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Evaluation of Hepatitis C virus (HCV) Genotypes and Prevalence of HCV Associated Sialadenitis in Patients with Chronic HCV

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Abstract: Objective and study design: HCV genotyping in serum and saliva as well as prevalence of the sialadenitis were determined in seventy Egyptian patients chronically infected with HCV. Histopathological examination in addition to HCV genotyping was also carried out on minor labial salivary gland biopsies in 15 of these patients to highlight the problem of reliance on serum alone to identify genotype associated with the increasingly recognized extrahepatic manifestations of HCV infection. Results: HCV genotype 4 was found in the serum of all 70 patients, in the saliva of 53 patients and in tissue samples of 11 patients (out of 15). Two cases were typed as having mixed infection (4+2a) in their saliva and tissue samples while type 4 only was found in their serum. There was a significant association between xerostomia and detection of HCV genotype 4 in saliva. The same association was found in the tissue showing histopathological destruction. Conclusion: Detection of HCV genotype 4 (the most common genotype in Egypt) in the saliva of HCV infected patients. In addition to the presence of HCV mixed genotypes (4+2a) in saliva and minor labial gland tissues, different from type (4) that found only in the serum of the same patient might denote active replication of HCV at the site of salivary secretion. This provides a biological evidence for the infectivity of saliva and this opens the question of the role of HCV replication in extra-hepatic sites.

Key words: Hepatitis C virus (HCV), Genotypes, Sialadenitis and Chronic HCV

INTRODUCTION

Hepatitis C virus is a major health problem affecting over 170 million people worldwide. It causes a wide spectrum of liver diseases, varying from asymptomatic to persistant infection Vejbaesya *et al* (2004). Among Egyptians, HCV infection is a major problem affecting 6-28% of the population which might be explained by unintentional iatrogenic from mass campaigns against endemic schistomaiasis with anti-schistosomal injections, which continued up to the 1980s Frank *et al* (2000). HCV is a heterogeneous virus composed of at least 6 major genotypes and multiple subtypes that are categorized based upon the variability in their genetic structure Forns and Bukh (1999). Parental exposure to infected blood is the most efficient mode of transmission of HCV. Permucosal exposure to infection via bodily secretions is documented but less effective. Sexual transmission, although uncommon, should be considered as a risk factor for HCV infection, especially in spouses who have had long-term intimate relationships with a chronic hepatitis C patient James and Gillerist. (1999), Tibbs and Smith (2001). High prevalence of HCV RNA in saliva of patients with chronic HCV infection was found by Febris *et al* (1999). A number of reports have suggested an association between HCV and Sjogren's syndrome while subsequent investigations reported conflicting results King *et al* (1994).

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The correlation between rate of HCV RNA detection in the serum, saliva and minor salivary gland tissues in chronic HCV patients was first proposed by Arrieta *et al* (2001) and Chernetsova *et al* (2003). These findings were encouraging for further research concerning the role of HCV in causing sialadenitis and the implication of HCV genotypes in the pathogenesis of HCV associated –sialadenitis. This study was done to show the correlation of HCV genotypes in serum, saliva and minor labial salivary gland of Egyptian patients to highlight the problem of reliance on serum alone to identify genotype associated with the increasingly recognized extrahepatic manifestations of HCV infection.

MATERIALS AND METHODS

This study was conducted on 70 Egyptian patients infected with Hepatitis C virus (HCV). They were selected among those were attending the Medical Service Unit, National Research Center. No patients had received antiviral or interferon therapy throughout the study. Blood and saliva samples were taken from all the 70 patients and additional minor labial salivary gland tissue samples were taken from 15 of them who gave informed consent.

- Full case history, using printed questionnaire, was taken from patients regarding mode of infection of the disease (if known), duration, complaint of xerostomia (if present) and if subjected to treatment or not.
- Clinical examination: Examination of the oral mucosa for determination of the degree of xerostomia (if present) was carried out according to Navazash *et al* (1992).

Lip Dryness:

Dryness and cracking of the corners and/or the vermillion border of the lip was scored as:

0 → Normal
1 → Dry vermillion border
2 → Dry chapped and/or fissured tissue
3 → Angular cheilitis , redness or fissuring at commissure, with lesions of traumatic origin excluded.

The dryness and fissuring were scored as present even if unilateral.

Buccal Mucosa Dryness:

0 → Normal
1 → Look dry but tissues do not stick to tongue blade.
2 → Dry and tissues stick to tongue blade.

