risk [(2)].

In the most rigorous epidemiological studies on HCV, the prevalence of patients in whom it is not possible to identify any risk factor for acquiring the infection ranges from 10% to 14% [(7)] .Therefore, in order to assess non-parenteral routes of transmission, the detection of HCV in body fluids other than blood is of great importance. Sero-epidemiological surveys indicate that saliva may be a potential source of infection [(1)].

The detection of HCV in saliva can be made indirectly by measuring antibody levels or by amplification of viral RNA. The efficacy of the detection of HCV antibodies in saliva by ELISA has been evaluated in several studies and has good sensitivity of 84.1 – 98.2 % and specificity of 99.1 – 100 % [(8- 12)] .Suggesting that ELISA methods for detection of HCV antibodies in saliva can be used in

epidemiological surveys [12 - 14] .Despite these high values, the low concentration of antibodies in saliva may result in false negative results [9,15].Also, immunocompromised patients fail to generate an immune response to HCV, which result in false negative results [9] . On the other hand, assays for testing salivary anti-HCV are not available commercially at present [12] .

HCV RNA has been detected in the saliva of HCV positive patients. Significant variability in HCV-RNA detection frequency in saliva samples was found in a careful review of the HCV literature from 1990 to 2003. Published data indicated a frequency of HCV RNA in saliva samples from 0 to 100% [5] .

Studies have indicated that there is extensive HCV contamination of dental surgeries after treatment of anti-HCV positive patients. If sterilization and disinfection techniques are inadequate, there is an increased risk of HCV transmission to exposed individuals [1] .

The aim of this study was to determine HCV RNA viral load in saliva from HCV RNA positive patients and correlate it to the serum viral load. The correlation between presence of HCV RNA and oral pathology was also studied.

2. Materials and methods

Study Population

The current study included a group of 40 HCV RNA positive patients; 18 males (45%) and 22 females (55 %) with a mean age of 45.95 + 8.9 years (range 28 – 70 years), attending the outpatient clinics in the Medical Research Institute, and the Maxillofacial Surgery Department, Faculty of Dentistry, Alexandria University over a period of 6 months; from September 2009 to February 2010.

The following information was recorded: age, gender, residence, occupation, history of blood transfusion, surgical operations, family history of HCV, bilharziasis and its treatment, and dental care status. Dental examination was performed for all patients.

Specimen Collection

Paired blood and saliva samples were obtained from each participant. The blood was collected in sterile tubes, and the serum was separated by centrifugation. Non-stimulated saliva samples were collected by spitting into a sterile falcon tube. Saliva was recovered by centrifugation and visually checked for the presence of blood cells.

Both saliva and serum were stored at -20 ^{o}C until tested by real time PCR.

Tissue biopsy was taken from suspected lesions and examined histopathologically by H&E stain.

Detection, and Viral Load of HCV in Serum and Saliva

RNA extraction. Total RNA was extracted from 140 µl of serum and 560 µl of saliva specimens using a QIAamp viral RNA kit (QIAGEN, Valencia, CA) following manufacturer's instructions.

Ten µl of extracted RNA was amplified by using 12.5 µl TaqMan universal PCR master mix 2-fold (AB applied Biosystem), RT-PCR reactions were performed in a final volume of 25µl. Real-time RT-PCR was performed using the TaqMan Gold RT-PCR kit (Applied Biosystems) with the MX3000 system.

Thermal Profile of amplification: 48°C – 30 min (reverse transcriptase step) followed by 95°C – 10 min, 95°C – 15 sec, 60°C – 60 sec for 40 Cycles.

The Student's t-test was used to compare continuous variables and the x^2-test or Fisher's exact test was used to compare proportions. Linear correlation was used to correlate the quantitative results between saliva and serum. A p value of 0.05 or less was considered significant.

3. Results

The 40 patients enrolled in the current study were all serum HCV RNA positive. The viral load in the study group was ranging from 1.1 x 10^4 IU/ml to 1.9 x 10^6 IU/ml. All the patients were HBsAg negative. None of them was on antiviral therapy.

Risk factors for HCV were identified in all patients. Dental treatment was the main risk factor reported (85.0 %), followed by previous surgeries (57.5%), history of parenteral anti-bilharzial treatment (27.5 %), positive HCV family member (22.5 %), and blood transfusion (20.0 %). None of the patients was a habitual alcohol consumer or an intravenous drug user.

