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Original Article

Incidence and clinical implication of TT virus in patients with hepatitis and its frequency in blood donors in India

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ABSTRACT

Background: Transfusion Transmitted Virus (TTV), also known as Torque Teno Virus is a new novel viral agent which appears to correlate with some acute and chronic hepatitis cases and may produce liver damage under specific circumstances. Aim of this study was to detect TT virus by real-time PCR, study its clinical implications and effects of its co-infection in HBV and HCV chronic liver diseases.

Methods: The study population comprised 50 acute hepatitis, 50 chronic hepatitis patients and 100 voluntary blood donors. All samples were tested for serum bilirubin, AST, ALT and alkaline phosphatase levels and for all available viral markers for hepatitis. The detection of TT viral genome was carried out by real-time PCR using TTV sequences as reported by Takahashi et al with modifications on the basis of database of the DDBJ/EMBL/GenBank (GenBank accession no. AB008394).

Result: Serum was positive for TTV in 72% of volunteer blood donors, 77.4% (24/31) of hepatitis A cases, 87.6% (36/41) of HBV-positive, 77% (10/13) of HCV-positive, and 92.8% (13/14) of non-B, non-C cases. Co-infection of TTV with other hepatitis viruses was detected in some patients.

Conclusion: TTV is a frequent virus detected in patients with various types of viral hepatitis, in cases of hepatitis without obvious viral agent, and from the healthy population in India. Rate of TTV was found to be significantly higher (92.8%) for Non A–E hepatitis group.

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Introduction

Torque Teno Virus (TTV) of genus Anelloviridae, is a single stranded, non-enveloped novel DNA virus of 30–50 nm

diameter with a small and covalently closed circular genome comprising of 3.8 kilo bp.¹ This virus was identified in 1997 in the serum of a Japanese patient (TT) having post-transfusion hepatitis. This acronym also stands for Transfusion Trans-

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mitted Virus.² TTV is genetically heterogeneous and has prompted many intensive studies to assess its molecular characteristics and whether its numerous genotypes cause liver disease. These studies have concluded that TTV has extraordinarily high prevalence of chronic viraemia in healthy individuals worldwide, a feature quite unusual among viruses.

The natural history, viral characteristics and pathogenic potential of TTV have been subject of active investigation. TTV shows high prevalence in haemodialysis (HD) patients from various countries throughout the world. TTV has been associated with various liver diseases, acute hepatitis and chronic viral hepatitis B and C. No defined aetiology is known in about 20% of community acquired hepatitis cases and 10% of transfusion associated hepatitis, suggesting the existence of an additional causative agent.

Clinical details and information regarding TTV prevalence in India is insufficient. The present study was carried out to detect TTV-DNA by real-time polymerase chain reaction (PCR) in patients with acute and chronic hepatitis and in voluntary blood donors who shall serve as a control group for Indian population. Clinical implications of TT virus and effects of its co-infection in HBV and HCV chronic liver diseases were elucidated after employing a sensitive and reliable PCR.

Materials and methods

The study population comprised 50 acute hepatitis patients and 50 chronic hepatitis patients. The control group comprised 100 voluntary healthy blood donors. Acute viral hepatitis (AVH) were those patients who had overt jaundice and/or increased alanine aminotransferase (ALT) levels, at least 3 times above the normal value with no history of pre-existing liver disease. Chronic hepatitis patients had liver disease persisting for longer than 6 months and histological evidence of chronic hepatitis on liver biopsy at the beginning of follow-up. Patients with autoimmune liver disease, drug-induced hepatitis and alcoholic liver injury were excluded. The patients with chronic active hepatitis and cirrhosis of liver were diagnosed by histopathological criteria laid down by International Study Group on chronic hepatitis. Cryptogenic cirrhosis meant cirrhosis of liver of undetermined aetiology when all other known causes of chronic liver disease had been ruled out.

For blood donors, inclusion criteria were negative results for all hepatitis virus serologic markers and absence of any acute or chronic infection.

Venous blood sample (8-10 ml) was collected in vacutainers, serum separated at 4 °C, made into aliquots and stored at -40 °C. Urine samples were tested the same day for bilirubinuria. All samples were tested for serum bilirubin, AST, ALT and alkaline phosphatase levels.

Viral markers for hepatitis tested in this study included Anti-HAV-IgM, Anti HBc-IgM, HBsAg, HBeAg, HBeAb, Anti-HCV, Anti-HDV-IgG/IgM, Anti-HEV-IgM by ELISA. PCR for HBV-DNA and HCV-RNA was carried out for confirmation in selected cases.

