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Detection of the Frequency of the Novel TT virus by PCR and Its Role in the Induction of Hepatic Injuries in Blood Donors in West Azerbaijan, Iran

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Abstract

Background: In 1997, a novel DNA virus was isolated from the serum of a patient in Japan, and it was named TT virus (TTV). As the virus is replicated in liver and has the ability to induce apoptosis in hepatocytes (Hepatocellular carcinoma cells) it is hypothesized that TTV is an opportunistic virus and in certain conditions causes liver damage. In this study the frequency of infection with TTV was detected in two groups of healthy and hepatitis B infected blood donors in the West Azerbaijan Province.

Material and Methods: Serum samples were collected from 100 healthy and 40 HBs Ag positive donors in west Azerbaijan Blood Transfusion Center. Patients' characteristics, sex, age and blood groups were recorded. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the sera were measured and then DNA was extracted and polymerase chain reaction (PCR) was performed using T801 and T935 consensus primers to amplify a 199 bp segment of a much conserved non-coding region of the genome (UTR).

Results: TTV was detected in 69% and 75% of healthy and hepatitis B infected blood donors respectively. No significant difference was observed in the frequency of the infection with TTV in different blood groups or age groups (P>0.05). In each group of blood donors the level of ALT and AST were not significantly different in TTV infected and non-infected individuals (P>0.05).

Conclusion: Regarding the high frequency of infection in healthy individuals and considering the level of hepatic enzymes in TTV infected individuals; it seems that the virus or at least its investigated genotypes have not been pathogenic for the infected individuals examined.

Keywords: TT virus, Blood donors, HBV, ALT, AST, PCR.

Introduction

In 1997, a novel human DNA virus, unrelated to the known hepatitis viruses, was isolated from the serum of a patient with post-transfusion hepatitis of unknown etiology in Japan.[1] Representational difference analysis (RDA) was performed for the specific amplification of nucleic acid sequences present in the serum of the patient during the period of his acute hepatitis.[1, 2] It was named Torque Teno virus (TTV). TTV is an unenveloped, single-stranded circular DNA virus containing a genome of 3900 nucleotides in the full length sequence. [3, 4]

TT viruses (TTV) are ubiquitous in nature and have been demonstrated in more than 90% of serum samples from healthy individuals where they persist over time.[4] Viral particles have been purified from feces [5] and are also excreted in saliva, breast milk and bile.[6,7,8] Furthermore, vertical and sexual transmissions have been reported .[9]

The cause of liver disease is still unknown in 50% of patients with fulminant hepatitis, 10%–20% of patients with acute hepatitis and 5%–10% of patients with chronic liver disease, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. The cases cannot be ascribed to A to E

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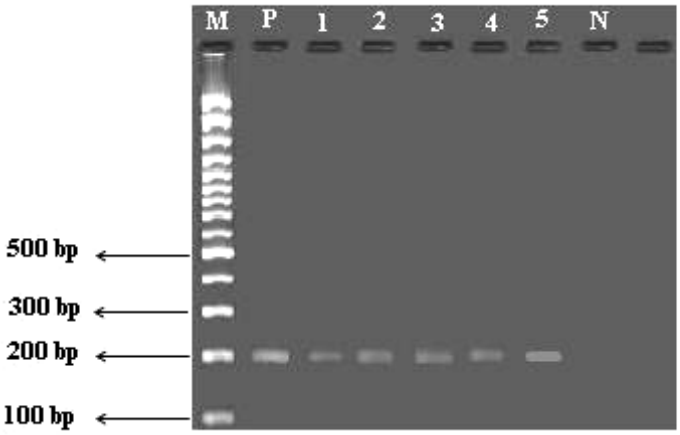


Figure 1. *The Products of PCR in 1.5% agarose gel electrophoresis stained with ethidium bromide. M, 100 bp ladder; P, positive control; 1, 2,3,4,5 positive samples and N, negative sample.*

Table1. *Frequency of TT Virus in West Azerbaijan blood donors*

Sample*	TTV Positive N (%)	TTV Negative N (%)
Healthy	69 (69)	31 (31)
HBs-Ag Positive	30 (75)	10 (25)
Total	99 (71)	41 (29)

