Detection of *Hepatitis C virus* RNA in peripheral blood mononuclear cells of patients with abnormal alanine transaminase in Ahvaz

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**Abstract**

**Purpose:** Hepatitis C virus (*HCV*) is an important agent for chronic and acute hepatitis. Occult hepatitis C remains a major health problem worldwide. Patients with chronic occult *HCV* may progress to cirrhosis and hepatocellular carcinoma. The aim of this study was to determine prevalence of occult hepatitis C by IS-PCR-ISH (*in situ* PCR *in situ* hybridisation) in the patients with abnormal ALT. **Materials and Methods:** The blood samples were taken from 53 patients including 17 females (32.1%) and 36 (67.9%) males who had abnormal alanine transaminase (ALT) for more than 1 year. The mean ALT and aspartate transaminase (AST) level were 41.02 ± 9.3 and 24.17 ± 7.3, respectively. The patients«SQ» age were between 4 and 70-years old with mean age 38 ± 13. All the patients were negative for HCV antibody, HCV RNA and HBs Ag. The peripheral blood mononuclear cells (PBMC) were separated with ficoll gradient from each blood sample, then the cells were fixed on slides by cold acetone and followed by IS-PCR-ISH for HCV RNA detection. **Results:** Seventeen (32%) patients including 6 (11.3%) females and 11 (20.7%) males showed positive results for HCV RNA by *in situ*-PCR *in situ* hybridisation. Ten (18.8%) positive cases were between 20 and 40-years old and 6 (11.3%) positive patients were between 40 and 60 years old. Ten (19.6%) patients who were positive for *IS-PCR-ISH* also had positive anti-HBc IgG and 7 (13.2%) patients were negative for HBc-IgG. **Conclusion:** In the present study high rate of 32% occult hepatitis C were found among the patients with elevated ALT.
Introduction

Hepatitis C virus (HCV) is an enveloped virus with a single-stranded positive sense 9.6-kilobase RNA nucleic acid. HCV classified in the family Flaviviridae and genus Hepacivirus. The major organ that can support HCV replication is liver; however, in recent years replicating form of HCV was found in the peripheral blood mononuclear cells (PBMC). The lack of an efficient vaccine against HCV makes it a global health problem. HCV is an important agent for acute and chronic hepatitis that approximately 180 million people get chronically infected with it and some of them lead to hepatocellular carcinoma. Presence of HCV RNA in hepatocyte and peripheral blood mononuclear in the absence of detectable HCV RNA in the serum specimen defined as occult hepatitis C infection. Patient with occult hepatitis C infection were divided in two types. Type A patients who were seronegative for anti-HCV antibody and the type B are the patients chronically infected who have sustained virological response to therapy and were seropositive. Detection of virus in biopsy of liver is the gold standard test for diagnosis of occult HCV infection but it is an invasive method. Lymphotropism of HCV was well known thus PBMCs are the other source of virus during HCV infection and could be used for HCV RNA detection. Based on other reports, the rate of positive mononuclear cells for HCV RNA in patients with persistent elevated ALT level were found to be 10-50%. The aim of present study is to evaluate the rate of occult HCV infection in patients with abnormal ALT.

Materials and Methods

In this study, we have collected 53 blood samples from patients including 17 females (32%) and 36 (68%) males who had abnormal ALT persistent for more than 1 year. All of the patients were registered in Danesh laboratory in Ahvaz city, Iran during 2011-2012. Ethic consent was taken from all the patients.

All the patients had abnormal ALT and AST with mean level of 41.02 ± 9.3 and 24.17 ± 7.3, respectively. The patients were divided in three age groups. The mean age of patients was 38.8 ± 13.77 with minimum 4 and maximum 70-years old. Patients with fatty liver disease, drug users, autoimmune hepatitis, genetic disease and metabolic disorder were excluded. The ficoll gradient (Stem Cell Technologies, USA) was used to separate PBMC. According to the instruction, briefly 3 ml of whole blood diluted with 6-ml PBS containing 2%
foetal bovine serum and then carefully 3 ml Ficoll Hypaque was added followed by centrifugation at 1200 \( \times \) g for 20 minutes at room temperature. The whitish layer was separated and washed three times with 1X hanks balance solution followed by final centrifugation at \( x2000 \) g for 20 minutes before the pellet was collected. The pellet was reconstituted to final concentration of 500000 cells/ml. About 100 \( \mu \)l of cells was smeared on glass slides and covered with cold acetone (-20) to fix cells. The PBMC of the 10 patients whom were positive for HCV antibody and RT-PCR used as positive control. The PBMC of the 10 patients whom had normal ALT, AST and were negative for HCV antibody and RT-PCR were used as negative control. {Table 2} {Table 3}

All the patient sera were tested for Anti HCV Ab, HBsAg, HEV IgM and HBc IgG using ELISA kit (Diapro, Italy), according to manufacturer. The samples which were negative for HCV antibody, HCV RNA, HBs Ag and HEV IgM, entered this study.