The detection of TT viral genome was carried out by real-time PCR. Chemagic Viral DNA/RNA Kit as well as QIAamp MinElute Viral Kit (Qiagen) were used for isolation and

purification of viral DNA. TTV sequences were prepared by Genome diagnostic Pvt Ltd as reported by Takahashi et al with modifications on the basis of database of the DDBJ/EMBL/GenBank (GenBank accession no. AB008394). These consisted of - Forward Primer (K 117) 1175-CACTTCCGAATGGCTGAGTT-3' (S) 98-117, Reverse Primer (K 233) 2335'-GCCTTGCCCA-TAGCCCGG-3' (A)250-232 and Probe TP (208)5'-TCCCGAGCCC-GAATTGCCCT-3' (A) 228-209.

The probe consisted of a fluorescent reporter dye (6-carboxyfluorescein) at the 5' end and a fluorescent quencher dye (6 carboxyfluorescein) at the 3' end. Rotor-Gene 3000, a four channel multiplexing system by Corbett Research, Australia was used for amplification and detection. Data acquisition was by cycling A FAM & JOE. Standards used when starting sample volume is 200 µl and Elution volume is 50 µl, QS 1: 10⁵ Copies/µl = 25,000,000 copies/ml, QS 2: 10⁴ Copies/µl = 2,500,000 copies/ml, QS 3:10³ Copies/µl = 250,000 copies/ml, QS 4: 10² Copies/µl = 25,000 copies/ml, QS 5: 10¹ Copies/µl = 2500 copies/ml. Fluorescence values were recorded during every cycle.

A standard curve was generated spanning the region of samples amplification. Direct conversion of copies/ml for the samples was obtained. Quantitation reports for various groups of isolates along with their Ct- value and calculated concentration (copies) were obtained.

Amplified products were sent to Bangalore Genei Ltd, Bangalore and automated DNA Sequencing was carried out using an automated DNA sequencer. Good quality value bars along with co-amplification was obtained from the samples.

Selected 12 patients of chronic hepatitis were given interferon (IFN) therapy. Inj Interferon-alfa was given for 24 weeks in 09 patients with chronic hepatitis B. Three cases of chronic hepatitis C were given combination therapy of Interferon-alpha (5 million IU/3 times a week) and Tab Ribavirin (800-1000 mg/day) for 24 weeks. The levels of TTV DNA before and after the IFN therapy were estimated.

Results

Results of Serum Bilirubin, AST, ALT and Alkaline Phosphatase levels and presence of bilirubinuria are shown in Fig 1. Positivity of viral markers for various hepatitis viruses are shown in Fig 2.

Results of real-time PCR giving Quantitation Reports for various groups of samples along with their Ct- Value and

Fig. 1 – Clinical chemistry profile in various groups.

Fig. 2 – Positivity of viral markers for various hepatitis viruses.

Calculated Concentration (copies) were obtained. Serum was positive for TTV in 72% of volunteer blood donors, 77.4% (24/31) of hepatitis A cases, 87.6% (36/41) of HBV-positive, 77% (10/13) of HCV-positive, and 92.8% (13/14) of non-B, non-C cases (Table 1).

Among the various chronic hepatitis cases, 70% showed TTV viraemia while all cases of cryptogenic hepatitis (100%) showed TTV positivity (Table 2). Co-infection of TTV with other hepatitis viruses was observed in patients with acute hepatitis A, B and C, in patients with chronic hepatitis B and C and in patients with non-A–E hepatitis (Fig 3).

The prevalence of TTV was not correlated to the levels of the hepatic ALT and AST between individuals evidencing dual infection with hepatitis A and C viruses and healthy blood donors. However, in the groups of patients with hepatitis B and non-A–E hepatitis, those having TTV viraemia had statistically significant higher levels of ALT and AST than those who were TTV negative (Fig 4). Complete clearance of TTV DNA was achieved in 30% of patients at 6 months after the cessation of IFN-alpha therapy. The levels of TTV DNA before IFN administration were found to be significantly lower in the viral eradication group than in non-eradication group.

Discussion

Torque Teno Virus is the most prevalent virus reported throughout the world. Several studies have been conducted to characterize this virus and explain its clinical implications.

Table 1 – Transfusion transmitted virus (TTV) viraemia among studied groups.

