



Evaluation of Saliva as an Alternative Specimen to Serum for Diagnosis of Hepatitis C Virus Infection

Dalia Saad EIFeky¹, Alaa Reda Awad^{1*}, Ahmed Fouad Soliman²
and Laila Rashed³

¹Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Egypt.

²Department of Infectious Diseases and Endemic Hepatic and Gastrointestinal diseases, Faculty of Medicine, Cairo University, Egypt.

³Department of Medical Biochemistry, Faculty of Medicine, Cairo University, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author DSE and ARA designed the study, participated in performing the practical part, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Author AFS participated in designing the study and collecting samples. Author LR participated in performing the practical part. All authors managed the analyses of the study and literature searches, read and approved the final manuscript.

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ABSTRACT

Aim: The study aimed at evaluation of saliva as an alternative specimen to serum for the detection of HCV Abs and HCV RNA.

Study Design: Comparative Study.

Place and Duration of Study: Department of Medical Microbiology and Immunology, and Department of Infectious Diseases and Endemic Hepatic and Gastrointestinal diseases, faculty of medicine, Cairo University, Cairo, Egypt. Between March 2013 and July 2013.

Methodology: The study was conducted on serum and saliva samples collected from 50 HCV-infected patients and 20 healthy controls. All serum and saliva samples were subjected to 3rd generation enzyme linked immune-sorbent assay (ELISA) for detection of HCV antibodies as well

*Corresponding author: E-mail: alaa.mreda1@gmail.com;

as real time RT-PCR assay for detection of HCV RNA. ELISA procedure for saliva samples was done according to a modified protocol, while 3 methods were used to calculate cut-off value (COV) above which saliva samples were considered positive.

Results: HCV antibodies were detected in all serum samples from patients but not in controls. Salivary HCV antibodies results for patients and controls differed according to the three methods used for determining the COV, with sensitivity ranged from 88 to 96% and specificity from 95 to 100%. No correlation existed between positivity of anti-HCV salivary Abs with either serum or salivary viral loads. Salivary real time RT-PCR had sensitivity and specificity of 100% for diagnosis of HCV infection with excellent significant correlation between the HCV viral loads in the saliva and serum.

Conclusions: Saliva can be used as an important substitute to serum for diagnosis of HCV infection either by detection of anti-HCV Abs or HCV RNA.

Keywords: HCV; saliva; ELISA; real time RT-PCR.

ABBREVIATIONS

HCV: Hepatitis C virus; Abs: antibodies; ELISA: enzyme linked immune-sorbent assay; COV: cut off value; RT-PCR: Reverse transcriptase polymerase chain reaction.

1. INTRODUCTION

Hepatitis C virus (HCV) infection is an important public health concern, affecting over 170 million individuals worldwide [1]. HCV diagnosis is typically based on detection of anti-HCV antibodies followed by identification of HCV RNA conducted using serum or plasma samples that have been obtained from individuals by venipuncture [2]. This procedure needs specialized personnel and may be difficult to perform in some individuals, such as children and intravenous drug users in addition to elderly and obese persons. In these situations, saliva collection can offer several advantages being easier to collect store and ship. Moreover, saliva collection is noninvasive which reduces anxiety and discomfort for the patient and is safer than blood tests for health professionals [3]. Furthermore, the analysis of saliva can provide a cost-effective approach for the screening of large populations [4]. Interestingly, reports have indicated that HCV-Abs and viral antigens not only exist in the saliva of infected subjects but also correlate well with blood samples [5-7]. These findings suggest a potential use for saliva as a non-invasive sample for HCV diagnosis and disease state monitoring [5-7]. This study aimed to evaluate the use of saliva as an alternative specimen to serum for the detection of anti-HCV antibodies and HCV RNA as well as to correlate positivity of saliva for HCV antibodies with salivary and serum viral load.