Salivary Flow Rate:

Low flow rate < 0.16 ml/min (unstimulated whole saliva was collected and a rate of < 0.16 ml/min was considered as low flow rate according to Navazash *et al* (1992).

Lab Investigations:

Prothrombin and partial thromboplastin time were estimated to be within the normal range before taking the biopsy.

HCV genotyping: Nested PCR amplification of HCV core gene using genotype specific primers according to Ohno *et al.* (1997) was carried out for all the samples (blood, saliva and minor labial salivary gland tissue biopsy).

Histological examination: was performed in hematoxylin and eosin-stained labial minor salivary gland biopsies.

Methodology:

- Blood sample: 5ml of blood were taken from each patient; serum was separated by centrifugation and divided into two portions before freezing at –80ºC.
- Saliva samples: Saliva collection was performed between 9:00 am and 2 pm so that the circadian influences would be minimized according to Dawes C (1974). Subjects were refrained from eating,

drinking, and smoking and oral hygiene procedures for 90 min before salivary collection. By spitting methods Unstimulated whole saliva was collected behind the closed lip and expectorated at the end of each minute in test tubes fitted with funnels for five minutes and stored at –80°C

- Tissue samples for biopsy were divided into two portions, one for RNA extraction for HCV genotyping by PCR and the other for histopathological examination.

All the samples (blood, saliva, minor labial salivary gland tissue biopsy) were subjected to the following techniques:

RNA Extraction:

Total RNA was isolated by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture. This method provides a pure preparation of undegraded RNA in high yield. All reagents used were molecular biology grade (Promega-USA).

HCV-RNA Detection by RT-PCR:

Reverse transcription-PCR (RT-PCR) was performed using primer sequences derived from the highly conserved non-coding region of HCV genomeEL-Awady *et al* (1999), using positive and negative controls within each run.

HCV Genotyping:

HCV genotyping was done for 70 patients that were subjected to HCV-RNA detection by RT-PCR in technique (II) to assure that the patient is infected with HCV virus so it could be genotyped and negative healthy patients excluded.

Procedure:

Nested PCR amplification of HCV core gene using genotype specific primers according to Ohno *et al.,* (1997). Denatured RNA was reverse transcribed into cDNA. Two rounds of PCR amplification were carried out on cDNA as follows: The first round of amplification utilized 2.5 uM of primers SC2 and AC2 (which are the sense and the anti-sense primers for the core region respectively), 0.2 mM dNTPs and 4 units of Taq DNA polymerase (Promega, USA). Thermal cycle of first PCR was as follows: 20 cycles of (94°C for 1 minute, 45°C for 1 minute and 72°C for 1 minute) followed by 20 cycles of (94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute). Two second-round PCR amplifications were performed for each sample: one with primer mixture (1) containing the S7, S2a, G1b, G2a, G2b and G3b. The other second PCR with primer mixture (2) contains the S7, G1a, G3a, G4, G5a and G6a. Thermal cycle of second PCR was as follows: 30 cycles of (94°C for 1 minute, 62°C for 45 seconds and 72°C for 1 minute). 10 µl of the second round PCR product was electrophoresed on a 2% agrose gel stained with ethidium bromide and evaluated under UV light. The expected sizes of the genotype-specific bands in primer mixture (1) are genotype (1b): 234 bp, genotype (2a): 139 bp and 190 bp, genotype (2b): 337 bp and genotype (3b): 176 bp. The expected sizes of the genotype-specific bands in primer mixture (2) are genotype (1a): 208 bp, genotype (3a): 232 bp, genotype (4): 99 bp, genotype (5a): 320 bp and genotype (6a): 336 bp.

RESULTS AND DISCUSSION

Results:

Table (1) shows descriptive statistics of results.

HCV Genotyping:

HCV genotype (4) (the most common genotype in Egypt) was detected in the serum of all patients.

- There were 53 cases with HCV genotype 4 in the saliva out of the 70 and 17 cases were untypable. From those 53 cases, 51 cases had genotype (4) and 2 cases had mixed genotypes (4+2a).
- Concerning the 15 cases that had minor salivary gland tissue biopsies, HCV genotype (4) were found in 11 cases while 4 cases were untypable. From those 11 cases, 9 were genotype 4 and 2 cases had mixed genotypes (4+2a). These two cases were the same cases of mixed genotypes in saliva.

Xerostomia Complaint:

Table (2) relates the presence of complaint of xerostomia (27 patients) and detection of HCV genotype in their saliva. There was a high significant association between the detection of HCV in the saliva and the complaint of xerostomia $p<0.01$.

