Prevalence of occult hepatitis C virus in patients with HCV-antibody positivity and serum HCV RNA negativity

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Introduction and aim
Chronic hepatitis C infection is a global problem with an increasing burden on healthcare, particularly in Egypt. Even with the advent of highly sensitive techniques, a subset of patients with positive hepatitis C virus antibody (HCV-Ab) and negative HCV-viremia remain challenging to treat. Therefore, we tried to determine the prevalence of occult HCV infection (OCI) in peripheral blood mononuclear cells (PBMCs) of patients presenting with a positive serologic test for anti-HCV-Ab and negative serum HCV-RNA-PCR (spontaneously cleared patients) and followed up those patients.

Patients and methods
Between March 2010 and March 2015, a prospective study was designed to include all consecutive patients with HCV-Ab positivity and HCV-RNA negativity who attended the Assiut Unit for treatment of viral hepatitis – the National Committee for Control of Viral Hepatitis. A total of 25 patients were recruited. Spontaneous clearance of serum HCV infection was approved on the basis of HCV-Ab positivity using two third-generation enzyme-linked immunosorbent assay tests and serum HCV RNA negativity on three consecutive occasions, each 6 months apart. Follow-up serum HCV RNA levels were evaluated for patients with OCI every 6 months. The RNA extraction step was performed by a protocol modified from that of the QIAamp viral RNA kits. Blood samples for separation of PBMCs were collected from all patients. PBMCs were obtained using Ficoll–Hypaque density gradient of EDTA anticoagulated blood according to the manufacturer's instructions (Lymphoflot). Detection of HCV viral load was performed with the kit supplied by Applied Biosystem (HCV RT-PCR Kit lot No.).

Results
A total of 25 patients (21 men, mean age 36.2 ± 9.1) cleared HCV spontaneously (HCV-Ab positive and serum HCV RNA negative). Genomic HCV RNA was detected in PBMCs of three (12%) of 25 patients. These three patients with OCI were followed up for 18 months by measuring their serum HCV RNA using highly sensitive real-time PCR every 6 months. Only one patient became overt HCV with a low level of viremia.

Conclusion
OCI was detected in a considerable prevalence in patients who cleared HCV spontaneously, that entails corporations of HCV-viral assay in PBMCs into the diagnostic algorithm.

Keywords:
HCV spontaneous clearance, OCI, PBMCs

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Introduction

Chronic hepatitis C infection is a global problem with an increasing burden on healthcare, particularly in areas with high endemicity like Egypt. The WHO estimates that ~200 million people worldwide are infected with hepatitis C virus (HCV). In Egypt, it was estimated that 15% of Egyptians had serologic evidence of HCV infection [1].

Since its discovery, the gold standard method for diagnosis of HCV has been serologic detection of both HCV antibodies (HCV-Abs) and the RNA viral load (HCV-RNA-PCR) in the serum [2,3]. However, and even with the advent of highly sensitive techniques, a subset of patients with positive HCV-Ab and negative HCV-viremia remain challenging to treat.

A new entity of HCV infection was first described in 2004 in patients with persistently elevated liver function tests and who were anti-HCV and serum HCV RNA negative; this clinical situation was termed OCI [4].

OCI has also been described in two other different clinical settings. One of these is in anti-HCV-positive, serum HCV-RNA-negative patients with persistently normal values of liver enzymes (asymptomatic HCV carriers), of whom nearly 90% have detectable viral RNA in the liver and in peripheral blood mononuclear cells (PBMCs) [5,6].

Therefore, the gold standard for the diagnosis of this occult situation depends mainly on the detection of HCV-RNA in liver cells. However, because of the invasive nature of liver biopsy, other alternatives were studied in an attempt to increase the sensitivity of the diagnostic tests related to serum by examining the HCV-RNA-positive and HCV-RNA-negative (replicative) strands as well as virus proteins in the PBMCs in patients with chronic hepatitis C, especially when liver biopsy is not feasible [7,8].

The question of whether HCV does persist as occult infection in individuals with clinically resolved hepatitis C is undoubtedly of tremendous epidemiological and pathogenic importance and deserves a thorough investigation. Therefore, we herein tried to determine the prevalence of OCI in PBMCs of patients presenting with positive serologic test for anti-HCV-Ab and negative serum HCV-RNA-PCR (spontaneously cleared patients) and also followed up those patients.

Patients and methods

Patient selection

Between March 2010 and March 2015, a prospective study was designed to include all consecutive patients with HCV-Ab positivity and HCV-RNA negativity who attended the Assiut Unit for treatment of viral hepatitis – the national committee for control of viral hepatitis. A total of 25 patients were recruited. Spontaneous clearance of serum HCV infection was approved on the basis of HCV-Ab positivity using two third-generation enzyme-linked immunosorbent assay tests and serum HCV RNA negativity on three consecutive occasions, each 6 months apart. Follow-up serum HCV RNA evaluation for patients with occult HCV infection (OCI) was done every 6 months. Patients with positive serum HCV RNA, combined HCV/HBV infection, autoimmune liver disease, and proven hepatocellular carcinoma were excluded.

