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Detection of the Hepatitis C Virus in Tear Fluid of Patients with Chronic Hepatitis C

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ABSTRACT

The highest prevalence rate of hepatitis C virus (HCV) infection in the world has been reported among Egyptians and up to 20 to 40% of patients chronically infected with HCV. The mode of transmission is still unknown. So the aim of this study was to detect the presence of HCV genome in the tear fluid of the patients with chronic HCV infection which may be a source of infection and to investigate the tear film function and stability in these patients. This study was done on 50 chronic active hepatitis C patients and 20 healthy subjects as a control group. The studied groups were subjected to full ophthalmological examination, liver function tests, qualitative detection of HCV-RNA in the serum and tear fluid by PCR and quantitative measurement of HCV-RNA in serum. Schirmer’s test and Break Up Time (BUT) show statistically significant decreased values in patients with +ve HCV RNA in their sera, while Rose Bengal scoring test show significant increased values. These results become more pronounced in patients with +ve HCV RNA in their tears. The viral RNA was detected by RT-PCR in the tear samples in 26 patients out of the 50 studied patients (52%) and most of cases with +ve HCV RNA in tears were associated with severe viraemia. We concluded that HCV-RNA was detected in the tear fluid quite frequently. This fluid therefore may be potentially responsible for non-blood transmission of the hepatitis C virus. This study also indicated that HCV-RNA play a role in development of the dry eye in those patients. So we recommended screening for HCV-RNA in patients with unexplained dry eye and appropriate disinfection of equipment in ophthalmological practice to avoid viral transmission.

INTRODUCTION

Hepatitis C virus (HCV) is a single stranded RNA virus related to the Flaviviridae family. It infects at least 200 million people worldwide.¹,²

High prevalence rates of hepatitis C virus were reported among Egyptian blood donors. The prevalence of antibodies to HCV were found in 18.1% of residents of a rural village, 22.1% of army recruits and 41% in adults greater than the age of 50 years.³

The hepatitis C virus is mainly transmitted by the parenteral route. However there is no identifiable source of infection in approximately 50% of patients with HCV infection. The virus may be transmitted via body fluids ⁴, ⁵, ⁶, but few studies show the presence of HCV RNA in tear fluid ⁷,⁸.

Lacrimal glands involvement was shown to be common in hepatitis C infection⁹-¹². Patients with chronic hepatitis C virus infection show both decreased tear volume and decreased tear lactoferrin concentration. These finding suggest that there may dysfunction of the lacrimal glands in patients with chronic hepatitis C, which may account for the mild dry eye.¹³,¹⁴

The diagnosis of dry eye was made by dry eye criteria, which are: (I) symptoms of dry eye (e.g. irritation, foreign body sensation, burning, stringy mucus discharge, transient blurring of vision and photophobia), (II) abnormalities of tear dynamics determined by Schirmer’s test and tear film break up time (BUT) and (III) abnormalities of ocular surface determined by Rose Bengal or fluorescein vital stain ¹⁵.

So the aim of the study was to detect the presence of HCV genome in the tear fluid of the patients with chronic hepatitis C virus infection which may be a source of infection among patients and doctors due to contamination. Also, this study aims to investigate the tear film function and stability in these patients.

SUBJECTS, MATERIAL & METHODS

This study was carded out on 70 subjects; they were classified into two groups: Group (I): 50 patients (28 males and 22 females), their age ranging from 30-70 years (mean 48.6 ± 12.1) with chronic active hepatitis C infection. They were selected from patients attending the internal medicine
outpatient clinic in Zagazig and Benha University hospitals according to the following criteria: elevation of serum levels of ALT and AST for at least 6 months, detectable anti-HCV antibodies, HCV-RNA and histopathological examinations of liver biopsy consistent with chronic active hepatitis.

Group (II): 20 apparently healthy subjects (11 males and 9 females), their age ranging from 29-69 years (mean 50.6 ± 11.3) as a control group. They were selected from patients attending the ophthalmology outpatient clinic in Benha University hospitals.

An informed consent was taken from all subjects after discussing with them the aim of the study.

Patients with history of chronic eye diseases and external ocular diseases were excluded from this study.