Table (3) shows the presence of xerostomia complaint and the detection of HCV genotype in the tissue biopsies (that were taken from 15 patients) 11 patients complained and their tissues were HCV genotype 4 (100%) while 4 patients did not complain and were untypable marked significant association between both measures $p<0.01$.

Table (4) reveals comparison between complaint of xerostomia and presence of histopathological lesions. There is a significant association between xerostomia complaint and the presence of histopathological lesions $p<0.05$.

Table 1: Descriptive statistics of results

Descriptive statistics		Number	%
Number		70	
Age		37.90 ±7.78	
Gender	M	54	77.10%
	F	16	22.90%
Serum HCV genotype	Type 4	70	100%
Saliva HCV genotype	-Type 4	51	72.9%
	-Mixed types (4+2a)	2	2.8%
	-Untybable	17	24.3%
Tissue Results (n=15)	Type 4	9	60%
HCV genotype	Type 4	2	13.3%
	Untybable	4	26.7%%
Histopath.	+ve	9 out of 15	60.00%
	-ve	6 out of 15	40.00%
Xerostomia complaint	+ve	27	38.6%
	-ve	43	61.4%
Clinical scores (n=27)			
Lip dryness	0*	11	40.7%
	1*	14	51.9%
	2*	2	7.4%
BM dryness	0*	18	66.7%
	1*	9	33.3%
	2*	-	
General symptoms	+ve	41	58.6%
	-ve	29	41.4%
Bilharziasis	+ve	7	10.0%
	-ve	63	90.0%

* Clinical scores for xerostomia

Table 2: Xerostomia Complaint in relation to detection of HCV-RNA in saliva

	Complaint +ve		Complaint -ve	
	No	%	No	%
Saliva +ve	26	96.3%	27	62.8%
Saliva –ve	1	3.7%	16	37.2%
Total	27	100%	43	100%
Chi-square	10.13		0.0015**	

Table 3: Complaint of xerostomia in relation to detection of HCV-RNA in tissue

	Complaint +ve		Complaint -ve	
	No	%	No	%
Tissue. RNA +ve	11	100.00%	0	0.00%
Tissue . RNA -ve	0	0.00%	4	100.00%
Total	11	100%	4	100%
Fisher's Exact P-value	0.0007***			

**** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05; ns = not significant (P>0.05)
+ve : Detection of typable HCV-RNA
-ve: Untypable HCV-RNA

Table 4: Complaint of xerostomia in relation to detection of histopathological lesion

	Complaint +ve		Complaint -ve	
	No	%	No	%
Tissue Histo +ve	9	81.8%	0	0.0%
Tissue Histo -ve	2	18.2%	4	100.0%
Total	11	100%	4	100%
Fisher's Exact P-value	0.0110 *			

**** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05; ns = not significant (P>0.05)
+ve : Detection of typable HCV-RNA
-ve: Untypable HCV-RNA

Fig (1) Shows HCV Detection in Some Patients:

Amplified PCR products were visualized on 2% agrose gel stained with eithiduim bromide. M represents ΦX 174 DNA-HaeIII digest marker. Lanes (1) shows band of 270 bp represents the amplified PCR product of positive control and Lane (6) represent the negative control. Lanes (2), (3) and (5) show band of 270 bp represents the amplified PCR products of some patients. Lane (4) shows no bands indicative of healthy patient.

Fig. 1: HCV Detection in Egyptian patients.

Fig. 2: HCV genotyping of a patient's saliva and serum (mixed infection (4 +2a) in saliva and genotype (4) in serum).

Fig (2) Shows an Example of HCV Genotyping in the Saliva and Serum of the Same Egyptian Patient:

Amplified PCR products were visualized on 2% agrose gel stained with eithiduim bromide. M represents ΦX 174 DNA-HaeIII digest marker. Lanes (1) and (2) represent the amplified PCR products of saliva of patient in multiplex

[1] and multiplex [2] respectively. Lane (1) shows 2 bands of 190 bp and 139 bp in multiplex [1] that is indicative of type 2a and lane (2) shows one band of 99 bp in multiplex [2] that is indicative of type 4. Lanes (3) and (4) represent the amplified PCR products of serum from the same patient in multiplex [1] and multiplex [2] respectively. Lane (3) shows no bands in multiplex [1], while lane (4) shows a band of 99 bp in multiplex [2] that is indicative of type 4.Thus, the patient whose samples were shown in fig (1) was typed as having mixed infection (4+2a) in saliva and type 4 only in serum.