2. MATERIALS AND METHODS

This study included 50 Egyptian patients with serological evidence of HCV infection and 20

age-sex matched healthy controls. The patients were recruited from the outpatient clinic of the Department of Infectious Diseases and Endemic Hepatic and Gastrointestinal diseases, Kasr Al Ainy hospital, Cairo University during the period from March to July 2013. Only individuals who were free of other concomitant liver diseases and were not immunosuppressed as evidenced by no history of immunosuppressive diseases or drugs were selected [8]. Previous antiviral treatment was an exclusion criterion in this study [4]. The study was approved by the local institutional review board of College of Medicine, Cairo University. All participants gave their informed consent prior to their inclusion in the study. Full history taking including past history of surgery, blood transfusion, body tattooing and piercing or previous dental visits; thorough clinical examination and laboratory investigations including liver function tests (ALT, AST) were performed for all the patients.

2.1 Laboratory Methods

2.1.1 Sample collection

- i) Serum samples: 5 mL blood was collected from each subject in sterile non-gel serum vacutainer tube under strict sterile conditions. Serum was separated by centrifugation and transferred to sterile Eppendorf tubes.
- ii) Saliva samples: A visual examination of the oral cavity was performed to exclude patients with evidence of ulceration, bleeding or inflammation of the gingival mucosa. Patients and controls were asked to spit into sterile plastic containers without

prior stimulation. Samples were then transferred to sterile Eppendorf tubes.

Both serum and saliva samples were divided into two aliquots and stored at -20°C until further analysis.

2.1.2 “EIA-ANTI-HCV” assay for detection of HCV antibody in serum and saliva samples using HCV antibody ELISA kit (DSI S.r.l., Italy)

2.1.2.1 Principle

A 3rd generation ELISA test based on an indirect solid-phase enzyme immunoassay. Strips composed of recombinant HCV antigens-coated wells were used to bind to HCV antibodies present in human serum or saliva samples. A horseradish peroxidase (HRP)-labeled antibody conjugate (mixture of anti-human IgG and anti-human IgM) will then bind to any human Ig captured on the wells.

2.1.2.2 Procedure

- i) ELISA procedure for serum samples was done as recommended by the manufacturer.
- ii) ELISA procedure for saliva samples was done according to a modified protocol adopted from previous studies. A Pilot study was performed to determine the modifications that yield the best results. It was performed as follows: documented two seropositive and two seronegative saliva samples were used. Two assays were compared; in the first one, the manufacturer's protocol was modified such that the saliva sample volume was increased to 110 µl (instead of 70 µl in serum) and 30µl of the sample diluent was added [9,5,2,10]. In the second assay, the saliva sample was increased to 100 µl and no sample diluent was added [8]. In both assays, the temperature of incubation was altered to room temperature instead of 37°C, while the duration of incubation of the samples was increased to 24 hours (instead of one hour) and also the duration of incubation of the conjugate was increased to 3 hours (instead of 20 minutes) [9,6,2,10]. The first assay gave more acceptable results. So, the first assay was used for the rest of the saliva samples.

2.1.2.3 Reading of the results

The optical densities (ODs) of both the serum and saliva samples were read spectrophotometrically at a wavelength of 450/ 620-680 nm. The color intensity is directly related to the concentration of HCV antibodies in a patient's sample.

2.1.2.4 Interpretation of results

- i) Serum samples:

The sample was considered as positive if the OD value was equal to or greater than the cut-off value. The cut-off absorbance value (COV) above which serum samples were declared as positive was calculated as per the manufacturer's instructions:

$$\text{CUT-OFF value} = \text{Mean OD value of Negative Control} + 0.180$$

- ii) Saliva samples:

Since saliva is not routinely used to screen patients, there are no standard guidelines to calculate the COV for saliva. The COV for saliva samples above which samples were considered positive, was calculated by three methods and compared. In the first method, the manufacturer's recommendation for calculation of COV was used (COV1). In the second method, 3 standard deviations above the mean saliva absorbance of HCV seronegative samples (mean + 3SD) was chosen as cut-off (COV2). In the third method, a receiver operating characteristic (ROC) curve analysis was done for saliva absorbance values. The absorbance value which yielded the maximum sensitivity and specificity was chosen as the cut-off value (COV3) [8,10].

