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ImmunoComb II Test in Detection of HCV Antibodies in Oral Fluid

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Clinical Relevance:
Scientific rationale for the study: HCV infection is a worldwide problem specially in developing countries. Accurate non invasive and rapid tests for screening and diagnosis of HCV are highly needed. Oral fluid ImmunoComb II HCV test was suggested to be helpful. Previous work by Yaari et al, demonstrated 100% sensitivity of the test in detection of HCV infection in hemodialysis patients. Studies on other populations specially in developing countries are not widely available. Principal findings: in the current study oral fluid ImmunoComb II HCV test showed 67.5 % sensitivity, 100% specificity in detection of HCV infection. Practical implications: oral fluid HCV testing by ImmunoComb II test is promising technique specially as a screening test.

Abstract:
Objectives: *a suitable, affordable, rapid and accurate test for HCV may be helpful in various clinical settings. The aim of the work is to evaluate the sensitivity, specificity and accuracy of ImmunoComb II HCV test in detection of HCV antibodies in oral fluid in comparison to serum 3rd generation ELISA test.*
Methods: *this case control study included 40 patients with positive anti-HCV antibodies in the serum and positive HCV RNA PCR (Group I), compared to 20 patients with elevated liver enzymes, negative anti-HCV antibodies in serum and negative HCV RNA PCR (Group II), and to other 40 healthy controls (Group III).*
Results: *ImmunoComb II HCV test of the oral fluid showed sensitivity: 67.5 %, specificity: 100%, PPV: 100%, NPV: 82% and diagnostic accuracy of 87% in diagnosis of HCV.*
Conclusion: *the usage of ImmunoComb II for detection of HCV antibodies in oral fluid is promising and has a lot of advantages but needs more effort and more researches to increase its sensitivity.*
Key Words: *Hepatitis C; Oral fluid; ImmunoComb II.*

Introduction

In 1989 the virus responsible for most transfusion-associated non-A non-B hepatitis was identified and cloned, and named hepatitis C virus (HCV). (1)(2)(3)

HCV infections are common worldwide. World Health Organization (WHO) estimates that about 3% of the world's population has been infected with HCV and that some 170 million are chronic carriers at risk of developing liver cirrhosis and/or liver cancer (4).

Egypt has a very high prevalence of HCV and a high morbidity and mortality from chronic liver disease, cirrhosis, and hepatocellular carcinoma(5). In Egypt, Anti HCV was found in 12% of rural primary children, 22.1% of army recruits and 16.4% in children with hepatosplenomegaly(6). Estimates of HCV prevalence in Egypt range from 11% to 14% with 8 to 10 million having anti-HCV and 5 to 7 million having active infections (i.e., HCV-RNA positive) (7).

Two classes of assays are used in the diagnosis of HCV infection: serologic assays that detect specific antibody to hepatitis C virus (anti-HCV) and molecular assays that detect viral nucleic acid, quantify, and/or characterize HCV RNA genomes within an infected patient (8).

Detection of antibody to a single epitope by ELISA "Enzyme Linked ImmunoSorbent Assay" (EIA "Enzyme Immune Assay") was the first test developed in 1990 to screen blood donors and to diagnose HCV infection in symptomatic patients (3). It had poor sensitivity and was not helpful early after infection since the antibody appears four to six months after infection (9). Second and third generation ELISA and Recombinant Immunoblot Assay (RIBA) tests have increased sensitivity and narrowed the window period between infection and viral detection. Second-generation EIAs detect antibodies to structural (core) and nonstructural (NS3 and NS4) proteins. Third-generation EIAs detect the same antibodies with better sensitivity, plus antibodies directed to NS5 (10). The currently available assay is in its 3rd generation; the test is highly sensitive but not specific; therefore it gives many false positive reactions (11).

To avoid the use of serum, which requires obtaining drawn blood, oral fluid collection has been demonstrated to be an alternative screening modality to screen populations for HCV (12).

The use of oral fluid in diagnostic tests provides many advantages over traditional serum-based analyses. Oral fluid collection is rapid and noninvasive and eliminates the risks of needle exposure. Furthermore, oral fluid can be collected by non medical personnel, thus relieving health care professionals of the time-consuming and economic burden of obtaining serum samples. Indeed, oral fluid-based assays may prove to be the preferred method of testing for infants and young children and in developing nations, as well as for patient groups where blood collection is difficult, such as intravenous drug users (13).