Histopathological Examination:

The tissue biopsies showed lesions in 9 out of the 15 patients characterized by the classical features of chronic lymphocytic sialadenitis: pericapillary infiltrate mainly composed of lymphocytes and plasma cells, enlarged intact ducts and acinar atrophy Intact serous demilunes and degenerated mucous cells were found (Fig 3-6).

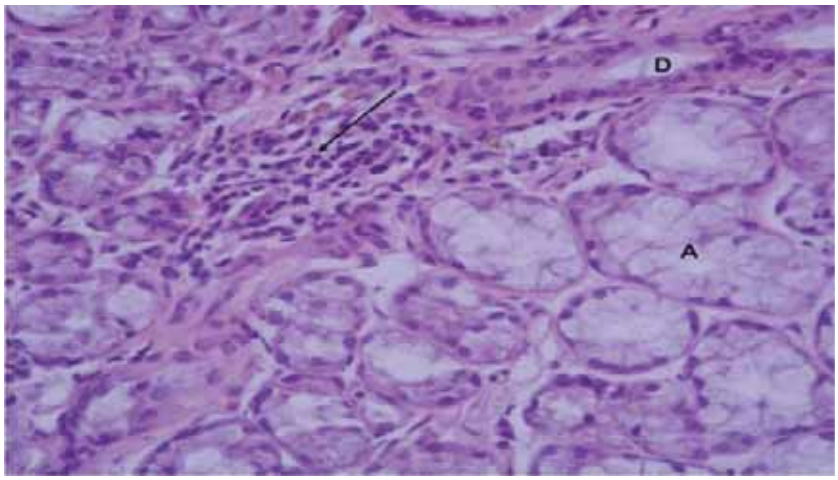

Fig. 3: Photomicrograph shows cellular infiltrate (pericapillary) (H&E stain, original, mag. X 400)

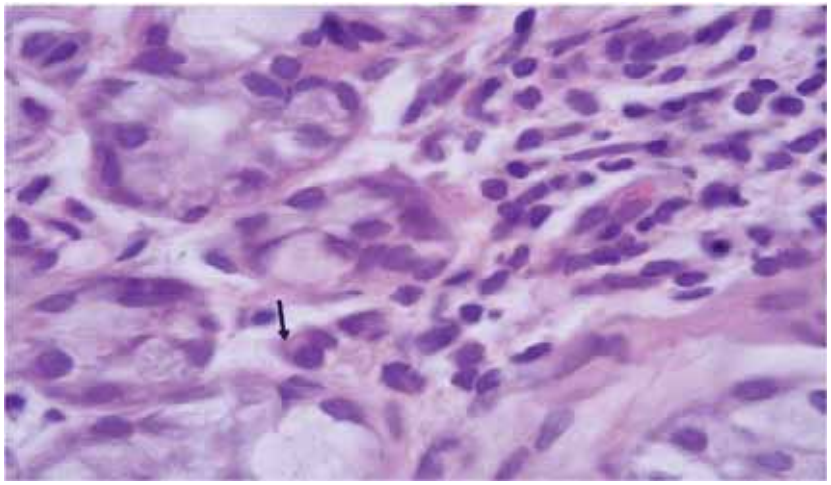

Fig. 4: Photomicrograph shows plasma cell (pericapillary) (H&E stain, original, mag. X 1000).

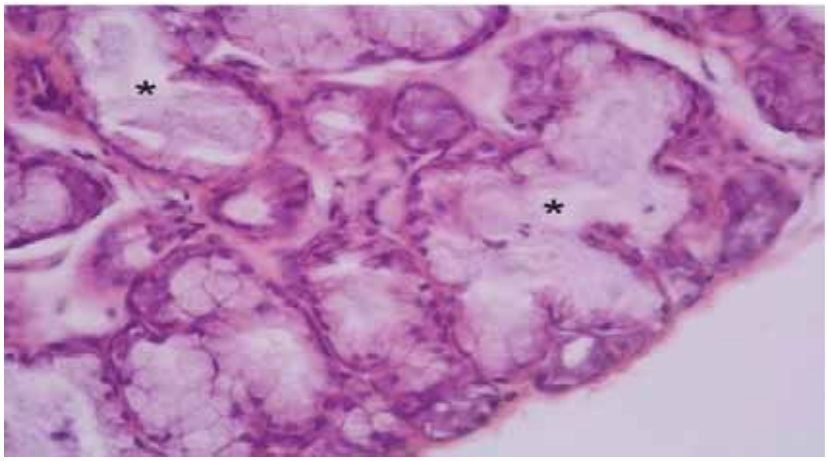

Fig. 5: Photomicrograph shows degenerated acinar cells (H&E stain, original, mag. X 400).

