Detection of TTV in peripheral blood cells from patients with altered ALT and AST levels

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This work analyzes the prevalence of TTV DNA in peripheral blood cells from patients with hepatic alterations and healthy blood donors and measures levels of sodium, potassium, urea, creatinine, phosphatase alkaline, total and direct bilirubin, gamma glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in certain randomly selected patients. DNA samples from 111 individuals were evaluated. They were divided into two groups, “A” (study) and “B” (control), including 54 patients with liver enzyme alterations (ALT/AST) presenting non-B-non-C hepatitis and 57 blood donors, respectively. TTV DNA was determined by nested PCR. Certain products of the second-round PCR were sequenced. Serum biochemical assay was performed and disclosed TTV in 31.48% (17/54) of patients in group A and 5.26% (3/57) in the control group B. TTV prevalence was significantly higher in patients with liver disease than in healthy donors. In group A, sodium, potassium, urea, creatinine, phosphatase alkaline, total and direct bilirubin, gamma glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were analyzed in certain randomly selected patients and no significant difference in biochemical levels (p>0.05) was found when TTV infected and noninfected individuals were compared. Knowledge related to TTV has rapidly increased, but many fundamental aspects remain unclear. This led us to question the role of TTV and doubt remains as to whether or not it is just a commensal virus. Further studies are necessary to confirm and extend these findings.

KEY WORDS: TTV, ALT, AST, GGT

INTRODUCTION

Throughout the world there still remain patients with liver diseases unexplained by hepatitis C and B viruses (HCV or HBV) or other classic hepatotropic viruses, such as hepatitis A, D and E. There has been speculation regarding the existence of another virus that would account for these unexplained cases and the recent discovery of TT virus (TTV) has rekindled the hope of a hepatotropic virus that may be able to explain the non-A-E hepatitis cases (Chattopadhyay et al., 2005; Das et al., 2004).

TTV was first described by Nishizawa et al., (1997) in a Japanese patient who exhibited elevated alanine aminotransferase (ALT) levels following transfusions. This agent was designated ‘TT’ virus, after the initials of the patient in whom it was discovered. More recently, however, taxonomists have further proposed that the full name for TTV should be Torque Teno Virus, within the genus Anellovirus (Abraham, 2005).

TTV is a nonenveloped virus (Okamoto, 1998b)
with a circular, negative-single-stranded DNA. Its genome varies in length between 3,808 nucleotides (SANBAN isolate) and 3,853 nucleotides (isolates TA278 and JA20); and is divided into a potentially coding region of ~2.6 kb and an untranslated region (UTR) of ~1.2 kb (Mushahwar, 1999). The former consists of two major potential protein-coding genes, ORF1 and ORF2, which correspond to a coding capacity of 770 and 150 amino acids (aa), respectively. Analysis of the sequences of 11 isolates revealed an additional small ORF (designated ORF3) with a coding capacity of 57 aa. The noncoding UTR of the TTV genome contains a G+C rich segment, as well as several regulatory sequences (Hijikata, 1999). In contrast to most DNA viruses, TTV isolates exhibit a high level of genetic heterogeneity: the existence of at least 16 genotypes (1 to 16) has been demonstrated by Okamoto et al. (1999). Therefore, depending on the PCR assay used, different TTV genotypes (or species) may or may not be detected (Vasconcelos, 2001).

In the decade since its discovery, TTV has been intensively studied to assess its molecular properties and whether it causes liver disease. The earliest reports of TTV suggested a causative link between TTV and liver disease. Okamoto reported 10 to 100-fold higher viral titers of TTV DNA in liver tissue, compared to serum in patients with non-A-G post-transfusion hepatitis. TTV was also more frequently seen in patients with fulminant hepatitis, chronic liver disease, liver cirrhosis and hepatocellular carcinoma than in chronic hepatitis (Okamoto, 1998a; Tanaka, 1998).

However, subsequent studies found TT viremia in hemophiliacs, thalassemics, hemodialysis patients (Das et al., 2004; Lopez-Alcorocho, 2001) and in apparently healthy individuals worldwide, particularly in certain geographic regions, such as African and South American countries (Prescott & Simmonds, 1998; Niel et al., 2000). Therefore the pathogenicity and clinical significance of TTV remain doubtful at this time.