Previous work showed that, in positive PCR patients the saliva assayed by ImmunoComb II kit had 100 % sensitivity (14).

ImmunoComb II HCV kit is a rapid and sensitive test for the differential detection of anti-HCV antibodies directed against structural and non structural viral proteins. It is useful tool for rapid diagnosis of suspected carriers of HCV, as well as for blood bank screening [15].

This study was conducted to answer the following questions:

Is oral fluid ImmunoComb II HCV test accurate in diagnosis of HCV infection?

What are the sensitivity and specificity of the test in comparison to serum ELISA antibody test?

Could the oral fluid test be the preferred route of diagnosis?

Study population and methodology

This case control study was conducted on one hundred participants; sixty of them are patients attending the Tropical Medicine, Internal Medicine Departments and Outpatient Clinics, Ain Shams University Hospitals, during the whole period of one year. The other 40 participants were collected as healthy controls attending the outpatient clinics for pre-employment assessment and from the blood donors from the blood bank. All participants gave oral and written informed consent and the study was approved by the ethical committee of Ain Shams University.

The study population had been divided into three groups:

Group1: included 40 patients with positive anti-HCV antibodies in the serum by third generation ELISA test and positive HCV RNA by PCR.

Group 2: included 20 patients with elevated liver enzymes and negative anti-HCV antibodies in serum by third generation ELISA test and negative HCV RNA by PCR.

Group 3: included 40 healthy volunteers with no past history of any liver disease or any risk factors of blood born viral infections, normal liver functions and negative anti-HCV antibodies in serum by third generation ELISA test and negative HCV RNA by PCR as control group.

Exclusion criteria

patients with autoimmune hepatitis, concomitant HBV, hemochromatosis, Wilson disease, drug history which can elevate liver enzymes and alcoholic hepatitis were excluded from the study.

Methodology

All the participants were subjected to the following

- ☒ Careful History taking with special stress on history of parental treatment of schistosomiasis, history of blood transfusion, surgical operations and/or dental procedures.
- ☒ **Thorough clinical examination.**
- ☒ **Laboratory investigations** including: Complete blood picture (CBC), liver profile: ALT, AST, total, direct bilirubin, serum albumin, PT, PTT, antinuclear antibody (ANA), anti smooth muscle antibody, anti mitochondrial antibody (AMA), hepatitis B surface antigen (HBs Ag), hepatitis B core antibody (HBc Ab), s. ceruloplasmin and iron study. Serum

HCV Ab test was done using ELISA (Abbott Diagnostics, Chicago, IL, USA) commercial kit in accordance with the manufacturer's instructions. HCV real time PCR was used for detection of positive stranded RNA in serum for which venous blood samples were drawn under aseptic conditions, centrifuged and 2 ml serum were collected and stored at minus 70ºC for real–time PCR tests for positive strand detection. The real time PCR test was done by Stratagene Mx3000P instrument.

- **Abdominal ultrasound** for assessment of liver size, echogenecity, spleen size, portal vein diameter and presence of ascites.
- All participants in the 3 groups had been subjected to oral fluid samples collection and samples were examined by **modified ImmunoComb II** kits for detection of HCV antibodies according to ***Yaari and co-workers*** [14]**.**

Steps of modified ImmunoComb II

Samples collection

All candidates were asked to give oral fluid sample in a clear container "cup", by spilling directly in the cup. Saliva samples were centrifuged immediately at 3000 rpm (round per minute), for 15 min, All the samples were kept at -70 C until assayed.

ImmunoComb test (P.B.S. Orgenics S.A., France)

The principle: immunoComb II is a rapid test for the differential detection of anti-HCV antibodies directed against structural (HCV core) and non-structural (NS3, NS4 and NS5) viral proteins.

The ImmunoComb HCV test is an indirect solid-phase enzyme immunoassay (EIA). The solid phase is a comb with 12 projections "teeth". Each tooth is sensitized at three spots:

- Upper spot - human immunoglobulin (Internal Control).
- Middle spot - HCV core antigen.
- Lower spot - HCV non-structural antigens.