HCV RNA was found in 17 out of the 40 saliva specimens (42.5 %), obtained from the patients. No statistical significant relation was found between the detection of HCV RNA in the saliva and age, sex, p values were > 0.05 and the different dental treatments as risk factors, p values were > 0.05. (Table 1).

Regarding the 17 cases of positive HCV RNA in the saliva, the viral load in the saliva was ranging from 1.1 x 10^3 IU/ml to 1.3 x 10^5 IU/ml (with a mean of 3.4 x 10^4 $\pm$ 3.3 x 10^4 IU/ml). There was no correlation between viral load in the serum and viral load in saliva, r = 0.242, p = 0.350,. Also, there was no statistically significant relationship between serum HCV RNA viral load and the delectability of HCV RNA in the saliva, p = 0.213. (Table 2)

Table (1): Relation between detection of HCV RNA in the saliva and different dental treatments.

Variables	HCV-RNA in saliva		*p* value
	Positive N = 17 (42.5 %)	**Negative N = 23 (57.5 %)**	
• ***No dental treatment***	1 (16.7 %)	5 (83.3 %)	
• ***Dental treatment***			
- Extraction	5 (35.7 %)	9 (64.3 %)	0.562
- Scalling	0 (0 %)	1 (100 %)	
- Restoration	2 (66.7 %)	1 (33.3 %)	
- Combined treatment	9 (56.3 %	7 (43.7 %)	

Table (2): Correlation between Serum HCV viral load and the detectability of HCV RNA in saliva

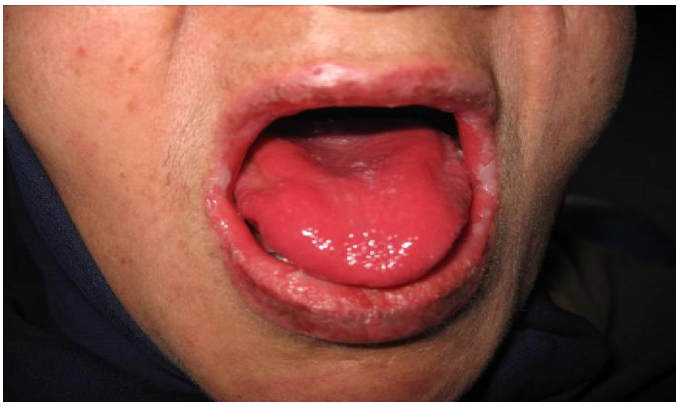
Variable	HCV-RNA in saliva		*p* value
	Positive N = 17	**Negative N = 23**	
• ***Range IU/ml (median)***	**1.1×10^4 - 1.5×10^6 (1.8×10^5)**	**1.6×10^4 - 1.9×10^6 (3.6×10^5)**	**0.213**

Out of the 40 HCV RNA positive patients, 17cases were HCV RNA positive in saliva of them 13 cases (76.5 %) had no oral lesions, 4(23.5%) cases had oral lesions. On the other hand, out of the 23 HCV RNA negative cases, 22 cases (95.6 %) had no oral lesions and one case (4.3 %) had oral lesions. (Table 3).

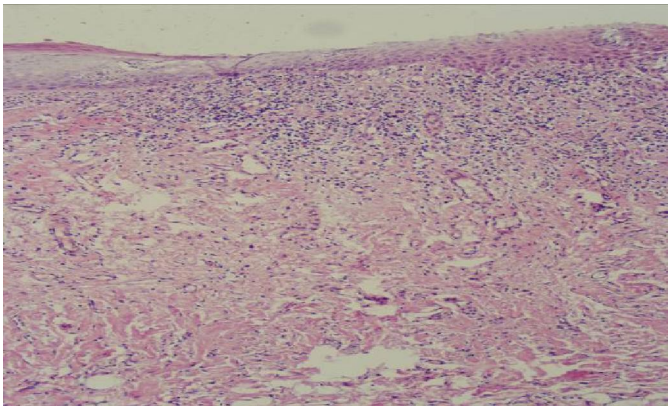
These results showed that there was no statistical significant relation between HCV detection in saliva and the presence of oral lesions, $x^2 = 4.516$, $p = 0.341$.

Table (3): Relation between detection of HCV RNA in the saliva and presence of oral lesions.