* P>0.05

Table 2. *Distribution of TT virus in healthy blood donors in different blood and age groups*

Infection status	Age groups, years*			Blood groups*			
	18-30	31-43	44-56	A	B	AB	O
TTV+, Number	30	28	11	29	18	9	13
TTV-, Number	17	11	3	14	8	3	6
Total, Number	47	39	14	43	26	12	19
TTV+, Percent	63	71	78	67	69	75	68

* P > 0.05

hepatitis viruses and GB virus C (also known as hepatitis G virus) or to certain enteroviruses, adenoviruses, parvovirus B19 and etc, which are suspected of occasionally causing liver damage. [1,10] Due to the history of TTV as a potential hepatitis virus, liver as a main target organ for TTV infection has also been studied extensively. TTV is found by *in situ* methods in the nucleus and/or the cytoplasm of hepatocytes in patients with liver damage [11, 12] without any cytopathological changes.[13, 14] Both acute resolving and chronic persistent hepatitis infection have been identified among TTV-infected individuals.[15] Furthermore, TTV has been detected in a variety of liver diseases including non A-G post-transfusion hepatitis, fulminant hepatic failure, chronic persistent hepatitis, cryptogenic liver disease and hepatocellular carcinoma. [4, 16, 17, 18] Evidence of potential hepatotropism of TTV was reported with TTV DNA titres shown to be 10-100 folds greater in liver tissue than in serum. [19] Although the virus was originally introduced as a novel hepatitis virus, the evidence has not been

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available for its causative role in any of the significant pathological entities.[20]

As TTV is transmitted by blood transfusion and due to the potential pathogenic effects predicted for some genotypes of the virus and also lack of information about the frequency of the virus in West Azerbaijan, the aim of this study was to detect the frequency of infection with TTV in two groups of healthy and hepatitis B infected blood donors in the area by amplification of a conserved region of the viral genome and the possible effects of co-infection in individuals infected with hepatitis B virus.

Materials and Methods

Serum samples

In this study two groups of healthy and hepatitis B positive blood donors of Urmia Blood Transfusion Center were tested. Serum samples were collected from 100 healthy donors (HBs Ag negative, Anti-HCV antibody negative, Anti-HIV antibody negative and negative for Syphilis) and 40 HBs Ag positive donors from June 2006 to May 2007. Patients' characteristics, sex, age and blood groups were recorded. Licenses from the Urmia Blood Transfusion Organization to receive blood samples and consent of the donors for the necessary tests were obtained. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined in all the samples with Man Transaminse Kit (Man, Iran). DNA was extracted and TTV DNA was detected by polymerase chain reaction (PCR) using T935 and T801 primers.

DNA extraction

Viral DNA was extracted using phenol/chloroform after treatment of 200 µl of serum with 0.5 mL of proteinase K in the presence of 0.2 M NaCl, 0.25 % Sodium Dodecyl Sulfate(SDS), for 2 h at 65°C. The pellet was dried and resuspended in distilled water or TE (Tris-HCL buffer (10 mM, pH 8.0) containing 1 mM EDTA) solution after precipitation with ethanol.

Detection of TT Virus DNA by PCR

The T801 and T935 primers were used for amplification of a highly conserved noncoding region (UTR) of the viral genome.[21, 22] The sequences of TT virus specific primers were: T801: 5′ - GCT ACG TCA CTA ACC ACG TG - 3′ (sense, 6 - 25 nt) and T935: 5′ - CTB CGG TGT GTA AAC TCA CC - 3′ (antisense, 185 – 204 nt). DNA was amplified by a single round of PCR performed with 3µL of DNA in a 25 µL reaction mixture containing 1 U of Smar *Taq* DNA Polymerase (Cinnagen, Iran), 0.4 µM of each primers, 240 µM of each dNTPs, 20 mM of Tris - HCL, 3 mM $MgCl_2$, 50 mM KCL and 20 mM ammonium sulfate. Thermal cycler conditions were as follows: denaturation at 94°C for 5 min followed by 40 cycles at 94°C denaturation for 20 sec, annealing at 57°C for 25 sec and extension at 72°C for 5 min. PCR products (10 µL) were loaded on 2% agarose gel (Sigma, Germany) containing ethidium bromide and electerophoresed.