2.1.3 Real time PCR assay for detection of HCV RNA in serum and saliva samples

HCV RNA was extracted from both serum and saliva samples of patients and controls enrolled in the study using Gene JET viral DNA and RNA purification kit (Thermo Scientific, USA) according to the manufacturer instructions. ProMag HCV Quantitative RT-PCR Diagnostic

Kit IVD Quick Protocol 144 (ProMag, Germany) was used to determine HCV-RNA loads in the saliva and serum samples according to the manufacturer instructions. A standard curve was generated using known concentrations of four HCV Standards (10×10^3 - 10×10^6 copies/ μ l). The HCV viral load levels for all of the unknown samples were calculated through the extrapolation of the standard curve.

2.2 Statistical Analysis

Data management and analysis were performed using Statistical Package for Social Sciences (SPSS) vs. 21. Numerical data were summarized as means, standard deviations and medians. Agreement between the three methods of calculating COV was measured by the Kappa measure of agreement. Spearman-rho method was used to test correlation between numerical variables. All p-values were two-sided. P-values < 0.05 were considered significant. Accuracy was represented using the terms sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

3. RESULTS AND DISCUSSION

3.1 Participant Characteristics

The cases included 35 males (70%) and 15 females (30%) while the controls were 14 males (70%) and 6 females (30%). The age of the cases ranged between 26-66 years with a mean value of 48.3 ± 11.9 years while that of the controls ranged between 28 - 66 years with a mean value of 45 ± 10.7 years, with no statistically significant difference between them ($P= 0.288$). Most of the studied patients discovered that they are infected with HCV during check up. However, the main complaint was dyspepsia and upper abdominal discomfort. The duration of illness in these cases ranged from one week to 10 years with a mean of 20.3 ± 28.1 months. In 34% of cases, no possible risk factor was found. The possible risk factors included: dental manipulations (15%), surgical procedures (13%), blood transfusion (13%), HCV positive close contacts (7%), haemodialysis (6%), barbershops (6%), intravenous anti-bilharzial treatment (4%) and previous drug addiction (2%). In 3 patients, more than one possible risk factor was found (blood transfusion, dentist, surgery and dealing with barbershops). Liver function tests' results (ALT and AST) of the studied cases ranged from normal values to four times the normal value.

3.2 ELISA Results

ELISA testing of the previously documented 50 positive and 20 negative serum samples enrolled in the study using "EIA-Anti-HCV, DSI" gave 100% sensitivity, specificity, PPV and NPV for the ELISA kit used in the study.

Concerning the saliva samples, three methods were used in this study to calculate COV above which samples were considered positive; COV1 (0.4) which was calculated using the manufacturer's recommendation, COV2 (0.22) which was calculated using mean saliva absorbance of HCV seronegative samples plus 3 standard deviations and COV3 (0.29) which was calculated using the ROC curve. The area under the ROC curve was 0.983 with standard error of 0.017, $P < 0.001$) and the 95% confidence interval was between 0.95 and 1.00.

Comparison of results for detection of HCV Abs in saliva samples using the 3 different COVs with results of HCV Abs detection in serum is shown in Table 1. There was a statistically significant strong agreement between the results of antibody detection in the saliva samples and the serum samples when using the three different COVs (Table 2).

Table 1. HCV Abs in saliva samples using the 3 different COVs methods compared with HCV Abs in serum

Saliva samples	Serum samples			
	Positive (n=50)		Negative (n=20)	
	N	%	N	%
COV1				
Positive (n=44)	44	88	0	0
Negative (n=26)	6	12	20	100
COV2				
Positive (n=49)	48	96	1	5
Negative (n=21)	2	4	19	95
COV3				
Positive (n=48)	48	96	0	0
Negative (n=22)	2	4	20	100

Anti-HCV detection in serum samples was used as a gold standard for calculation of the accuracy indices of the salivary anti-HCV assay using the 3 different COVs (Table 2). Comparing the accuracy indices and the measures of agreement of serum versus salivary HCV-Ab detection using COV1, COV2 and COV3, the use of COV3 was found to yield the best results compared to COV1 and COV2.