Category	n (%)	TTV positive n (%)	TTV negative n (%)
Blood donors	100 (100%)	72 (72%)	28 (28%)
Hepatitis A	27 (54%)	21 (77.4%)	06 (22.6%)
Hepatitis B	41 (82%)	36 (85.7%)	05 (14.28%)
Hepatitis C	13 (26%)	10 (77%)	03 (23%)
Non A–C	14 (28%)	13 (92.8%)	01 (7.14%)

Table 2 – Prevalence of TTV infection among chronic hepatitis.

Groups	TTV positive rate (n)	TTV negative rate (n)	Total
Cryptogenic hepatitis	100 (03)	0 (0)	03
HBsAg asymptomatic carrier	69.6 (16)	30.4 (07)	23
Chronic hepatitis B	70.0 (07)	30.0 (03)	10
HBV related active liver cirrhosis	50.0 (01)	50.0 (01)	02
HBV related hepatic failure	100 (01)	0 (0)	01
Hepatitis C	63.6 (07)	36.4 (04)	11
Total	70.0 (35)	30.0 (15)	50

Hepatotropism was indicated by findings of increased TTV-DNA levels in hepatic tissue, appearance of TTV infection in post-transfusional hepatitis and its close correlation with raised hepatic enzyme levels. Analysis of earlier reports suggested TTV to be associated with hepatitis of unknown aetiology. However the exact link between TTV infection and cryptogenic hepatitis or a given pathology has not been established.

During this study, reliable PCR protocols that target all regions of TTV genome were used to detect TTV-DNA in acute A, B and non A–E hepatitis cases, chronic hepatitis B and C cases and apparently healthy blood donors. In our study, the prevalence of TTV viraemia among healthy blood donors was found to be 72%. This finding is in agreement with the remarkable high prevalence of chronic TTV viraemia in healthy people in many countries. TTV prevalence ranging from 10.0% to 70.0% has been reported in healthy adults in different epidemiological studies.³ People who have received multiple blood transfusions are almost universally positive for TTV, with many subtypes in each individual.

In Indian population, TTV-DNA was detected significantly more in patients on haemodialysis (HD) than in voluntary blood donors. Asim et al⁴ reported TTV-DNA in 45.3% volunteer blood donors whereas rate of TTV-DNA infection reported by Chattopadhyay et al⁵ was 83% in patients on HD in New Delhi. The clinical background, age, sex, duration of HD and ALT levels did not differ significantly between TTV DNA-positive and negative HD patients. These studies concluded that North Indian patients on maintenance HD show very high rate of TTV positivity and these patients did not show clinical or biochemical signs of liver disease.

Fig. 3 – Co-infection of TTV with other hepatitis viruses.

Fig. 4 – Comparative study of liver function among blood donors and patient groups with positive and negative Transfusion Transmitted Virus (TTV) viraemia.

Frequent TTV positivity in volunteer blood donors is independent of previous blood or blood products transfusion.⁶ Such high prevalence in healthy blood donors with no history of blood transfusion points towards existence of other routes of transmission. Besides parental transmission, faeco-oral route is suspected for TTV transmission. Ali et al⁷ and Asim et al⁴ studied the prevalence of TTV infection in acute and chronic hepatitis in comparison with that in blood donors and in patients receiving blood products. They found that the prevalence of TTV infection was higher in hepatitis patients than in healthy blood donors. In their study, clinical and biochemical characteristics were found to be similar between TTV positive and TTV negative patients. Therefore the virus could not be aetiologically linked to any specific hepatic disease.

In our study, TTV co-infection with hepatitis C was 77% which is quite comparable to co-infection reported in previous studies.⁸ Similarly 85.7% of hepatitis B patients showed co-infection with TTV which is similar to co-infection reported by Liwen et al⁹

Irshad et al¹⁰ investigated TTV infection in various liver diseases in North India and reported TTV co-infection in 27.1, 18.9, 48.4 and 9.4% cases with acute viral hepatitis, chronic viral hepatitis, cirrhosis and fulminant hepatic failure respectively. They identified TTV-DNA positivity in 27% of healthy blood donors which is in contrast to findings of our study where we found 72% positivity in blood donors. Desai et al¹¹ reported abnormal liver function profiles in TTV positive Indian subjects. They also found higher mortality rate in acute hepatitis patients having superimposed TTV infection. However, exact pathogenicity of TTV could not be explained.