RNA isolation from serum

The RNA extraction step was performed by a protocol modified from that of the QIAamp viral RNA kits (Qiagen, Courtaboeuf, France) as follows: 200 µl of serum sample was suspended in 600 µl of AVL lysis buffer (Qiagen). After an incubation step of 10 min at room temperature, 600 µl of absolute ethanol was added and the whole mixture was centrifuged through a QIAmp column at 6000*g* for 1 min. After two successive washing steps with AW1 buffer for 1 min at 6000*g* and AW2 buffer for 3 min at 12 000*g*, the RNA extracts were removed from the column by addition of 200 µl of AVE elution buffer and were centrifuged at 6000*g* for 1 min.

Preparing of peripheral blood mononuclear cells lysate

Blood samples for separation of PBMCs were collected from all patients. PBMCs were obtained using Ficoll–Hypaque density gradient of EDTA anticoagulated blood according to the manufacturer's instructions (Lymphoflot; Biotest, Dreleich, Germany). Cells were washed three times with Mg^{2+}-free and Ca^{2+}-free PBS and resuspended to 1×10^6 cells/ml in PBS.

RNA isolation from PBMCs: to isolate RNA from PBMCs, 500 ml of Trizol reagent was added in each Eppendorf tube containing white pellets of PBMCs and slightly shaken to get the mixture of the cells. Then chloroform (100 ml) was added in each Eppendorf tube containing 500 ml Trizol reagent and cell mixture, shaken for 2–5 times by hand to mix, and centrifuged at 13 200 rpm for 15 min at 4°C. The upper layer containing the RNA (leaving behind the precipitated protein-DNA pellet) was pipetted out into a clean 1.5 ml tube containing 500 µl of 100% isopropanol and centrifuged at 13 200 rpm for 15 min; the RNA was visible as a small, translucent pellet. The supernatant was poured off and the tube was drained briefly on clean absorbent paper. A measure of 500 µl of 70% ethanol was added and the tube was inverted several times to wash the RNA pellet and then centrifuged at 13 000 rpm for 10 min. The ethanol was then carefully poured out. The tubes were inverted and drained on clean absorbent paper and allowed to air-dry 10–15 min. Hydration solution (20 µl) was added to the RNA pellets to dissolve them. The solution was then kept on ice for 30 min before a short spin.

Hepatitis C virus amplification by real-time PCR

The HCV viral load was detected using a kit supplied by Applied Biosystem (HCV RT-PCR Kit lot No., Qiagen,courtaboeuf, France), which constitutes a ready-to-use system for the detection of HCV RNA using PCR on a 7500 fast analyzer supplied by Applied Biosystem. The HCV quantitative real-time PCR kit is based on RNA reverse transcription process and consequent cDNA fragment amplification with PCR. The amplification process lies in repeated cycles: thermal DNA denaturing, primer annealing with

complementary sequences, and completion of further polynucleotide chains by Taq-polymerase. An internal control (IC) sample corresponding to a stabilized RNA fragment was added to a sample being examined at the stage of nucleic acid isolation and intended for estimation of the efficacy of all the examination stages. The HCV quantitative real-time PCR kit and DNA probes, each of which contains a fluorescence label and fluorescence quencher, were included in the PCR mix. In case of specific cDNA product formation, a probe gets destroyed and that leads to fluorescence level growth registered by special appliances. DNA probes used for sought nucleic acid and IC PCR product detection are labeled with FAM and HEX fluorescence probes accordingly. That allows separate registration of HCV cDNA and IC sample PCR results.

Statistical analysis

Data were entered, cleaned, and analyzed using SPSS, version 16 (SPSS Inc., Chicago, Illinois, USA). Descriptive statistics such as frequencies and means and SD were calculated.

Ethical aspects

The study protocol was approved by the local ethics committee in the Faculty of Medicine, Assiut University. All patients were informed about the study in detail and written consent was obtained.

Results

A total of 25 patients (21 men; mean age 36.2 ± 9.1) who cleared HCV spontaneously (HCV-Ab positive and serum HCV RNA negative) were recruited into the study. Genomic HCV RNA was detected in PBMCs of three (12%) of 25 patients (Tables 1 and 2).

Results of follow-up

The three patients with OCI were followed up for 18 months by measuring serum HCV RNA using highly sensitive real-time PCR every 6 months. Only a single patient became overt HCV with a low level of viremia.