The studied groups were subjected to the following:

- Full history taking with special emphases on symptoms suggestive of dry eye (e.g. irritation, foreign body sensation, stringy mucus discharge, transient blurring of vision) and symptoms suggestive of chronic liver diseases (e.g. ascitis, jaundice or lower limb edema).
- Full ophthalmological examination and recording of dry eye signs\(^{15}\) such as:
  - Slit lamp examination
  - Schirmer's test No I (without topical anesthesia).
  - Tear film break up time (BUT).
  - Rose Bengal staining scores.
- Liver functions tests: including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin using Bayer Advia 1650 Chemistry Analyzer.
- Qualitative detection of HCV-RNA in serum by reverse transcriptase polymerase chain reaction (RT-PCR) using Amplicor HCV PCR diagnostic Kit (Roche Diagnostics, USA) It is based on five major processes: specimen preparation, reverse transcription of the target RNA to generate complementary DNA (cDNA), PCR amplification of the target cDNA using HCV specific complementary primers, hybridization of the amplified products to oligonucleotide probes specific to the target and detection of the probe-bound amplified products by colorimetric determination.\(^{16}\)
- Quantitative measurement of HCV-RNA in serum using Quantiplex HCV RNA 2.0 assay (bDNA) (Chiron Diagnostic, USA).

Principle: It is a sandwich nucleic acid hybridization procedure for the direct quantification of HCV-RNA in human serum. After HCV genomic RNA is released from the virion, the RNA is captured to microwell by a set of specific, synthetic oligonucleotide capture probe. A second set of target probes hybridizes to both the viral RNA and the pre-amplifier probes. The capture and target probes bind to the 5'untranslated and core regions of the HCV genome. The amplifier probe subsequently hybridizes to the pre-amplifier forming a branched DNA (bDNA) complex. Multiple copies of an alkaline phosphatase conjugated probe are then hybridized to this immobilized complex to amplify the signal. Detection is achieved by incubating the complex with a chemiluminescent substrate and measuring the light emission which is directly proportional to the amount of HCV RNA present in each sample.\(^{17}\) A standard curve is defined by light emission from standards with known concentrations of recombinant bacteriophage. Concentrations of HCV-RNA in samples are determined from this standard curve. Patients in this study were classified according to level of viraemia into:

  - Mild viraemia (rang of viral load was from 600to 100,000 IU/ml).
  - Severe viraemia (rang of viral load was more than 100,000 IU/ml).
- Qualitative detection of HCV-RNA in tears: After the instillation of 50 μm of sterile saline to the patient's eye, tears were collected with a micropipette. The samples were immediately transferred to a microtube containing the RNase inhibitor (Promega, Madison, USA), then stored at -70°C until analysis.\(^{8}\) RNA was extracted by QIAamp Viral RNA mini Spin (Qiagen, USA). cDNA was synthesized in presence of 1μM of the primer (Innogenetics, NV, Ghent, Belgium) located in the 5' untranslated region of the viral genome (5'-CAC TCG CAA GCA CCC TAT CA-3') in a mixture containing 200 U RT-MLV, 0 mM of each dNTP, 200 mM DTT and RT buffer (Qiagen, QIAamp, USA) for 1 hr at 37°C. The cDNA was amplified with Tag polymerase in thermal cycler (Biometra, Germany) in presence of the primers (26S; 5'-TGT GGT ACT GCC TGA TAG GG-3') and (J85A; 5'-AGG AAG ATA GAG AAA GAG CA-3') (Innogenetics, NV, Ghent, Belgium); the PCR consisted of 3 min at 94°C, then 40 cycles of 1 min each of 94°C, 50°C and 72°C and final incubation at 72°C for 10 min. A second round of amplification was done with a different set of nested primers (27S; 5'- GAT AGG GTG CTT GCC AGT GC-3') and (J82A; 5'- AAA
TIC CCT GTT GCA TAG TT-3’ (Innogenetics, NV, Ghent, Belgium). The final amplified products were visualized under UV light after separation by 2% agarose electrophoresis. The DNA Molecular weight marker (Bioron-Germany) which gave bands ranging from 100-3000bp. Florescent bands of products equivalent to the MW of 552 bp were recorded as positive for HCV-RNA.

• **Statistical analysis:** Statistical analysis was done using the Statistical Package for Social Sciences version II (SPSS Inc., Chicago, USA). For quantitative data, they were summarized by mean ± standard deviation; comparison of two means was calculated by t test. For qualitative data; they were presented as number and percentage and analyzed by Chi Square test, Fisher exact test was recommended when expected cell is less than 5. P value of <0.05 was considered significant.