The Developing Plate has 6 rows (A-F) of 12 wells, each row containing a reagent solution ready for use at a different step in the assay. The test is performed stepwise, by moving the Comb from row to row, with incubation at each step.

The concentration of the saliva specimens were increased by discarding the specimen diluents and the incubation period was prolonged to overnight, at low temperature (4°C), instead 10 min at room temperature. According to the manufacturer's instructions this test is considered as positive when anti-core or anti-NS3, NS4 & NS5 HCV antibodies are positive.

Interpretation of the Results:

- Appearance on a tooth of only the upper spot (Internal Control) indicates that the specimen is non-reactive for antibodies to HCV.

- Appearance on a tooth of even faint spots on both the middle spot (Core) and the lower spot (NS) indicates that the specimen is reactive for antibodies to HCV.
- Appearance on a tooth of a very faint spot only on the middle spot (Core) may correspond to a non specific reaction to the presence of antibodies to HCV or to an early infection and must be further investigated.
- Appearance on a tooth of a very faint spot only on the lower spot (NS) may indicate the presence of antibodies to HCV and must be further investigated.

☒ **Statistical methodology**:

Data were analyzed on an IBM personal computer, using Statistical Package for Special Science (SPSS) software computer program version 15. Data were described using mean ± standard deviation (SD) and frequencies according if they are quantitative or qualitative respectively. Chi-square test was used for comparison of qualitative variables. Student's t-test: of two independent samples was used for comparison of quantitative variables. One-way ANOVA test was used to compare more than two groups as regard quantitative data.

Results

This study was conducted on 100 subjects who were divided into 3 groups:

Group 1: included forty HCV patients "positive HCV RNA by PCR" with positive anti-HCV antibodies in serum by third generation ELISA test **(N=40)**. They were 31 males (77.5%) and 9 females (22.5%). Their ages ranged between 20 and 79 years (Mean ± SD: 53.18 ± 11).

Group 2: included twenty patients with elevated liver enzymes and negative anti-HCV antibodies in serum by third generation ELISA test with negative HCV RNA PCR **(N=20)**. They were 5 males (25%) and 15 females (75%). Their ages ranged between 15 and 55 years (mean ± SD: 29.2 ± 8.5).

Forty healthy volunteers were enrolled as control group **(Group 3)** who were negative for anti-HCV antibodies in serum by third generation ELISA test and with negative HCV RNA by PCR (N=40). They were 21(52.5%) males and 19 females (47.5%). Their ages ranged between 18 and 66 years (mean ± SD: 37.35 ± 14.59).

Results of oral fluid HCV antibodies in relation to serum HCV antibodies are shown in **table 1**. The sum of the results of the three groups demonstrates the total positive case of salivary antibodies (27 cases) in relation to the positive cases in the serum (40 cases) which represent 67.5 %, on the other hand, the total negative cases of salivary antibodies were 60 cases in relation to 60 negative cases in the serum which represent 100%.

The interpretation of the results of testing oral fluid antibodies by ImmunoComb II kits shows that the 27 cases (67.5 % of positive serum cases) were true positive, no cases (0 % of negative

serum cases) were false positive, while 60 cases (100% of the negative serum cases) were true negative and 13 cases (32.5 % positive serum cases) were false negative. **(Table 2)**

By evaluation the specificity and sensitivity of testing oral fluid antibodies by modified ImmunoComb II kits, the sensitivity of the test was 67.5 %, the specificity was 100 %, positive predictive value was 100 %, negative predictive value was 82 %, and its diagnostic accuracy was 87%. **(Table 3)**

Table 1: results of testing of both salivary and serum antibodies in all groups.

Group	Serum Abs		Salivary Abs	
	Positive	Negative	Positive	Negative
Group I n=40 (100%)	40 (100%)	0 (0%)	27 (67.5%)	13 (32.5%)
Group II n=20 (100%)	0 (0%)	20 (100%)	0 (0%)	20 (100%)
Group III n=40 (100%)	0 (0%)	40 (100%)	0 (0%)	40 (100%)

Table 2: interpretation of the results of testing the oral fluid antibodies.