The aim of the present study was to evaluate the presence of TTV infection in patients presenting altered ALT and AST levels and blood donors and, in the patient group, determine whether infection is correlated with alterations in sodium, potassium, urea, creatinine, phosphatase alkaline, total and direct bilirubin, gamma glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

**MATERIAL AND METHODS**

**Patients**

Following approval from the Human Ethics Committee from Londrina State University, DNA samples from 111 individuals attending the University Hospital of Londrina State University, Paraná, Brazil, were evaluated. They were divided into two groups, “A” (study) and “B” (control), including 54 patients with liver enzyme alterations (ALT/AST) presenting chronic liver disease like hepatitis and cirrhosis, gastric cancer, lupic nephropathy. Individuals for the control group including 57 blood donors were required to be in good health, defined as the absence of chronic diseases affecting the immune system such as human immunodeficiency virus infection (HIV), hepatitis B and hepatitis C infections. Also, for inclusion in the study the normal healthy blood donors were required to have normal alanine aminotransferase (ALT).

**TTV DNA Detection**

Genomic DNA was isolated from 5 mL of peripheral blood cells (PBCs) extracted from total blood in the presence of 0.2M NaCl, 0.25% SDS, for 4h at 37°C. After precipitation with ethanol, the pellet was dried and resuspended in 50 µL of milli Q water. The presence of TTV DNA was determined by nested PCR (Polymerase Chain Reaction) using a set of four primers, as described by Nishizawa et al. (1997). In this system, PCR product is a DNA fragment of 197bp localized in a region of the large open reading frame (ORF-1) that has been named region N22. Reaction conditions for the two PCR rounds were the same (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 mM dNTP and 1.25 units of Taq polymerase) and consisted of an initial denaturation step of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min and a final extension of 72°C for 10min using a thermocycler (PCRSprint ThermoHybaid). PCR products of 197bp were analyzed by electrophoresis on acrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method.
Sequencing
After purification, certain products of the second-round PCR were sequenced with MegaBACE1000 (Amersham Pharmacia Biotech, NJ, USA) using DYEnamic™ ET Dye Terminator Kit (Amersham Pharmacia Biotech, NJ, USA). Sequence analysis of TTV was performed by comparing the results with the NCBI-NIH database (Blastn), available at http://www.ncbi.nlm.nih.gov/blast.

Biochemical assay
The blood samples were centrifuged at 2700 g for 10 minutes. The serum was collected and stored in a refrigerator for a maximum of 24 h prior to use. These assays were carried out in a biochemical auto-analyzer Dade XL® (USA), using Dade Behring® kits (Dade Behring, Inc., Newark, NJ).

Statistical analysis
Statistical analysis was performed using the t test and odds ratios (OR) with 95% confidence intervals (95% CI), using Microcal Origin™ software, version 6.1. A P value ≤0.05 was considered statistically significant.

RESULTS
The prevalence of TTV DNA in patients with hepatic alteration (group A) and healthy blood donors (group B) was analyzed. TTV was detected in 31.48% (17/54) of patients in group A and 5.26% (3/57) in the control group B (Figure 1). TTV prevalence was significantly higher in patients with liver disease than in healthy donors (OR: 8.27; 95% CI: 2.26-30.25).

To prove primer specificity, three samples were

![Figure 1](image_url)

**FIGURE 1 - Detection of TTV virus.** PCR products were submitted to electrophoresis in 10% silver-stained acrylamide gels. Amplicons of 197 bp correspond to TTV-DNA. Group A (9, 10, 12, 13, 15, 26, 32, 62, 65, 66, 68, 71, 72, 73, 75, 77, 79) and Group B (14, 23, 28) L - ladder 100 bp (Invitrogen); Bl - blank reaction; C - positive control for TTV.

<table>
<thead>
<tr>
<th>Biochemical Assay</th>
<th>TTV- (n=33)</th>
<th>TTV+ (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/I) (n=50)</td>
<td>145.24</td>
<td>47.14</td>
</tr>
<tr>
<td>ALT (U/I) (n=52)</td>
<td>250.09</td>
<td>97.05</td>
</tr>
<tr>
<td>ALP (U/I) (n=38)</td>
<td>235.05</td>
<td>44.42</td>
</tr>
<tr>
<td>GGT (U/I) (n=41)</td>
<td>433.49</td>
<td>81.92</td>
</tr>
<tr>
<td>Total Bilirubin (U/I)</td>
<td>6.83</td>
<td>2.19</td>
</tr>
<tr>
<td>Direct Bilirubin (U/I)</td>
<td>5.44</td>
<td>1.91</td>
</tr>
<tr>
<td>Sodium (mEq/l) (n=32)</td>
<td>135.45</td>
<td>1.46</td>
</tr>
<tr>
<td>Potassium (mEq/l) (n=32)</td>
<td>4.30</td>
<td>0.25</td>
</tr>
<tr>
<td>Urea (mg/dl) (n=34)</td>
<td>63.56 ± 9.86</td>
<td>42.45 ± 12.18</td>
</tr>
<tr>
<td>Creatinine (mg/dl) (n=38)</td>
<td>1.50 ± 0.23</td>
<td>1.57 ± 0.51</td>
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randomly chosen, sequenced and compared with the NCBI-NIH database. Analysis demonstrated that the amplified fragment was compatible with a sequence in the GenBank, access AB008394. After analyzing all the individuals in both groups (n=111) to determine the existence of TTV infection, it was verified that 58% of the TTV negative individuals were male (51/91) and among TTV infected patients, 70% (14/20) were male. No significant difference was found (OR=0.55; 95% CI: 0.19-1.55).