Variables	HCV-RNA in saliva		p value
	Positive N = 17	**Negative N = 23**	
• ***No oral lesions***	13 (76.5 %)	**22 (95.6 %)**	**0.341**
• ***Oral lesions***	4 (23.5%)	**1 (4.3 %)**	



Macrograph (1) Showing severe atrophic glossitis and angular chelitis in erosive lichen planus atient.



Micrograph (2) Showing erosive lichen planus showing thin srface epithelium and subepithelial lymphoctic band (H&E x100).

4. Discussion

The potential role of human saliva as a source of HCV infection has raised a 10-years debate, implying that dentists have an increased risk of exposure to HCV [16]. Detection of HCV RNA is indicative of the presence of HCV particles in the saliva, which may establish saliva as a potential vehicle of infection and confirm the possibility of a non-parenteral route of HCV transmission [1].

In the study group, the main risk factor for HCV infection was the dental treatment (85.0%), followed by history of previous surgery (57.5%). history of parenteral anti-bilharzial treatment (27.5 %), positive HCV family member (22.5 %), and blood transfusion (20.0 %). None of the patients was a habitual alcohol consumer or an intravenous drug user. HCV RNA was detected in the saliva of 17 out of them (42.5 %). Studies on chronic HCV patients revealed detection rate ranging from 48 – 65% % that was in agreement with the result of the current study [16-19].

Grossmann *et al*, [20] demonstrated no statistical significance between stimulated and unstimulated methods for HCV RNA detectability in the saliva. In the current study whole unstimulated saliva was collected by spitting technique.

The majority of the studies to detect HCV in saliva used whole saliva to detect HCV RNA [1,16,19,20] .Whereas, information about the presence of HCV RNA in the different fractions of saliva (whole, supernatant and cell fraction) has been provided in few studies [14,17,21].It was reported that there was no significant difference in virus detectability between different saliva fraction. In the current study HCV RNA was detected in the supernatant fraction.

The relation between HCV RNA titers in serum and the presence of HCV RNA in saliva has not been completely clarified; as few reports reported that the best predictive factor for the high levels of HCV in saliva was the high levels of serum HCV RNA [17,19,22] .In the present study no correlation was found between the quantitative level of HCV RNA in serum and the presence of HCV in saliva. This is in agreement with Roy *et al*. [18,21] and Lins *et al* [1] .

In the present study no correlation was found between serum viral load and HCV viral load in saliva. These findings were in agreement with Rey *et al* [16] .On the other hand, they found a correlation between serum viral load and HCV RNA positivity in saliva, such correlation was reported by other workers [17,19,22] .In the current study no correlation was found between serum viral load and HCV RNA positivity in saliva. A possible explanation could be attributable to the large volume 560 µl used for RNA extraction from saliva samples which increased the possibility of HCV RNA detection in the saliva of patients with low serum viral load.

Despite these contradicting results, the most common finding was a lower HCV RNA titer in saliva compared to that in serum [17] .This could designate the source of HCV RNA in the saliva, as there might be continuous transudation of serum into the mouth from the gingival crevice *via* concentration gradient [1] .A finding that was supported by the presence of HCV RNA in the gingival crevicular fluid [23,24] .This theory was supported by the findings that salivary genotypes were the same as that of the blood[1,5] .(transudation from general circulation, or active replication in salivary gland epithelial cells) .However, viral spillover from blood is another confirmed source. HCV RNA was detected in all saliva fractions tested and from homeostatic gauze that was used to stop bleeding in chronic HCV patients after surgery and scalling [14, 25]. The issue of intraglandular replication of HCV remains controversial. Laskus *et al,* [26] found HCV

quasispecies in different tissue samples compatible with independent replication at extrahepatic sites which could include major salivary glands.

The present study did not report significant relation between the presence of oral lesions and the detectability of the virus in the saliva. In contrast, Farghaly *et al*.[27] demonstrated that periodontitis and the bad oral hygiene of the Egyptian population are associated with the detectability of hepatitis markers in the saliva.

The present study investigated the prevalence of OLP in HCV infected patients, and it revealed 3 patients out of 40 (7.5 %) had OLP. The studiesthat investigated the frequency of OLP among HCV positive patients showed that from 0 to 20% of the HCV patients may have OLP [28-31]. This wide variation in the studies results may be attributed to geographic distribution of HCV infection worldwide. It would be expected that OLP prevalence will be high in countries where HCV is endemic.