In this study, attempt was made to study dynamics of persistent hepatitis B infection with TTV co-infection. HBsAg carrier, chronic hepatitis B disease and HBV related cirrhosis showed TTV-DNA in 50%–70% cases whereas 100% TTV co-infection was found in HBV related chronic hepatic failure. It suggests positive correlation between TTV co-infection and severity of liver disease. The difference in levels of total bilirubin between TTV positive and TTV negative patients was not statistically significant, as also accorded with another

report.¹² However, ALT and AST levels were found to be higher in hepatitis B cases co-infected with TTV indicating greater liver damage. Our findings were similar to findings of Kasirga et al¹³ who found that children co-infected with hepatitis B virus and TTV had greater liver damage.

In chronic hepatitis C patients, TTV co-infection was detected in 72.7% cases. Levels of ALT and AST enzymes in hepatitis C patients co-infected with TTV were correlated with those in TTV positive healthy blood donors. It was found that TTV co-infection had little or no effect on severity of biochemical parameters of liver damage in hepatitis C patients. Zhao et al¹⁴ and Moriyama et al¹⁵ reported that TTV co-infection did not modify the serology or biochemical markers of chronic hepatitis B and C. However, few workers have reported increased severity of biochemical and histologic parameters of liver damage in hepatitis C patients co-infected with TTV.¹⁶

In our study, TTV viraemia was associated with raised serum bilirubin, AST, ALT and alkaline phosphatase levels in only 1%, 2%, 4% and 1% healthy individuals respectively. Dai et al¹⁷ investigated the prevalence and effects of TTV infection on liver function tests in patients with negative hepatitis B and hepatitis C viral markers. In their study, the prevalence of TTV-DNA was found to be 24.9% and all these cases showed significantly raised ALT levels. They attributed the raised ALT levels to TTV infection.

Attempt was made to investigate role of TTV in causation of non-A to E viral hepatitis. Among such 11 patients with acute hepatitis, 10 patients (90.90%) had TTV viraemia. Also in these patients of non-A to E acute hepatitis, serum ALT levels were found to be significantly higher than those not infected with TTV. In our study, frequency of TTV infection in hepatitis of unknown aetiology was significantly high (92.88%). These findings suggest that TTV may account for some cases of acute and chronic cryptogenic hepatitis. Earlier studies demonstrated that TTV-DNA levels were higher in liver tissue than in serum¹⁸ and liver enzymes were higher in TTV positive cases.¹⁹ Keffe²⁰ speculated that the extent of TTV replication determines the liver damage.

Currently TTV detection can be done by amplification of viral DNA by PCR. There are no other commercial detection systems available for screening of TTV-DNA. Since this virus shows great genetic heterogeneity, detection rate depends on the region of genome amplified and results vary depending on the set of primers used. Different PCR protocols to amplify various genotypes of TTV have assessed high prevalence of TTV in healthy populations around the world. Depending on primers used for screening, the prevalence of TTV has been reported to vary from 10% to 92% in various studies. Even in the same set of population, divergence of TTV prevalence has been reported when different DNA sequences and primers are used.^{21,22}

In the present study more accurate and specific real-time PCR with the primers described by Takahashi et al²³ has been used to identify genotypes of TTV-DNA. The primers were so designed that their sequences were predictable with reference sequences of TTV and also based on the published database of the DDBJ/GenBank. During this study Taqman probes were used for TTV-DNA detection. Primers derived from N22 region of the virus genome have been extensively used in earlier

studies. Primer sets derived from the UTR(A) region [NG132/134 and NG133/147] have been reported to be more sensitive and specific than the primers derived from other regions. UTR [A] primers were used in the present study to investigate prevalence of TTV in the Indian population and to analyze pathogenic potential of this virus.

Due to small sample size and use of one set of primers for TTV-DNA amplification the present study has some limitations. It is possible that other genotypes of TTV-DNA existed in our samples that were not detected using this set of primers. Also, the long term impact of this virus on liver function, histopathologic changes and efficiency of interferon could not be assessed in all the patients.

To conclude, very high prevalence of TTV-DNA has been detected in hepatitis patients as well as in healthy blood donors in Indian population. Real-time PCR using UTR [A] primers can detect TTV with high sensitivity and specificity. Exact pathogenicity of TTV has not been established and it is suggested that TTV may not have serious clinical implications in humans, both with and without viral hepatitis. TTV co-infection in hepatitis B may increase the severity of hepatic damage and TTV infection may play some role in hepatitis of unknown origin. Presently, screening of TTV may not be recommended for transfusion safety. Future investigations on important aspects of clinical virology and possible disease association of TTV will unravel etiological links and avenues in the field of transfusion medicine.

Conflicts of interest

The authors have none to declare.

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