Transfusion-transmitted virus in association with hepatitis A-E viral infections in various forms of liver diseases in India

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INTRODUCTION

In 1997, a novel DNA virus was identified in serum of a Japanese patient (TT) having post-transfusion hepatitis[1]. This virus was designated as transfusion-transmitted virus (TTV). Later studies demonstrated TTV to be a non-enveloped single-stranded DNA virus, whose genome consists of 3739 bp and two large overlapping open reading frames (ORF-1 and ORF-2) encoding 770 and 202 amino acids and several small ORFs (22-105 amino acids)[2]. The virus has little sequence similarity with any known virus and so, it is presently not clear to which family it belongs. It is likely that TTV represents a new family. These are dense and 40-nm diameter particles. Individual genome sequences vary by up to 40%. Comparison of the genomic sequence, encoded proteins and the biophysical characteristics of the virus suggests that TTV is closely related to the Circoviridae. The genome exhibits high diversity and is classified into 6 genotypes[3]. As the prevalence of TTV in populations that require frequent transfusion of blood products is higher than that in healthy blood donors, TTV was supposed a parenterally transmissible virus. However, since TTV has been reported to be excreted in feces[4] also, it was felt that virus might also be transmitted non-parenterally. Using PCR with a new set of primers derived from a highly conserved region of the TTV genome, TTV DNA was detected in 92% of the general population in Japan[5], demonstrating that TTV is indeed a common virus.

Since its discovery, it has become clear that TTV infection is present worldwide among blood donors and is common in patients with liver diseases, including cryptogenic cirrhosis and fulminant hepatic failure. In one study, TTV DNA was detected in 46% and 12% of non-A to G hepatitis patients and healthy blood donors of non-A to G hepatitis patients and healthy blood donors.
donors, respectively,\(^4\), and it was initially suggested that TTV was a new hepatitis virus. The association of TTV with cryptogenic chronic liver diseases, post-transfusional hepatitis\(^5\) and acute hepatitis of unknown etiology suggested an etiological role of this agent in the development of both acute and chronic hepatitis. Preliminary data from Japan and the United Kingdom indicated that TTV sequences were detectable in 25%-47% of patients with fulminant or chronic hepatitis of unknown origin, 27%-68% of hemophiliacs, and 1.9%-22% of apparently healthy blood donors\(^6\).\(^10\). Moreover, TTV-DNA titres were found to be 10-100-fold greater in liver tissue than in serum. All these findings have been interpreted as demonstrating its hepatotropism. Evidence for potential hepatotropism of TTV was supported by the presence of high TTV-DNA concentration in liver\(^1\) and by a correlation between TTV-DNA titres and aminotransferase levels in post-transfusional non-A-G hepatitis patients\(^12\). In some studies, TTV-DNA has been found more frequently in patients with liver cirrhosis and hepatocellular carcinoma than in those with chronic hepatitis. However, virus DNA is not integrated in tumor cells, which may suggest that the virus is a passenger rather than a cause of the tumor. And therefore, further studies are required to determine the role of TTV. In fact, the significance of TTV infection in liver disease is, at present, analogous to that of HGV.

The aim of this study was to evaluate the prevalence of TTV-DNA in patients with different liver diseases in north India, and at the same time, find out the possible relation between TTV and other hepatitis viral infections in causation or progression of different liver diseases.