RNA extraction and cDNA synthesis

The total RNA was extracted from serum samples using a high pure viral RNA kit (Roach, USA). The cDNA was prepared using cDNA synthesis kit (Thermo science, USA). Briefly, 6 \( \mu \)l of each sample was mixed with 1 \( \mu \)l random hexamer and 5 \( \mu \)l DEPC water was added to make mixture (A). The solutions were incubated at 65° C for 5 minutes and immediately chilled on ice. To make mixture (B), 4 \( \mu \)l of \( \times 5 \) reaction buffer was mixed with 1-\( \mu \)l RNase inhibitor (20 unit), 2-\( \mu \)l dNTP (1 mM) and 2 \( \mu \)l reverse transcriptase (40 unit). Mixtures A and B were mixed together and incubated at 37°C for 60 minutes and finally the reaction was terminated by heating at 70°C for 5 minutes.

Nested RT-PCR

The nested RT-PCR was done for detection HCV RNA in serum samples was done with following primers from 5' untranslated region (UTR) of Hepatitis C virus genome:

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\begin{align*}
\text{BKP-7,} & \text{-CACTCCCCTGTGAGGA} & \text{ACTGTC (nucleotides 38 to 62) as the outer sense,} \\
\text{BKP-8,} & \text{-ATGGTGCACGGTCTACGAGACCTC} & \text{C (nucleotides 319 to 343) as the outer anti-sense;} \\
\text{BKP-9,} & \text{-TTCACGCAGAAACGTCTACGAGATC} & \text{CATG (nucleotides 63 to 87) as the inner sense;} \\
\text{BKP-10,} & \text{-GC} & \text{CAGCTCGCAAGCACCCTATCAGG (nucleotides 292 to 314) as the inner anti-sense primer.}
\end{align*}
\]

For first round: 2.5 \( \mu \)l 10x PCR buffer (Roach) was mixed with 0.5-\( \mu \)l dNTP mix (0.2 mM), 0.25 \( \mu \)l of BKP-7 and BKP-8 primers (0.5 \( \mu \)M), 0.2 \( \mu \)l (1 unit) Taq polymerase, 5 \( \mu \)l of template and water up to 25 \( \mu \)l. The PCR was performed over 25 cycles followed by 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec with final extension at 72°C for 10 minutes. The second round was carried out like the first round with the inner set of primers (BKP-9 and BKP-10) with the same PCR mixture and programme. The first PCR product was 306 bp for the outer set and the second product was 254 bp for the inner set. [7]

Is-PCR-ISH In situ-PCR-In situ Hybridisation)

The IS-PCR-ISH method was carried out as described previously [8],[9] with a little modification. Briefly, the fixed mononuclear cells on slides were dehydrated through graded ethanol (70%, 80%, 90% and 100%) for 10 minutes, and then covered with RNase-free water for 5 minutes. Slides then were covered with 0.02 M HCL, washed in PBS and then covered with PBS/Triton X-100 for 3 min. The mononuclear membrane was permeabilised by incubation with
2.5 μg/ml pepsin in 0.01 M HCl at 37°C for 30 minutes. The slides were washed in PBS/glycine, and then dehydrated through graded alcohols for 10 min. Gene frames were attached around the sections and RNase-free DNase solution (100 U/ml) was added and incubated overnight at 37°C. Then the slides were incubated at 94°C for 2 minutes to inactivate DNase. The gene frames were removed and slides were washed in water and then in 100% ethanol. New gene frames were attached and cDNA was synthesised as previously described. Gene frames were removed and the slides washed with distilled water, PBS and dehydrated through graded ethanol. New gene frame was attached and the nested PCR was done as follow: for the first round a 25-μl reaction was prepared consisting of 2.5-μl 10x PCR buffer, 2 μl (4 mM) mgcl 2, 0.5 μl (0.2 mM) dNTP, 0.25 μl each primers, 0.2 μl taq polymerase (1 unit) and DPEC water up to 25 μl. The slides were placed in in situ PCR thermal cycler (Techne TC-512, UK) and programmed as previously described. After amplification, the sections were washed in PBS, water and dehydrated through graded alcohol over 10 min and air dried. The second round was done like to first round with the inner set of primers (BKP-9 and BKP-10) and same PCR mixture and program. The gene frames were removed and the slides were washed in PBS, distilled water and dehydrated through graded alcohol over 10 min and air dried. In situ hybridisation was performed using a novel 65-bp biotin-labeled probe, BKP-probe [5’CCA CG AAAGGACCCGGTCATCCTGGCAATTCCGGTTATCACCCGTTCCGCAGACCACTATGGC]. A new gene frame was attached and 25-μl hybridisation buffer containing 200 ng/ml of BKP probe was added to the sections. The slides were placed in the thermo cycler to run a denaturing cycle of 94°C and hybridisation was carried out overnight at 37°C. The slides were washed with ×2 SSC solution (saline-sodium citrate). The slides were covered with Streptavidin Alkaline Phosphatase conjugate diluted at 1:5,100 in buffer 1 and incubated at room temperature for 15 min. Slides were washed in buffer A (0.5 M Triton x-100, 2mM MgCl 2 , 0.1 M NaCl, 0.1 M Tris Hcl = 7.5) and then in buffer B (0.1 M Tris Hcl PH: 9.5, 1 M NaCl, 50 mM MgCl 2 ). The slides were covered with dye solution NBT-BCIP (Roach) and incubated in 37°C for about 1 h. Finally, the slides were washed in PBS and water, air dried and microscopy observation was prepared.