Table 2. Accuracy indices and measures of agreement for the three methods of COV determination

	COV1	COV2	COV3
Sensitivity %	88	96	96
Specificity %	100	95	100
PPV %	100	98	100
NPV %	76.9	90.5	90.9
Accuracy %	91.4	95.7	97.1
Kappa	0.807	0.897	0.932
Standard Error	0.074	0.058	0.047
P-value	<0.001	<0.001	<0.001

3.3 Real Time RT-PCR Results

HCV-RNA was detected in both saliva and serum samples of all HCV-seropositive patients, while it was undetectable in samples from healthy controls. Considering real time RT-PCR of the serum as the gold standard method for diagnosis of HCV infection, real-time RT-PCR for detection of HCV in the saliva had a sensitivity and specificity of 100%.

In HCV-seropositive patients, the range of viral RNA levels was 10,850-1,850,000 copies/μl with a median level of 317,665 (3.17665x10⁵) copies/μl in the serum, while the range was 970-485,000 copies/μl with a median level of 40,685 (4.0685x10⁴) copies/μl in the saliva. It was observed that, the salivary HCV viral load was significantly lower than the viral load in the serum ($P < 0.0001$). Also there was an excellent significant correlation between the HCV viral loads in the saliva and serum ($r=0.846$, $P=0.001$).

In this study, 19 out of the 50 HCV-seropositive patients (38%) showed low salivary viral load (970-15,780 copies/μl). Despite of having low viral load, 17 out of these 19 (89%) was positive for salivary anti-HCV antibodies. There was no significant correlation between salivary anti-HCV with either salivary ($r=0.079$, $P=0.586$) or serum ($r=-0.006$, $P=0.969$) HCV viral loads.

4. DISCUSSION

The diagnosis of HCV infection is based on the detection of anti-HCV antibodies in the patient's serum followed by identification of HCV RNA [2]. To enable greater access of patients for HCV infection screening, other non-invasive body fluids as saliva and urine are being investigated as alternatives to blood [11]. The current study aimed to evaluate the use of saliva as an

alternative specimen to serum for the detection of anti-HCV antibodies and HCV RNA. This study included 50 HCV-seropositive patients and 20 healthy volunteers. From the history of the participant patients, several risk factors for infection with HCV were reported, however, 34% of the patients reported no possible risk factor. These findings were in accordance with other studies [2,12] which reported that about 40% of the patients didn't present any risk factor for acquiring the infection suggesting that unknown routes of transmission may exist.

Liver function tests (ALT and AST) were done for the cases and the results ranged from normal values to four times the normal value. Similarly, several studies had reported the fluctuating nature of serum ALT levels in HCV-infected patients [13,14].

Saliva has been suggested as a convenient specimen for the detection of antibodies to various infectious disease agents. HIV occupies a prominent place in this regard with numerous studies reporting a favorable sensitivity of saliva for HIV antibody detection [15,16]. Former studies have also investigated saliva as a possible alternative to serum for the detection of antibodies for hepatitis A and B, Epstein Barr, and rubella viruses [16,17,18]. Similarly, whole saliva has been employed by many research groups for the detection of antibodies to HCV [2,6,8,9,11,16,17,19-24]. Most of these studies gave a sensitivity ranging from 71 to 100% and specificity from 92 to 100%.

A commercially available test that can rapidly identify HCV-Abs in saliva by using an EIA was developed (OraSure Technologies Inc. 2010). Although the results obtained from this test draw parallels with those of serum immunoassays (97.5%), it hasn't obtained yet FDA approval and is currently not available in the United States. Even so, it is widely available in Europe and, if employed effectively, could possibly have a substantial impact on the early detection and management of HCV infections [24,25,26].