![Fig (1): A 2% agarose gel containing the RT-PCR products](image)

Lane M, 100-bp molecular weight marker, lanes 1,3,4,5 and and 7 positive samples (each has one DNA band of approximately 552 bp), lanes 2 and 6 negative samples (No DNA band)

**RESULTS**

The comparison of Schirmer’s test, BUT and Rose Bengal stain among patient and control groups was studied as shown in table (!). Schirmer’s test values were found to be significantly lower among chronic hepatitis C patients (mean 15.8 ± 4.5 mm/5min, P =0.001) when compared with healthy control group (mean 20.0 ± 3.5mm/5min).

Statistical analysis showed significant decrease in BUT value among chronic hepatitis C patients (10.16 ± 2.8 sec) when compared with control group (15.8 ± 1.4 sec). Also Rose Bengal staining results among chronic hepatitis C patients showed statistically significant higher scores (mean 84.0%) than the healthy control group (mean 0.0%).
Table (1): Comparison of Schirmer's test, BUT and Rose Bengal stain among patient and control groups.

<table>
<thead>
<tr>
<th>Test</th>
<th>Patient group No=50</th>
<th>Control group No=20</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sch. test (mm/5min)</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>0.001</td>
</tr>
<tr>
<td>BUT test (seconds)</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>0.001</td>
</tr>
<tr>
<td>RB stain</td>
<td>No %</td>
<td>No %</td>
<td>0.001</td>
</tr>
<tr>
<td>+ve</td>
<td>42 84%</td>
<td>0.0 0.0</td>
<td></td>
</tr>
<tr>
<td>-ve</td>
<td>8 16%</td>
<td>20 100%</td>
<td></td>
</tr>
</tbody>
</table>

Sch= Schirmer's test. BUT= Break Up Time. RB =Rose Bengal.

The HCV-RNA was detected in the serum samples of all patients and none of control group. Also the viral genome was detected in the tear samples of 26 patients out of the total 50 studied patients (52 %) (Results were not presented).

As regard the relation between the presence of HCV in tears and tear film function, there was no statistically difference between Schirmer's test, BUT test or Rose Bengal staining test in patients with HCV PCR +ve in tears and patients with HCV PCR -ve in tears (P>0.05) (Table 2).

Table (2): The relation between presence HCV RNA in tears and tear film function among patient group.

<table>
<thead>
<tr>
<th>Test</th>
<th>Patient group No=50</th>
<th>Control group No=2</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sch. test (mm/5min)</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>0.12</td>
</tr>
<tr>
<td>BUT test (seconds)</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>0.27</td>
</tr>
<tr>
<td>RB stain</td>
<td>No %</td>
<td>No %</td>
<td>0.013</td>
</tr>
<tr>
<td>+ve</td>
<td>24 92.3%</td>
<td>18 75%</td>
<td></td>
</tr>
<tr>
<td>-ve</td>
<td>2 7.7%</td>
<td>6 25%</td>
<td></td>
</tr>
</tbody>
</table>

Sch.= Schirmer's test. BUT= Break Up Time. RB=Rose Bengal.

The relation between detection of HCV-RNA in tears and viral load of HCV in serum among patient group was studied as shown in table (3). This table shows that HCV-RNA was detected in tear samples of 18 patients out of 22 patients with severe viraemia (81.8%), while it was detected only in 8 patients out of 28 patients with mild viraemia (28.6%) with statistical significant difference (P=0.001).

Table (3): Relation between presence of HCV-RNA in tears and viral load of HCV in serum among Patient groups

<table>
<thead>
<tr>
<th>Test</th>
<th>HCV RNA +ve in tear No=26</th>
<th>HCV RNA -ve in tear No=24</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral load of HCV in serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild viraemia (No=28)</td>
<td>8 28.6%</td>
<td>20 71.4%</td>
<td>0.001</td>
</tr>
<tr>
<td>Severe viraemia (No=22)</td>
<td>18 81.8%</td>
<td>4 18.2%</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The HCV RNA is mainly transmitted by the parenteral route. However, different epidemiological studies have revealed that for up to 20-40% of patients clinically infected with HCV, no known risk factors for HCV transmission can be demonstrated. Risk factors for transmissibility of hepatitis C virus include exposure to blood products by means of intravenous drug use, transfusion, needle sticks and maternal-fetal transmission. However, the isolation of hepatitis C virus from tear fluid and aqueous humor raises the possibility of transfer of hepatitis C during the course of an ophthalmologic examination, that is, Goldmann tonometry and trial contact lens fitting.