Studies from Japan reported high OLP prevalence (8.5%) in one of the hyperendemic region of HCV infection [30] .This is in agreement with the current study as Egypt has the highest seroprevalence of HCV worldwide. However, the prevalence repoted in the current study is higher than expected, especially with low OLP prevalence in the general Egyptian population reported by Ibrahim *et al*, [32] 0.169 % in 1999. Other factors, such as sample size, diagnostic criteria of OLP and the age of the examined HCV patients should be taken into consideration.

Epidemiological studies need large sample size to generalize their results on the population. Another relevant issue is that the antiviral therapy of HCV infected patients. It has been suggested that interferon-α may induce or modify the manifestations of OLP [33] .Thus, to exclude possible false positive results, the present study did not enroll patients on antiviral therapy.

The possibility of the saliva to be a potential vehicle for non-parenteral transmission of HCV remains also unclear. Multiple factors contribute to the infectivity of saliva, 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. Epidemiologic data do not support the concept that HCV is efficiently transmitted through saliva. However, toothbrushes have been implicated as a vehicle for HCV transmission.

Corresponding Author:
Medical Research Institute, Alexandria University.
Alexandria, Egypt.
dr.dalia.ragab@hotmail.com.

References

1. Lins L, Almeida H, Vitvisk L, Carmo T, Parana R, and Reis M.(2005): Detection of Hepatitis C virus RNA in saliva is not related to oral health status or viral load. Journal of Medical Virology, 77:216–220.
2. Daniel Lavanchy. (2008):Chronic viral hepatitis as a public health issue in the world. Clinical Gastroenterology, 22(6) 991–1008.
3. Yumiko Nagao, Michio Sata, Seiji Noguchi, Tamiko Seno', Moritoshi Kinoshita, Tadamitsu Kameyama, Takato Ueno. (2000): Detection of hepatitis C virus RNA in oral lichen planus and oral cancer tissues J Oral Pathol Med., 29: 259–66.
4. Vincent Agnello, Francesco G. De Rosa. (2004): Extrahepatic disease manifestations of HCV infection: some current issues. Journal of Hepatology, 40 :341–352.
5. Patrícia L. Gonçalves, Carla B. Cunha, Solange C. U. Busek, Guilherme C. Oliveira, Santo1, Vitória, ES; René Rachou, Rodrigo Ribeiro-Rodrigues and Fausto EL Pereira. (2005): Detection of Hepatitis C Virus RNA in Saliva Samples from Patients with Seric Anti-HCV Antibodies. The Brazilian Journal of Infectious Diseases, 9(1):28-34.
6. Asaad Tonsi, Azam Jah Samdani. (2005): Association of lichen planus with hepatitis C virus infection. Ann Saudi Med.,25(3) 243-246.
7. MC Ferreir1, PD Dios, C Scully. (2005): Transmission of hepatitis C virus by saliva? Oral Diseases. 11: 230–235.
8. Elsana S, Sikuler E, Yaari A *et al.* (1998): HCV antibodies in saliva and urine. J Med Viro., 55: 24 - 27.
9. Bello PY, Pasquier C, Gourney P, Puel J, Izopet J. (1998): Assessment of a hepatitis C virus antibody assay in saliva for epidemiological studies. Eur J Clin Microbiol Infect Dis., 17: 570 - 572.
10. Cameron SO, Wilson KS, Good T *et al.* (1999): Detection of antibodies against hepatitis C virus in saliva: A marker of viral replication. J Viral Hepat., 6: 141 - 144.
11. Judd A, Parry J, Hickman M *et al.* (2003): Evaluation of a modified commercial assay in detecting antibody to hepatitis C virus in oral fluids and dried blood spots. J Med Virol., 71: 49 - 55.
12. De Cock L, Hutse V, Verhaegen E, Quoilin S, Vandenberghe H, Vranckx R. (2004) :Detection of HCV antibodies in oral fluid. J Virol Meth., 122:179 - 183.
13. 10-Coates EA, Brennan D, Logan RM, Goss AN, Scopacasa B, Spencer AJ, Gorkic E. (2000):