The result	*Explanation*	*No.*
True Positive	Positive in oral fluids, Positive in serum.	27
False Positive	Positive in oral fluids, Negative in serum.	0
True Negative	Negative in oral fluids, Negative in serum.	60
False Negative	Negative in oral fluids, Positive in serum.	13

Table 3: sensitivity and specificity of testing oral fluid antibodies by modified ImmunoComb II

Assessment	*Explanation*	*Percentage*
Sensitivity	The ability of the test to detect those who have the antibodies.	67.5 %
Specificity	The ability of the test to detect those who are free of antibodies.	100 %
Positive Predictive	The proportion of patients with positive test results who are correctly diagnosed.	100 %
Negative Predictive	The proportion of patients with negative test results who are correctly diagnosed.	82 %
Diagnostic Accuracy	Describe the quality and usefulness of a test.	87 %

Discussion

There is great need to develop or to find a suitable, affordable, sensitive and accurate test for screening of HCV especially in mass screening campaigns, blood donors in blood donation campaigns and blood banks and in screening of HCV in children.

Assays which were developed to utilize oral fluid instead of serum have shown promise in the detection of virus-specific antibodies. It seems to be better way to avoid the use of serum, which requires obtaining drawn blood. Because of this knowledge; oral fluid collection has been evaluated as an alternative modality to screen populations for HCV [(12)].

The current study aimed to evaluate the ImmunoComb II test and its sensitivity, specificity and accuracy in diagnosis of HCV in Egyptian patients.

Our results showed statistically significant difference between the studied groups as regard age and sex. In the current study, higher infection incidence was in men. This is in agreement with ***Armstrong and colleagues*** who found that anti-HCV prevalence was significantly higher in men than in women [(16)]. Men have consistently lower clearance rates than women. The reason for this difference, however, is unclear. Possible factors include the age at which a man is infected with hepatitis C, whether he has other infections, such as HIV, and the route of infection (blood transfusion, sexual contact, drug use, etc.) [(16)]. As regard the age differences in the current study, patients with positive anti-HCV were of higher age than other studied groups. This is also in agreement with ***Armstrong and colleagues*** who found peak prevalence in individuals between 40 and 49 years of age. It is also similar to results of ***Hanafiah et al,*** who found higher prevelance in older age patients [(17)]

Yaari and co-workers evaluated the detection of HCV Abs in the oral fluid by using ImmunoComb II and showed that all patients with positive HCV RNA by PCR were positive by saliva ImmunoComb II test with 100% sensitivity [(14).]

The current study showed sensitivity 67.5 %, specificity 100 %, positive predictive value 100 %, negative predictive value 82 %, and diagnostic accuracy of ImmunoComb II testing of 87%. These results are mildly different from the results of **Yaari and co-workers** who showed that the modified ImmunoComb II kits had 100% sensitivity & 95% specificity[(14)].

Although the same technique of sample collection, the same concentration method and interpretation were used as **Yaari and co-workers,** the results showed some differences in the sensitivity and specificity of the test. This could be explained by the difference in the study populations of the two studies. **Yaari and co-workers** study included 37 chronic haemodialysis patients, 48% of them were HCV-PCR positive. Haemodialysis patients may carry other infections which may stimulate formation of antibodies that cross react with the tested antigens or in contrary inhibit its detection which interfere with the results.

Another cause of the difference in the results may be due to the difference between the genotype of the infecting virus. This is confirmed by **Beld and colleagues**, who reported that individuals infected with HCV genotype 1 have significantly higher median antibody responses to core and NS4 as compared with those infected with other genotypes [(15)]. Genotype 1 is more predominant in Western Countries while genotype 4 was found in Middle Eastern countries such as Egypt [(18)].

Although there were differences in the technique of sample collection, results of the current work are near to results of ***McIntyre and colleagues*** with sensitivity 72% & specificity 98% [(19).] Also ***Judd and co-workers*** study results showed sensitivity 74.1% & specificity 99% [(20)]. In those studies, samples were collected using oral fluid collection device "Salivette". Other study done by ***Sherman et al., 1994***, showed sensitivity 98.2 % & specificity 99.1% ***(12)***, while ***De Cock et al., 2004*** study showed sensitivity 83.6 % & specificity 100% [(21)].