To use saliva for ELISA tests, the collection technique is essential to obtain a reliable sample for viral antibody detection because the IgG concentration among saliva samples is 4–15 times lower than those present in serum samples [27,10]. In this study, we opted for the use of the simple spitting method without prior stimulation for saliva samples as it demonstrated excellent performance in previous studies [11,22,10]. Also

the simplicity as well as the low cost of this method may support the use of EIA on saliva samples for epidemiological studies of HCV in the near future.

Development of an assay for detection of antibodies in saliva requires careful optimization of numerous parameters to maximize sensitivity and specificity. The various approaches include decreasing the dilutional effect of the sample buffer [11,19,16], exclusion of dilution step entirely [17], increasing the sample input [9], increasing the sample incubation time [19,20,9], increasing the time for conjugate incubation [20] and modification of the conjugate to detect antibodies besides IgG [21]. Also the calculation of the cut-off value was an important step for assay optimization because commercial EIAs have been developed for serum samples. The different methods used to determine cut-off absorbance included: (i) reduction to an absorbance of 0.200 [19], (ii) calculation of standard deviations from the mean saliva absorbance of HCV seronegative samples (Mean + xSD, where x varied from 1 to 6) [21,9,8,10] (iii) lowering the value of reactivity rate (sample/COV) by 20% [16], (iv) use of a formula based on mean ODs for negative and positive samples [11] and ROC analysis [21,8,10].

In the current study, the modifications adopted included increasing the duration of incubation of the samples to 24 hours at room temperature, increasing the duration of incubation of the conjugate to 3 hours at room temperature as well as increasing the saliva sample volume (110 μ l instead of 70 μ l) and adding sample diluent [9,6,2,10]. Also in the present study, three methods for calculating the COVs (COV1, COV2 and COV3) were adapted from the work of Moorthy et al. [8] and Cruz et al. [10] and further evaluated. These results were compared to the serum results where a very good performance and a statistically significant ($P < 0.001$) very good agreement were demonstrated. Also the accuracy percentage for the three methods was above 90% with the highest for COV3.

Our study showed comparable results to those performed by Elsana et al. [11] which had also shown 90.4% sensitivity, 100% specificity, PPV of 100% and NPV of 90.7%. Elsana and colleagues also collected samples by the spitting method but used a second generation ELISA kit, this may explain our slightly better results.

Our results were also very close to that of Lee et al. [25] with specificity of 100% but it showed a

much better sensitivity of 99.2% which could be explained by their use of 4th generation ELISA kit that is optimized to be used with oral fluid specimens.

It is also worth noting that in almost all previously mentioned studies, the specificity of the results was over 95% and many shared the exact same specificity of 100% as in Cameron et al. [19]; De Cock et al. [16] and Gonzales et al. [6]. This further confirms that the detection of HCV antibodies in saliva is a reliable method to detect the prevalence of HCV in the population.

On the other hand, the sensitivities of most of the studies as in Cameron et al. [19]; Judd et al. [9]; De Cock et al. [16]; González et al. [6] and Moorthy et al. [8] were much lower than the sensitivity of this study which were 85.2%, 91.7%, 89%, 86.7% and 81.6%, respectively despite using 3rd generation ELISA kits and performing nearly the same modifications. This result may be due to the difference in the conjugate used because the ELISA kit used in this study (EIA-Anti-HCV, DSI) uses IgG and IgM conjugates while the kits used in the other studies [Monalisa anti-HCV plus (Sanofi Pasteur) and Ortho 3.0 ELISA (Ortho-Clinical Diagnosis)] employs only IgG conjugate. The difference in the conjugates' content of the ELISA kit made a great difference in the work of Zmuda et al. [21] who tested the use of a marketed anti-IgG, anti-IgA and anti-IgM antibody cocktail as conjugate solution with the original Ortho HCV 3.0 kit and they achieved, in the same work, a sensitivity of 100% instead of 81% when detecting solely IgG.

In our study, the false-negative saliva results obtained may be explained by the low concentration of antibodies in the saliva samples [28,3]. Also HCV genotypes were not available for our patients, so we cannot exclude the possibility of the effect of this factor on the results. While the use of COV2 yielded one false-positive saliva test result among the controls which may have been attributed to non-specific interaction between HCV antigen and saliva elements, as has been demonstrated in previous studies [11,29]. Also it can be attributed to the concomitant infections as HIV, syphilis and HBV which was not investigated in this study.