Involvement of lacrimal glands was shown to be common in hepatitis C infection and the majority of chronic hepatitis C patients show symptoms and signs of dry eye.

In order to decide whether pathways other than blood can play a role in HCV transmission, the serum and the tear fluid from patients with chronic hepatitis C virus were screened for the presence of the viral genome by polymerase chain reactions (PCR).

In the present study Schirmer's test and BUT values showed a significant decrease in chronic hepatitis C group when compared to the control group.

The results of Schirmer's test value in the present study came in accordance with those obtained by Uno et al. and Wenke et al. who reported that Schirmer's test values are lower in the chronic hepatitis C patients due to reduced lacrimal gland function.

Fukuda and Wang gave an explanation to the decreased BUT as it may be due to mucin deficiency and lipid abnormalities. Also they stated that non invasive BUT showed a reasonable level of accuracy, so it is better to be performed to determine the precorneal tear film stability.

Significant higher staining scores of Rose Bengal of corneal and conjunctival epithelium were recorded in chronic hepatitis C group of the present study when compared with control group due to tear deficiency states. These results came in accordance with those obtained by Pflugfelder et al. who reported that Schirmer's test results were correlated inversely with Rose Bengal staining scores.

Abe et al. and Tohru et al. concluded that chronic hepatitis C patients showed both decreased tear volume and decreased tear lactoferrin concentration. These findings suggest that there may be dysfunction of the lacrimal glands in patients with chronic hepatitis C that may account for mild dry eye. Also Jacobi et al. and Chen et al. concluded that dry eye syndrome is the most frequently observed ocular feature in HCV infection.

The results of the present study can be explained by the findings of Goncalves et al. who reported that various immunologic and clinical abnormalities can occur in chronic hepatitis C patients including vasculitis. While Martinez et al. reported that chronic hepatitis C is associated with a broad range of autonomic manifestations. Ramos et al. stated that the production of different auto-antibodies and cryoglobulines are responsible for systemic vascular and various organ damage as extrahepatic manifestations of hepatitis C virus, such as lacrimal glands which reported by Abe et al. to be affected in patients with hepatitis C virus.

In this study, we screened for the presence of HCV genome in the tear fluid of 50 patients with chronic hepatitis C who were seropositive for HCV RNA. The viral RNA was detected in the tear fluid of 26 patients (52.0%).

Feucht et al. conducted a study in Germany in which plasma, tear fluid and swabs from the eye of 33 patients were examined for the presence of hepatitis C virus (HCV) RNA by polymerase chain reaction. All samples from plasma, tear fluid and eye swabs were found to show a positive reaction in HCV RNA PCR.

Shimazaki et al. conducted another study in Japan in which ten (10) tear samples and five (5) aqueous humor samples were collected from ten patients with anti-hepatitis C virus antibody as determined by a second-generation enzyme immunoassay. HCV RNA was detected by RT nested PCR in nine (9) of the tear sample and all five of the aqueous humor sample. Although the infectivity of the samples was not determined, the authors suggested that tear fluid and aqueous humor may represent an additional route of hepatitis C virus transmission. The authors also recommended that tonometers and contact lens trial sets should be disinfected after their use for all patients; they also suggested that the corneas from hepatitis C virus antibody positive donors are contaminated by the hepatitis C virus.

These studies agreed with our results although the percentage of positive cases is even higher, in addition it included samples obtained from the aqueous humor.
Feucht et al.\(^8\) conducted a study on 76 patients chronically infected with HCV who were positive by HCV antibody testing; these samples were examined for the presence of HCV RNA by RT-PCR. All 76 patients chronically infected with HCV were positive by RT-PCR for tear fluid and plasma. Furthermore tear fluid regularly showed a stronger RT-PCR signal compared with that of blood plasma. The authors also demonstrated that tear fluid is a suitable diagnostic material for detection of HCV RNA.

On the other hand lower percentage of positivity of HCV RNA in tear fluid were detected by Mendel et al.\(^9\) and Jacobi et al.\(^12\) who detected HCV RNA in tear samples by RT-PCR in about 10% of patients with chronic HCV infection.

Although there are great difference in the percentages of positive HCV RNA detected by PCR in the tear fluid of chronic hepatitis C (10%) in the studies of Mendel et al.\(^9\) and Jacobi et al.\(^12\) compared to (52%) in our study and 90% and 100% in the studies performed by Shimazaki et al.\(^8\) and Feucht et al.\(^7,8\) respectively, all these studies proved the presence of the viral genome in the tear fluid, moreover they suggested that this tear fluid is potentially responsible for non-blood transmission of hepatitis C virus.