Hepatitis C infection and associated oral health problems. Aust Dent J., 45:108–114
14. 11-Chen M, Yun ZB, Sallberg M, Schvarcz R, Bergquist I, Berglund HB, Sonnerborg A. (1995): Detection of hepatitis C virus RNA in the cell fraction of saliva before and after oral surgery. J Med Virol.,45:223–226.
15. De Cock L, Hutse V, Vranckx R. (2005): Correlation between detection of antibodies against hepatitis C virus in oral fluid and hepatitis C virus RNA in serum. Eur J Clin Microbiol Infect Dis., 24: 566 - 568.
16. Rey D, Fritsch S, Schmitt C, Meyer P, Lang JM, Stoll-Keller F. (2001): Quantitation of hepatitis C virus RNA in saliva and serum of patients coinfected with HCV and human immunodeficiency virus. J Med Virol., 63: 117 - 119.
17. Fabris R, Infantolino D, Biasin MR *et al*. (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 (2): 86 - 91.
18. Roy KM, Bagg J, McCarron B, Good T, Cameron S, Pithie A. (1998): Predominance of HCV type 2a in saliva from intravenous drug users. J Med Virol., 54: 271 - 275.
19. Mariette X, Loiseau P, Morinet F. (1995): Hepatitis C virus in saliva (Letter). Ann Intern Med.,122: 556.
20. Grossmann SM, Teixeira R, Oliveira GC, Vieira 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., 109: 851 - 856.
21. Roy KM, Bagg J, Bird GL *et al.* (1995): Serological and salivary markers compared with biochemical markers for monitoring interferon treatment for hepatitis C virus infection. J Med Virol., 47: 429 - 434.
22. Hermida M, Ferreiro MC, Barral S, Laredo R, Castro A, Dios PD. (2002):Detection of HCV RNA in saliva of patients with hepatitis C virus infection by using a highly sensitive test. J Virol Meth., 101: 29-35.
23. Suzuki T, Omata K, Satoh T *et al.* (2005): Quantitative detection of hepatitis C virus (HCV) RNA in saliva and gingival crevicular fluid of HCV-infected patients. J Clin Microbiol., 43 (9): 4413 - 4417.
24. Maticic M, Poljak M, Kramar B *et al*. (2001): Detection of hepatitis C virus RNA from gingival crevicular fluid and its relation to virus presence in saliva. J Periodontol., 72 (1): 11 - 16.
25. Hasegawa H, Yamada T, Esumi M. (2003): Detection of hepatitis C virus antibody and RNA in hemostatic gauze used for dentistry. Infect Control Hosp Epidemiol., 24: 137 - 139.
26. Laskus, T., Radkowski, M., Wang, L.F., Nowicki, M., Rakela, J. (2000): Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication. J. Virol.,74: 1014–1017.
27. Farghaly AG, Mansour GA, Mahdy NH, Yousri A. (1998): Hepatitis B and C virus infections among patients with gingivitis and adult periodontitis: Seroprevalence and public health importance. J Egypt Public Health Assoc., 73 (5 - 6): 707 - 735.
28. Giuliani M, Lajolo C, Miani CM, Lodi G, Minenna P, Mangia A. (2007): Hepatitis C virus chronic infection and oral lichen planus: An Italian case-control study. Eur J Gastroenterol Hepatol., 19: 647 - 652.
29. Mico-Llorens JM, Delgado-Molina E, Baliellas-Comellas C *et al*. (2004): Association between B and ⁄ or C chronic viral hepatitis and oral lichen planus. Med Oral., 9: 183 - 190.
30. Nagao Y, Tanaka J, Nakanishi T *et al*. (2002):High incidence of extrahepatic manifestations in an HCV hyperendemic area. Hepatol Re., 22: 27 - 36.
31. Mignogna M, Fedele S, Lo Russo L, Ruoppo E, Lo Muzio L. (2001): Unexpected low incidence of oral lichen planus in an HCV hyperendemic area of southern Italy. Gastroenterol.,121: 1528 - 1529.
32. Ibrahim HA, Baddour MM, Morsi MG, Abdelkader AA. (1999):Should we routinely check for hepatitis B and C in patients with lichen planus or cutaneous vasculitis? East Mediterr Health J., 5: 71 - 78.
33. Nagao Y, Sata M, Ide T *et al*. Development and exacerbation of oral lichen planus during and after interferon therapy for hepatitis C. (1996): Eur J Clin Invest., 26: 1171 - 1174.

4/2/2011