In this work, the results of using a quantitative PCR assay to determine HCV viral load levels in the saliva and serum of patients were in agreement with Lins et al. [30] who reported that HCV-RNA was detected in all of

the saliva samples from the HCV-infected patients but not in the samples of the control group. In addition, others [31] have reported that HCV RNA was present and often persistent in the saliva of HCV-infected individuals. Another study [32] showed that salivary HCV was detected in 64% of the studied HCV infected patients, however, they reported that the main factor associated with the presence of HCV in saliva was the high viral load in serum. While contrary to our result, one study [20] reported that although HCV RNA was detected in 75% of HCV- seropositive patients, it was not detected in any saliva sample of these patients. In the previous study, the serum viral load was not determined and the investigators explained their results by the possibility of low HCV RNA serum titers as some studies reported that salivary HCV RNA was related with high viral load in serum [33]. In another study [2], 70% and 57% of non-stimulated and stimulated saliva samples, respectively, obtained from patients with confirmed chronic HCV were negative for HCV RNA.

In our study, the salivary HCV viral load was significantly lower than the viral load in the serum ($P < 0.0001$). This was in accordance with some studies which demonstrated that the HCV viral load was higher in serum than in saliva in HCV-infected patients [34,35,4]. In addition, in the latter study [4], salivary viral load (median viral RNA levels was 2.1×10^3 copies/mL) was significantly lower than the viral load in the serum (median viral RNA levels was 1.21×10^6 copies/mL) ($P < 0.0001$). The lower salivary HCV viral load may augment the notion of low potential of HCV transmission through saliva.

Our results showed excellent significant correlation between the HCV viral loads in the saliva and serum ($r=0.846$, $P=0.001$). This means that saliva can be used as a substitute for serum in the detection of HCV RNA. In agreement with these findings, some studies showed that salivary HCV RNA detection was associated with serum HCV RNA load [31,32]. However, Menezes et al. [4] reported no significant correlation between the HCV viral loads in the saliva and serum, however, they related this most likely due to the small number of available samples.

In the present study, there was no significant correlation between salivary anti-HCV Abs with either salivary or serum HCV viral loads. This was in agreement with Moorthy et al. [8] and Caldiera et al. [2] who reported that there was no

correlation found between salivary positivity for HCV Abs with either HCV viral load in plasma or in non-stimulated saliva of patients with chronic hepatitis C, respectively. However, Van Doornum et al. [20] reported the existence of significant correlation ($P=0.01$) between the presence of salivary HCV antibody and HCV RNA in serum, while Caldiera et al. [2] showed that statistical analyses revealed a significant association ($P = 0.035$) between detection of anti-HCV Abs and HCV RNA in stimulated saliva but he didn't discuss a possible influence of this sampling collection method in his results.

In this study, salivary anti-HCV antibodies were detected in 89% of patients having low salivary viral load. This was in accordance with Moorthy et al. [8] who showed that salivary antibodies can be detected even in patients with low viral load and he explained this considering that since the viral load generally remains almost unvariable during the course of the disease in non-treated patients, it seems reasonable that other factors modulate the level of anti-HCV Abs in saliva.

5. CONCLUSION

In conclusion, saliva can be used as a substitute to serum for diagnosis of HCV infection either by detection of HCV RNA or anti-HCV Abs as detected by the high accuracy indices. However, salivary detection of HCV-Ab assay needs further optimization before it can be recommended as a screening test in the general population. No correlation existed between positivity of anti-HCV salivary Abs with either serum or salivary viral loads.

6. LIMITATIONS

Limitations of the study include:

1. HCV genotype was not specified.
2. The presence of HIV infection, syphilis or HBV which may affect the diagnostic accuracy of HCV tests was not investigated in both patients and controls.

CONSENT

All patients' consents were taken before inclusion in the study.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore

been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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