Lee and colleagues made a study in 2011, in which they used a new HCV rapid antibody test device (OraQuick® HCV Rapid Antibody Test). They found that sensitivity was slightly lower for oral fluid at 98.1% though the upper CI (99.0%) was equal to the lower CI for venous blood and finger-stick blood. Most of the HCV positive subjects who gave nonreactive results in oral fluid had serological and virological results consistent with resolved infection [(22)].

In a more recent study done by ***Cha and colleagues***, the clinical sensitivity and specificity of the OraQuick HCV test using oral fluid were 97.8% and 100% respectively supporting the supplementary use of rapid HCV testing using oral fluid in various medical and non-medical settings [(23)].

The minor differences between the results may be referred to the use of different kits or different tests in each study. This is confirmed by ***McIntyre***

et al., 1996 who stated that the choice of test is crucial, because not all manufacturers' tests are equally reliable when oral fluid is used [19].

All the previous studies used collection devices for collection of the oral fluid samples such as Orasure, Salivette and Oracol. These are commercial devices for oral fluid sampling, not available in the Egyptian market. In the current study no device was used to collect samples and the patient was asked to give oral fluid sample in a clear container "cup" by spilling directly in the cup- like the technique used by ***Yaari, et al., 2006*** [14]**. Van Doornum et al. 2001** did not find any significant statistical difference between the sensitivity and specificity of the salivary anti-HCV testing using two different collecting systems [24].

Another explanation of the above mentioned differences in the sensitivity and specificity of the different studies, may be due to the type of antibodies detected in the oral fluid by each test. Most of the serum anti-HCV antibody tests are designed to detect immunoglobulin G (Ig G) class of antibodies.

Oral fluid is considered to be a mixture of secretions from the salivary glands and plasma components derived by passive transudation from the capillaries in the mucosa of the mouth, particularly the gingival crevicular fluid. This fluid is rich in Ig G and Ig M. The latter fluid is passively transuded into the mouth across the mucosa and through the gingival crevices; the IgG concentration is approximately 1/800th of that found in serum, but higher than that in whole saliva, which contains mainly Ig A and only traces of Ig G and Ig M [25][26]**.**

The decreased concentrations of antibodies in oral fluid may be responsible for the decreased detection sensitivity of anti-HCV antibodies in oral fluid. Serum-based immunoassays which are modified to test for HCV in oral fluid utilize tracer antibodies that recognize only antibodies of the IgG class while other classes of antibodies remain undetected. With the relatively low levels of antibodies present in oral fluid, it is likely that many of the false negatives results obtained using modified serum-based assays to test oral fluid are the result of HCV-positive patients possessing levels of anti-HCV Ig G in their oral fluid that are so low as to be detectable by immunoassays recognizing only Ig G class antibodies [13].

Thus, ***Zmuda et al., 2001*** modified the oral fluid test for detection of HCV antibodies to detect "antibody cocktail" that recognizes not only IgG but Ig A and Ig M as well. These modifications attained 100% specificity and sensitivity with oral fluid samples in comparison to only 81% in detection of Ig G alone.

By effectively increasing the pool of antibodies detectable in oral fluid samples, it may be possible to overcome the intrinsic difficulty of detecting the extremely low levels of antibodies in oral fluid and allow the generation of novel non-blood-based immunoassays [13].

The finding of alternative non invasive method to serum detection of HCV Abs is considered by many researchers, ***Elsana et al., 1998*** tried to detect HCV Abs in urine in 73 patients with HCV

related liver diseases. Urinary anti-HCV could be detected in 36 (49%) only of the anti-HCV sero-positive patients [26].

Elsana et al., 1998 also compared the presence of HCV Abs in urine and saliva in the same patient group (73 patients), salivary anti-HCV could be detected in 66 (90%) in comparison to urinary anti-HCV which is the Ab detected only in 36 (49%) [26].

Those results suggest that saliva, (but not urine), can be better a substitute for serum for the determination of anti-HCV positivity [27].

Conclusion

The current study conclude that the usage of oral fluid sample in alternative to serum is promising and has a lot of advantages but needs more effort and more researches to increase its sensitivity.

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