A positive control which was already sequenced with accession number of DQ 133465 in Gene Bank was used. [23]

Statistical analysis

One-way ANOVA, Tukey-Kramer, Kruskal- Wallis, Dunn's and Fisher's Exact tests were used for statistical analyses using GraphPad Instat Software version 3.05 (GrapPad, USA). P value <0.05 was considered statistically significant.

Results

In PCR 199 bp product was detected in 99 out of 140 (72%) blood donors in whom 69% and 75% were detected in healthy and hepatitis B infected blood donors respectively (Figure 1 and Table 1). No significant differences were observed in the frequency of the infection with TTV in different blood and age groups (P>0.05) (Tables 2 and 3). In each group of blood donors the level of ALT and AST were not significantly different in TTV infected and non-infected individuals (P>0.05) (Tables 4 and 5). Significant differences were observed in the level of hepatic enzymes among the healthy and HBV infected groups (P<0.05). As the number of female blood donors was only a few, we did not compare their results with males due to the lack of statistical power.

Discussion

TTV is a widespread virus, having been found in a large proportion of general population. The high prevalence of TTV in healthy blood donors in different countries poses a quandary for the transfusion services. Its incidence varies in different countries. [4]

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Table 3. *Distribution of TT virus in HBV positive donors in different blood and age groups*

Infection status	Age groups*, years			Blood groups*			
	18-30	31-43	44-56	A	B	AB	O
TTV+, Number	14	11	5	9	10	5	6
TTV-, Number	6	2	2	4	3	1	2
Total, Number	20	13	7	13	13	6	8
TTV+, Percent	70	84	71	69	77	83	75

* P > 0.05

Table 4. *Comparison of ALT serum levels (IU/L) in healthy and HBV infected donors*

Comparison groups	Mean±SD		P-Value
Healthy TTV+ versus Healthy TTV-	16.52±10.62	17.25±9.08	N.S
Healthy TTV+ versus HBV+ TTV+	16.52±10.62	37.95±25.49	P<0.001
Healthy TTV+ versus HBV+ TTV-	16.52±10.62	36.31±20.72	P<0.01
Healthy TTV- versus HBV+ TTV+	17.25±9.08	37.95±25.49	P<0.001
Healthy TTV- versus HBV+ TTV-	17.25±9.08	36.31±20.72	P<0.01
HBV+ TTV+ versus HBV+ TTV-	37.95±25.49	36.31±20.72	N.S

N.S, not statistically significant; SD, standard deviation

Table 5. *Comparison of AST serum levels (IU/L) in healthy and HBV infected donors*

Comparison groups	Mean±SD		P-Value
Healthy TTV+ versus Healthy TTV-	18.71±9.84	20.68±10.64	N.S
Healthy TTV+ versus HBV+ TTV+	18.71±9.84	41.09±22.21	P<0.001
Healthy TTV+ versus HBV+ TTV-	18.71±9.84	43.38±22.76	P<0.001
Healthy TTV- versus HBV+ TTV+	20.68±10.64	41.09±22.21	P<0.001
Healthy TTV- versus HBV+ TTV-	20.68±10.64	43.38±22.76	P<0.001
HBV+ TTV+ versus HBV+ TTV-	41.09±22.21	43.38±22.76	N.S

N.S, not statistically significant; SD, standard deviation

When the original TTV isolate was discovered, TTV (genotype 1) detectable by N22 PCR was found in three of five patients with post-transfusion acute hepatitis of unknown etiology and the presence of TTV genotype 1 was closely associated with the serum ALT level .[1] Infection with TTV is characterized by persistent lifelong viremia in human beings, with circulating levels of up to 10^6 copies/ml in general population.[24] Furthermore TTV double-stranded replicative intermediates are detected in the liver, as documented by in situ hybridization and/or quantitative PCR.[10,14]

However further researches have revealed contradictory results showing that TTV is not associated with ALT levels or with any form of hepatitis (post-transfusion, chronic idiopathic, acute or fulminant). [25, 26, 27] At present, despite evidence for hepatic replication of TTV, TTV does not fulfill the criteria for being a hepatitis virus. For TTV to be characterized as a hepatitis virus, direct causal evidence of cytopathology or specific inflammatory changes associated with replication as well as statistical difference in comparison with controls in terms of TTV prevalence, loads, sequence variation, genotype distribution or co-infection among liver disease patients have to be demonstrated in future studies.