**MATERIALS AND METHODS**

**Patients and blood samples**

One hundred and thirty seven patients of both sexes and in adult age group were included in the present study. The patients consisted of 37 patients with acute viral hepatitis (AVH, age range: 21-48 years), 37 patients with chronic viral hepatitis (CVH, age range: 19-48 years), 31 patients with liver cirrhosis (age range: 34-57 years) and 32 patients with fulminant hepatic failure (FHF, age range: 28-46). All these patients consisted of 37 patients with acute viral hepatitis (AVH, age range: 21-48 years), 37 patients with chronic viral hepatitis (CVH, age range: 19-48 years), 31 patients with liver cirrhosis (age range: 34-57 years) and 32 patients with fulminant hepatic failure (FHF, age range: 28-46). All these patients attended either Outpatient Department or were admitted to the Liver Unit of All India Institute of Medical Sciences, New Delhi, from June 2001 to February 2004. They were evaluated clinically and biochemically and their sera were tested for hepatitis viral markers. The diagnosis of different types of liver diseases was based on accepted clinical, biochemical and histological criteria as outlined elsewhere\(^12\). AVH was diagnosed when patients exhibited overt jaundice and/or increased alanine aminotransferase levels (at least 3 times above the normal value) documented at least twice at a 1-week interval without any history of pre-existing liver disease. None of the patients had a past history of alcohol intake or using any drug. We also could not find any clinical or serological evidence of autoimmune diseases or biliary infection in these patients. The patients with CVH and cirrhosis of liver were diagnosed by histopathological criteria laid down by International Study Group on Chronic Hepatitis\(^13\).

All these CVH patients had persistent elevation of transaminases level (at least twice the upper limit of normal range) for more than six months and histologic evidence of chronic hepatitis on liver biopsy at the beginning of follow-up. Fulminant hepatic failure was diagnosed if the patients developed hepatic encephalopathy within 4 wk of the onset of acute hepatitis as outlined elsewhere\(^12\). One hundred age- and sex-matched healthy subjects were used as controls.

From each of the above patients, 6-10 mL of venous blood was drawn and aliquoted in plain tubes without anticoagulant. Serum was separated after centrifugation and then stored at -70°C until further analysis. Repeated freezing and thawing of serum was avoided as far as possible. These sera samples were used to analyze various hepatitis markers, liver function tests and hepatitis C virus (HCV) core protein.

**Hepatitis viral markers**

Sera were investigated for hepatitis B surface antigen (HBsAg) and IgM antibodies to hepatitis A virus (IgM anti-HAV), hepatitis B core antigen (IgM anti-HBc), hepatitis D virus (IgM anti-HDV) and hepatitis E virus (IgM anti-HEV). Similarly, all these sera were also tested for total antibodies against hepatitis C virus (anti-HCV). The serological analysis was done using enzyme immunoassay kits of high sensitivity and specificity. Kits for HBsAg, IgM anti-HBc and IgM anti-HAV were purchased from Abbot Laboratories, USA. Anti-HCV was tested using highly sensitive third generation ELISA kit from Ortho Diagnostics. This anti-HCV kit used peptides versus core, NS3, NS4 and NS5 regions of HCV genome, as antigen to coat the ELISA plate. IgM antibody to hepatitis D virus (HDV) was tested using an enzyme immunoassay kit from Wellcome, UK. Similarly, IgM anti-HEV was tested using third generation ELISA kit from Genelabs and Diagnostics, Biotechnology, Singapore.

**Detection of HCV-RNA by RT-PCR**

Total RNA was isolated from 100-μL serum or plasma using High Pure Isolation kit from Roche, Germany according to manufacturer’s instructions. Five micrograms of the isolated RNA were applied to reverse transcription and nested PCR with primers located in the highly conserved 5′ noncoding region (5′ NCR) using BIOHCV kit (B&M Labs., Madrid, Spain). The reverse transcription mixture was incubated for 1 min at 85°C, followed by 30 min at 60°C. First PCR was performed in whole content after adding 40 μL of HCV amplification mixture. Thermal cycler was programmed as follows: 85°C for 30 s, 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and then further incubation of samples for 5 min at 72°C. Five microliters of first PCR product were subjected to nested PCR using nested PCR mixture containing second round primer and enzymes. The protocol on thermo cycler was the same as mentioned in first PCR. The PCR product was electrophoresed on 20 g/L agarose containing ethidium bromide and visualized under UV. A positive control provided in the kit was used as control. All positive and negative controls were tested in parallel with test samples throughout the entire procedures.