Table 1 Characteristics of patients in the studied groups

Category	Spontaneous clearance (*n*=25) (*n* (%))
Sex	
Male	21 (84)
Age	
Mean±SD	36.2 ± 9.1
BMI	
Mean±SD	25 ± 2.9
Diabetes	2 (8)
LFTs	
ALT	
Normal	22 (88)
Increased	3 (12)
AST	
Normal	24 (96)
Increased	1 (4)
Bilirubin	
Normal	25 (100)
Increased	0 (0)
GGT	
Normal	25 (100)
Increased	0 (0)
Albumin	
Normal	25 (100)
Decreased	0 (0)
INR	
Normal	25 (100)
Increased	0 (0)
CBC	
WBC	
Normal	25 (100)
Increased	0 (0)
Decreased	0 (0)
HB	
Normal	23 (92)
Decreased	2 (8)
Platelets	
Normal	25 (100)
Decreased	0 (0)
AFP	
Normal	25 (100)
Increased	0 (0)
Liver ultrasound	
Normal	23 (79.3)
Abnormal echopattern	2 (6.9)
Cirrhosis	0 (0)

AFP, α-feto protein; ALT, alanine transaminase; AST, aspartate transaminase; CBC, complete blood count; GGT, γ-glutamyle transferase; Hb, hemoglobin; INR, international normalization ratio; LFT, liver function test; WBC, white blood cell.

Discussion

Egypt has the largest epidemic of hepatitis C in the world, making it the most challenging public health problem facing the country. In The Egyptian Demographic Health Survey (EDHS 2008), the overall prevalence of HCV was 14.7% and HCV-viremia was detected in 9.8%, which means almost 10% of the total population is infected and infectious to other people [1].

In the past years, a new entity of HCV infection was identified and defined as OCI, in which HCV-RNA is undetectable in serum by conventional assays, but patients have HCV-RNA in the liver or PBMCs [9]. The long-term presence of HCV infection after

Table 2 Results of HCV RNA detection in serum and PBMCs

Category	Spontaneous clearance (*n*=25) (*n* (%))
Serum HCV RNA	
Positive	0 (0)
Negative	25 (100)
PBMCs HCV RNA	
Positive	3 (12)
Negative	22 (88)

HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cell.

spontaneous resolution of hepatitis C was also described with recurrent infection in kidney transplant recipients treated with immunosuppressive therapy [10].

Persistence of HCV as occult infection following natural clearance or successful antiviral therapy clinically has epidemiological and pathogenic importance and deserves a thorough investigation. This may confirm not only a new view on the natural history of HCV infection but may also warrant considerations of new preventive and therapeutic practices against HCV infection, which continues beyond a clinically apparent recovery [11].

In our study, genomic HCV RNA was detected in PBMCs of 3/25 patients (12%) who cleared HCV spontaneously and test positive for HCV-Ab and negative for serum HCV RNA. A total of three patients discovered to have OCI in our study were followed up with highly sensitive real-time PCR in serum every 6 months for a period of 18 months. Only one case became overt HCV with HCV RNA detected in serum. This patient cleared HCV spontaneously. This patient achieved sustained viral response after treatment with sofosbuvir 400 mg daily plus pegylated interferon weekly and weight-based ribavirin for 12 weeks. The patient also tested negative for HCV RNA in PBMCs.

As mentioned before, although the detection of HCV RNA in liver biopsy specimens is the gold standard method for diagnosis of OCI, detection of HCV RNA in PBMCs is an alternative procedure in the absence of liver biopsy. HCV RNA was detected in the PBMCs of 70% of patients with occult infection [4,9]. Therefore, detection of HCV RNA in PBMCs does not identify all cases with OCI.

Contrary to our study findings, several investigators did not find the presence of OCI [12,13], which might be due to the use of commercial assays and the sensitivity of assays not described in some studies. Also, all the previous studies included a small number of patients, ranging from 5 to 27.

Our findings as regards the detection of OCI in patients who spontaneously cleared HCV matched with the studies conducted by Carreño *et al.* [8], Pham *et al.* [14], Falcón *et al.* [6], Radkowski *et al.* [5], and Veerapu *et al.* [15], but with different prevalence rates.

Several Egyptian studies were conducted to determine the prevalence of occult HCV in different groups of patients, such as in healthy spouses of patients with HCV infection [16], in patients with chronic lymphoproliferative disorders [17], those with nonalcoholic fatty liver disease [18], and hemodialysis patients [19]. To the best of our knowledge, the current study was the first Egyptian study to investigate the prevalence of occult HCV in patients with so-called spontaneous HCV recovery. Despite this, the present study had some limitations, such as absence of confirmed OCI in liver tissue and small sample size. Therefore, we recommend a large prospective cohort study to overcome these limitations.

Conclusion

OCI was detected in a considerable prevalence in patients with apparent clearance of HCV-viral load, that entails incorporations of HCV-viral assay in PBMC into the diagnostic algorithm. Also OCI may changed to an overt infection.

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Conflicts of interest

There are no conflicts of interest.

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