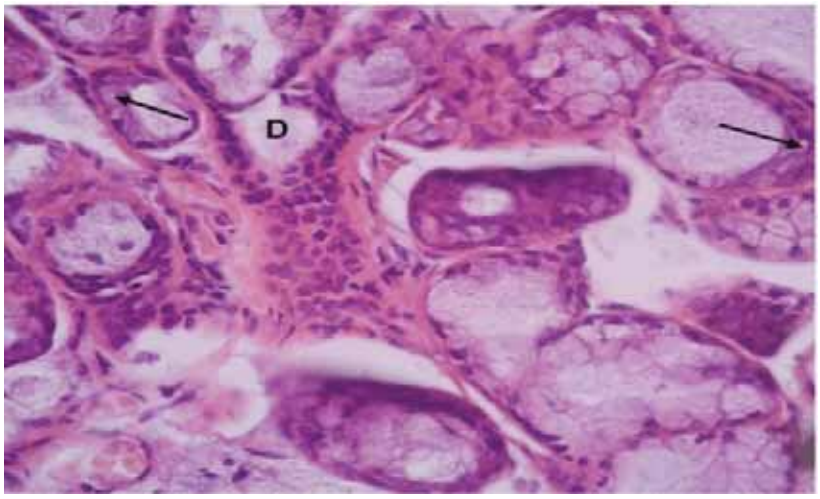

Fig. 6: Photomicrograph shows serous demilune and degenerated mucous cells (H&E stain, original, mag. X 400).

Discussion:

The current study showed that HCV genotype 4 was detected in the serum of all patients. This is in accordance with the studies that found a striking geographical shift in genotype distribution between Europe and countries in the Middle East, northern and central Africa where type 4 predominates Halim *et al* (1999), Frank *et al* (2000), and Pybus *et al* (2001).

Three out of the 53 patients with HCV-RNA positive saliva in the present study, were edentulous which is in agreement with the results of Roy *et al.,* (1998) who stated that if the source of salivary HCV-RNA is serum transudation through the gingival crevices, we would expect the same ratio of positive serum and saliva samples. Furthermore, the presence of HCV in saliva would have been likely to be influenced by the presence of teeth and their number which is not the case in edentulous patients.

In the present study, minor salivary gland histopathologic lesions were found to be characterized by the features of chronic lymphocytic sialadenitis: pericapillary infiltrate mainly composed of lymphocytes and plasma cells and enlarged intact ducts with acinar atrophy. These findings were in accordance with Haddad *et al* (1992), Poet *et al* (1994), Boscagli *et al* (1996), Verbaan *et al* (1999) and Ohoka et al (2003). However, intact serous demilunes with degenerated mucous cells were found in our study which was not previously noted.

The 27 patients complaining of xerostomia were among the 53 patients with HCV genotype 4 (50.9%) this was supported by Roy *et al.,* (1998) who found that half of patients with HCV-RNA positive saliva had a complaint of xerostomia which was the only significant oral feature that differentiated patients with or without HCV in their saliva. On the other hand Haddad *et al.,* (1992), Boscagli *et al.,* (1996) reported that xerostomia complaint represented (35.7%) and (30.7%) respectively.

In the present study we also tried to correlate the findings of the 15 biopsies with the complaint of xerostomia. Our results are supported by Rotti *et al.* (2001) who found the prevalence of xerostomia among 45 patients to be 62% with histopathological evidence of sialadenitis, representing 80% of cases.

Among our data, there were 2 cases out of 15 with genotype (4+2a) in tissues and saliva which was different from their serum genotype (4), it could be concluded that HCV-RNA might reside and propagate in salivary glands leading to mutation during replication resulting in different genotypes in tissues and saliva compared with serum, this was supported by Toussirot *et al* (2002).

To sum up, Isolate genotypes of HCV were identified in paired serum and saliva samples of HCV infected patients. A different HCV genotype was found in serum compared to saliva specimen suggesting that in some cases, the salivary HCV may not be serum transudation along the periodontal membrane or a cross damaged mucosa and that an alternative local source, possibly the salivary glands themselves.

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