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starting with RNA extraction.

**Detection of TTV-DNA**

Total DNA was extracted from 200 µL of serum using DNA isolation kit from Roche Diagnostics GmbH, Germany. This isolation method utilizes the ability of nucleic acids to absorb to silica (glass) in the presence of a chaotropic salt. Serum sample was treated with buffer containing proteinase K and silica particles where nucleic acids are bound to silica surface of magnetic particles. Since the binding process is specific for nucleic acids, the bound nucleic acids are purified from salts, proteins and other impurities by washing. A low salt buffer is used to elute the DNA. Using 5 µL of the DNA solution as a template, TTV DNA of the open reading frame (ORF-1) sequence, was detected by PCR employing the semi-nested primers reported by Okamoto et al.\(^{[2]}\). The first-round PCR was carried out for 35 cycles, each cycle consisting of denaturation at 94°C for 45 s, primer annealing at 60°C for 45 s and extension at 72°C for 60 s, followed by an additional extension at 72°C for 7 min, using the primers NG059 (sense: 5'-ACAGACAGAGGAGAAGGCCAAC ATG-3') and NG063 primer (antisense: 5'CTGGCATT TTACCATTTGCAAGTT-3'). Thereafter, the second-round PCR was carried out using 1 µL of the first-round PCR product, NG061 primer (sense: 5'-GGCAACATG YTRTTGGGATAGACTGG-3', where Y=T or C; R=A or G), and NG063 primer for 25 cycles under the same aforementioned conditions. The PCR product (10 µL) was electrophoresed on 20 g/L agarose gel containing ethidium bromide, and observed under ultraviolet light. The product of the first-round PCR was of 286 bp and that of the second-round PCR was of 271 bp.

**Diagnosis of viral hepatitis**

Liver function tests, including transaminase levels (AST and ALT) in serum, were performed on autoanalyser Hitachi-917 using the established techniques. Similarly, hemogram and coagulation profiles were performed using routine assays established in our laboratory. The diagnosis of different types of viral hepatitis was established as follows: The diagnosis of hepatitis A virus (HAV) infection was confirmed by the presence of IgM anti-HAV in serum. Hepatitis B virus (HBV) infection was established by presence of IgM anti-HBe in sera of AVH and FHF patients and by the persistent HBsAg antigenemia in sera of CVH and cirrhosis cases. Similarly, anti-HCV and IgM anti-HDV in sera samples were used for the diagnosis of HCV and HDV infections, respectively. All anti-HCV sera were also tested for HCV-RNA using nested PCR as described above. However, final diagnosis of HCV was based on anti-HCV antibodies in serum. Active or recent hepatitis E virus (HEV) infection was diagnosed by the presence of IgM anti-HEV in serum. Sera positive for HBsAg but negative for all other viral markers were labeled as HBV-carriers. Absence of all the markers including HBsAg labeled the patients with hepatitis non-ABCDE infection. HCV-RNA in serum was used to confirm active HCV infection.

**RESULTS**

Analysis of sera for different hepatitis viral markers demonstrated the presence of hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis E virus (HEV) infections in these patients. None of the sera analyzed could demonstrate the presence of markers related to hepatitis A virus (HAV) and hepatitis D virus (HDV) infections. HBV infection was detected in 19 of 37 (54.1%) patients with AVH, 30 of 37 (80.1%) with CVH, 18 of 31 (58.1%) with cirrhosis and 13 of 32 (40.6%) with FHF. HCV infection, as indicated by the presence of anti-HCV in serum, was detected in 8 of 37 (21.6%) patients with AVH, 15 of 37 (40.5%) with CVH, 5 of 31 (16.1%) with cirrhosis and 2 of 32 (6.3%) with FHF. All anti-HCV-positive sera were also tested for HCV-RNA which was found positive in 96.98% sera samples. The percentage of HCV infection was based on anti-HCV positivity in these cases. HEV infection was observed in 14 of 37 (37.8%) cases with AVH and 5 of 32 (15.6%) cases with FHF.