Statistic

Data (ALT, AST, age) are expressed as the mean ± standard deviation, or n (%) as appropriate. SPSS version 17 and independent sample T test was used for data analysis to find out any significant relationship between the two groups of patients who were positive or negative by IS-PCR-ISH. The P < 0.05 is significant.

Results

Out of 53 patients, 17 (32%) including 6 (11.3%) female and 11 (20.7%) male showed positive HCV RNA by IS-PCR-ISH, 36 (68%) showed negative result [Figure 1]. Most positive cases were between 20 and 40 years (10 patients) and 40 and 60 years (6 patients). Ten (18.8%) patients who were positive HCV by IS-PCR-ISH were also positive for anti-HBc IgG. The means of ALT and AST were higher in patients with occult HCV infection compared to patients with negative HCV infection. The distribution of occult HCV within the age groups was not found significant (P > 0.05) [Table 1]. All the patients were negative for HCV antibody, HCV RNA, HBs Ag, HEV IgM and HAV IgM. [Figure 1]

Discussion
The occult hepatitis C, as a new concern of public health worldwide, occurs in two different forms, anti-HCV Ab and HCV RNA-negative patients with abnormal ALT or anti-HCV Ab-positive and HCV RNA-negative patients with normal ALT level. Occult HCV is responsible for recurrent hepatitis C infection in patients who were subjected to liver transplantation. [10],[11] For diagnosis of occult HCV, detection and localisation of HCV in liver tissue is a gold standard for diagnostic purposes and clinical management of HCV-infected patients. [10],[11],[12] However, invasive aspects of liver biopsy for patients, make it an hard and unpractical method. In other studies, it is mentioned that PBMCs could be used for HCV RNA detection by IS-PCR-ISH. [10],[13] In other report, HCV RNA was detectable in PBMC of 70% of patients, positive for HCV RNA in their liver biopsies. [14] In this study, acetone was used for cell fixation on glass slide instead of formaldehyde which have negative effect on the fixed cells. [15],[16] We also used pepsin enzyme to facilitate permeabilising cell membrane to permit PCR components enter to the cytoplasm that is a critical stage for IS-PCR-ISH. Pepsin is a better option than protease K for permeabilisation process and do not need heat for inactivation process. [8] RNase-free DNase was used to remove cellular DNA to increase specificity and decrease the chance of non-specific amplification and false positive result. In our study, 17 (32%) patients had positive result for HCV RNA by IS-PCR-ISH and recognised as occult hepatitis C patients, our result is close to other reports (10-50%) in patients with persistent elevated ALT level. [1],[5],[6],[13],[17],[18] 10 (18.8%) patients were positive anti-HBc IgG which may indicate dual infection. HCV coinfection with hepatitis B virus in southeast-Asia and Mediterranean countries is common. [19],[20] Some reports suggest that in dual infection with HCV and HBV, HCV can inhibit the replication of Hepatitis B virus. [19],[20] Sagnelli reported that presence of anti-HCV and anti-HBc Antibody in patients without HBsAg and anti-HBs, might be duel HBV and HCV infection. [21] Based on these facts in our study, those patients with occult hepatitis C infection whom their sera were negative for HBsAg, might have occult hepatitis B infection. However, dual infection with Hepatitis B and C virus and their interaction is a complex concept and requires further investigation.

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