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Due to the great genetic variability of TTV, the choice of the viral DNA segment targeted for amplification has an enormous impact on PCR assay sensitivity, possibly more so than for most viruses. For historical reasons, the N22 region of ORF1 has been extensively targeted in nested or heminested PCR protocols. The primers designed on this region have been considerably improved over time, but nonetheless, they still may fail to amplify TTV isolates other than types 1 to 6, especially if the virus content in test samples is low. This limit has been solved only partly by using multiple sets of primers. Due to its higher conservation, the UTR of the TTV genome is much more suitable for primer design. [4] Indeed, generally UTR PCR has proven satisfactory at detecting most if not all the 16 TTV genotypes currently recognized and compared to ORF1 PCR, has greatly increased the rates of plasma or serum samples that give a positive reaction to TTV DNA: from 23 to 92% in the study by Takahashi et al [21], from 9 to 50% in the study by Irving et al [28] and from 20 to 95% in the study by Itoh et al. [22]

The results of this study also revealed a high prevalence of the virus in the population of the West Azerbaijan province. The results obtained in West Azerbaijan by using T801 and T935 consensus primers that are able to identify all the genotypes of this virus, like other parts of the world, is high. [4] The prevalences of 85, 93, 96, 82, 100, 98, 73, 57 and 82 percent were reported in Egypt, Japan, Myanmar, Nepal, Saudi Arabia, Singapore, Finland, Britain and Bolivia respectively. [4] In Isfahan and East Azerbaijan the prevalences were 79.5 and 65 percent respectively. [23, 29]

Meanwhile, the prevalences reported using primers designed for N22 regions of the virus in Egypt, Japan, Saudi Arabia, Finland, UK, USA and Bolivia were 29, 58, 19, 17, 10, 11 and 20 percent respectively. [4] Such prevalences were 41% [30], 4.4% and 11% [31] and 22.4%[32] in Tehran and 23.7% in Ahvaz [33].

This shows that primers designed for coding regions (N22) of the virus are more specific (detect a limited number of specific genotypes) and does not show the actual prevalence of the virus. [4,30,31,32,33] So, the high frequency of the virus in this study compared to the lower frequencies reported in other studies in Iran could be correlated to the methodology and the PCR primers used.

In the present study, TTV infection was detected in 69% and 75% of the healthy and hepatitis B infected blood donors respectively. No significant difference was observed in the frequency of the infection with TTV in different blood and age groups ($P>0.05$). In each group of blood donors the levels of ALT and AST were not significantly different in TTV infected and non-infected individuals ($P>0.05$). This fact indicates that TTV does not have any role in increasing of the levels of the hepatic enzymes. Significant differences were observed in the levels of hepatic enzymes between the healthy and HBV infected groups ($P<0.05$). This could be correlated with the effect of HBV rather than TTV. In conclusion, regarding the high frequency of infection in healthy individuals and considering the level of hepatic enzymes in TTV infected individuals; it seems that the virus or at least its investigated genotypes have not been pathogenic in the infected individuals examined. Although the precise relationship between TTV infection and viral hepatitis remains to be established, recent studies indicate that further clinical studies of TTV infected patients, in relation to TTV genotype, viral load, and quasispecies complexity are warranted.

Conclusion

As the blood donors tested positive for HBs Ag had no acute or chronic clinical signs, they may have been in early stages of the infection, so detecting the frequency of infection in patients with acute and chronic hepatitis with clinical signs is suggested. As certain genotypes may be pathogenic, determining the genotypes of the virus is also suggested.

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