Presence of TTV-DNA was detected in 10 of 37 (27.0%) cases with AVH, 7 of 37 (18.9%) cases with CVH, 15 of 31 (48.4%) cases with cirrhosis and 3 of 32 (9.4%) cases with FHF (Table 1). TTV-DNA was also detected in 27 of 100 (27%) healthy blood donors. In order to investigate TTV coinfection with different hepatitis viral infections, we observed TTV-DNA with IgM anti-HBc in 16.2% cases with AVH and none with FHF. TTV-DNA with HBsAg, indicating TTV-HBV co-infection in CVH and cirrhosis, was observed in 13.5% and 32.3% cases, respectively. TTV-HCV coinfection, indicated by simultaneous presence of TTV-DNA and anti HCV, was present in 10.8% case with CVH, 6.5% with cirrhosis and 3.2% cases with FHF. None of AVH patients had TTV-HCV coinfection. TTV-HEV coinfection could be demonstrated in 2.7% cases with AVH only. No other disease group had HEV-TTV coinfection (Table 2).

When TTV-DNA positive cases were analyzed for ALT level in sera, we found that 60% AVH patients had ALT level up to 3 334 nkat/L, 50% up to 6 668 nkat/L and only 2.7% more than 10 002 nkat/L. In CVH, all TTV-positive cases had ALT level less than 3 334 nkat/L. Similarly, in cirrhosis patients, ALT level was found up to 3 334 nkat/L in 93.3% cases and up to 6 668 nkat/L in 6.7% cases. In FHF, all three cases had ALT level more than 10 002 nkat/L. ALT level in serum was used as an index of liver damage in different disease groups (Table 3).

**DISCUSSION**

To investigate the status of TTV coinfection in relation...
to other hepatitis infections in different liver diseases, we tested TTV-DNA simultaneously with markers related to hepatitis A-E infections. Results are shown in Table 2. Analysis of data showed that TTV-HBV coinfection was detected in all the groups except FHF. Similarly, TTV-HCV coinfection was also recorded in all except AVH group. TTV-HEV coinfection was found in a minor proportion of AVH group. Attempts were also made to study the impact of TTV infection on liver damage both with or without other hepatitis viral infections. For this, serum level of ALT was tested in all the cases. The results showed that TTV infection did not cause severe liver necrosis in these groups of patients. This was indicated by a very moderate elevation in ALT level in the disease groups except FHF, which otherwise always showed a high ALT level.

While reviewing our findings in reference to various other studies, we found that our data are in agreement with several other reports. Earlier reports indicate that a majority of individuals who become TTV-DNA-positive after blood transfusion usually have normal ALT level and do not develop chronic hepatitis, although TTV viremia persists for years. We found TTV-DNA in 27% normal population with normal ALT level and at the same time no significant increase in ALT in hepatitis A-E, TTV-positive patients, thereby suggesting that TTV alone does not cause much change in ALT level. This raises the possibility that TTV is merely an innocent bystander, both with and without non A-E hepatitis viral infections, and does not add to the damage caused by other hepatotropic viruses. Also, as such it does not appear to be the primary cause of hepatitis, though many more studies are still needed to prove this theory. The results by Vimolket et al demonstrated that in non A-E hepatitis cases, mean ALT level was comparable among TTV-positive and negative cases. There was no consistent relationship between ALT and TTV-DNA level among these patients. These results also support our findings.

In conclusion, TTV infection is prevalent both in normal as well as patients populations in India. It shows its presence in all types of liver diseases, though comparable to the normal population and presenting no evidence of increasing liver damage both with or without other hepatitis viral infections. TTV infection has been found to be coinfected with HBV, HCV and HEV infections, but does not significantly increase ALT level in these patients. It appears as if TTV is a benign virus acting as a bystander in the body without causing any damage of the liver.

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