The relation between presence of HCV-RNA in tears and viral load of HCV in serum among patient group was studied. It was found that the most of cases with positive HCV RNA in tears were associated with severe viraemia. These results were in agreement with the results of Feucht et al.\(^7\) and Shimazaki et al.\(^8\). In contrary, Mendel et al.\(^9\) found that the presence of RNA in tear fluid was independent of the severity of the hepatitis and of the viral load as measured by branched DNA assay.

From all the previously discussed studies as well as the current study we concluded that there is a relatively high probability of the presence of infectious viral particles in the tear fluid of chronic hepatitis C patients which might be a potential source for the transmission, and in a country like Egypt where seropositive patients for HCV antibodies form about (9.2%) of the general population\(^4\) this conclusion is of a particularly high concern.

Further investigations are needed to determine the source of HCV-RNA in the tear fluid: transudation or active replication? And to determine its potential infectivity.

This study also indicates that HCV RNA plays a role in development of dry eye in those patients. So, screening for HCV RNA in patients with unexplained dry eye is recommended.

Also appropriate disinfection of equipment in ophthalmological practice would appear to be necessary in view of the presence of HCV in ocular fluid.

Acknowledgment
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REFERENCES


اكشف عن الفيروس الكبدى سي في السائل الدمى في مرضى الالتهاب الكبدى المرئى (سي)

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كلية الطب البشري، جامعة الزقاق(21) وجامعة بنها(3)

وقد قام الفيروسات الكبدى (سي) بانتشار في كل أنحاء العالم وأن معدل نتائجهما ما زال غير معروف، ولكن أكبر انتشار له موجود في مصر. ويعد استخدام الإبرة الملونة والمولدة والقليل من أكثر الأسباب انتقال عندما فيروس (سي) إلا أن حوالي 20-40% من حالات الالتهاب الكبدى (سي) المزمن لا يوجد لها سبب واضح ومعروف للعنوى.

ونجد نتائج هذا البحث عن إنزيمات فيروس في السائل الدمى الذي يمكن أن يكون مصدر للإيبير فايروسات المرئى والأطباء.

وإلى ذلك، لبيهذ البحث أيضًا تقييم وظيفة السائل الدمى في مرضى الالتهاب الكبدى (سي).

قد أجرب هذا البحث على مرضى من مرضى الالتهاب الكبدى (سي) المزمن وعشرين شخصًا من الأسماء كمجموعة ضابطة وقد قام بفيروس وثباث السائل الدمى المزمني لدى استخدام اختبارات شيرير واسبغ والروز بنجال وقابلات تشابه السائل الدمى. كذلك، نجد أن عدد في حالات السائل الدمى لكربخ وحظى تلك العينات البحث عن الحمض النووي (آي. إن. آي.) للفيروس والفيروسات الكبدى في مرضى السائل الدمى بفاعل إنزيم الليازة السائل المزمني والثاني.

وقد تم الكشف عن الفيروس في 64 عينة (54%) من عينات السائل الدمى لمرضى الالتهاب الكبدى (سي) وكان أكثرهم من مرضى في المزمنى الكلي العالمي للفيروس في الدم، كما أنه وجدنا أن هناك خلل في وظيفة وثباث السائل الدمى المزمني كبير من مرضي وثباثة في مرضى الذين تم الكشف عن الفيروس في عينات السائل الدمى لهم. وهذه النتيجة تحد من إمكانية أن يكون السائل الدمى مصدرًا للإيبير فيروسات الالتهاب الكبدى (سي) خاصة من طرق عيون العيون، وأيضًا

بين أن هناك علاقة بين إيبير فيروس الالتهاب الكبدى (سي) وجودة سطح العين.

لكن ونحن في دولة نحن لم يلزمنا التأكد مننلكن، ونحن نحب أن ندرك أن لا توجد أسلوب للمعالجة، وذلك لتقييد كافية على ضخ العين، ونقدر من الأجهزة التي تحسن سطع العين بالإضافة إلى تعيين العدسات اللاصقة التجريبية داخل عيون.

طب العيون وعملية التمديدات لاصقة بين الأشخاص.

وكذلك، نشكرك على البحث الذي يمكنا أن tutoring فيروسات الالتهاب الكبدى (سي) بفاعل إنزيم الليازة المتبلاسل لجميع عيان العين الغير مصحوبة بآسيا واضحة لها.