In group A, sodium, potassium, urea, creatinine, phosphatase alkaline, total and direct bilirubin, gamma glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were analyzed in certain randomly selected patients. In this group, no significant difference in biochemical levels (p>0.05) was found when TTV infected and noninfected individuals were compared (Table 1).

DISCUSSION

In the present study, TTV DNA was detected much more frequently among patients with liver disease compared to the control group, in agreement with previous studies that detected TTV more frequently in hepatitis of unknown etiology (Ikeda et al., 1999) or in co-infection with other hepatotropic viruses (Chattopadhyay et al., 2005; Hu et al., 2002; Jeon et al., 2002). TTV detection in both groups (A=31.48% and B=5.26%) was lower than that previously reported. Das et al. (2004) showed a prevalence of 52% among blood donors and Hu et al. (2002) of 15%; in patients presenting liver disease of unknown etiology, a prevalence of 47% was found (Ikeda et al., 1999). This difference was also observed among Brazilian studies, where 31% infection was verified among healthy donors (Jeon et al., 2003).

TTV DNA has been detected in liver and bile specimens at concentrations 10-100 times higher than in the corresponding plasma samples and in fecal extracts at lower concentrations (Okamoto et al., 1998; Rodríguez-Inigo et al., 2000). Maggi et al. (2001) demonstrated TTV virus loads associated with different peripheral blood cell types and evidence for TTV replication in activated mononuclear cells.

Results reported by Itoh et al. (1999) indicate that the selection of PCR primers influences TTV DNA detection. Furthermore, these results suggest that restricted TTV genotypes detected by N22 PCR could be associated with liver damage among blood donors. Recently, it was verified that TTV infection is prevalent both in patients and healthy individuals in India. However, no significant correlation with other hepatitis viral infections was found, nor any evidence of severe liver patients, suggesting that TTV does not cause severe liver damage (Irshad et al., 2006).

TTV replication has been demonstrated in hepatocyte and leukocyte cell lines (Desai et al., 2005). Barril et al. (2000) verified the presence of TTV in serum and peripheral mononuclear cells from continuous ambulatory peritoneal dialysis using primers for the ORF1 and ORF2 regions of TTV. It has been suggested that concealed as a “Trojan horse”, TTV in peripheral blood cells might serve as a TTV reservoir inducing infection and transmission chronicity in some clinical and epidemiological settings (Zhong et al., 2002).

Large differences in the positive rate for TTV in healthy donors have been reported, ranging from 1 to 93%, sometimes in the same region (Hu et al., 2002). The difference in TTV prevalence between the present study and those reported by other authors is mainly the result of the type of primer sets used in the study. The set of primers derived from the UTR region, which were used in the other studies, have been found to be highly conserved and are able to detect TTV at a high frequency than primers from the N22 region used in the present study. However, in this study detection was made in PBCs whereas in previous studies detection was performed on serum. According to Okamoto et al. (1999), N22 PCR determines a much greater frequency of TTV DNA in peripheral blood mononuclear cells (PBMCs) than in plasma. The type of primer sets used in the study derived from the N22 region, could be associated with liver damage among blood donors. TTV was detected in 31.48% (17/54) of patients in group A and 5.26% (3/57) in the control group B. In the study group, TTV was detected in samples from patients with AIDS, gastric cancer, autoimmune diseases and B,C hepatitis.
Gender and age distribution were not significantly different between the TTV-positive and TTV-negative groups, a fact also verified in other studies (Jeonet et al., 2003; Lyra et al., 2005; Ikeda et al., 1999; Okamoto et al., 2000; Chattopadhyay et al., 2005).

In their initial description, Nishizawa et al. (1997) detected TTV in sera from three out of five patients with posttransfusion non-A-G hepatitis, in which the TTV DNA titers were closely correlated with aminotransferase levels. After this discovery TTV was intensively investigated as a hepatotropic virus. The analysis of biochemical parameters that indicate liver disease, such as ALT, AST, ALP, GGT and bilirubin showed no significant differences between TTV-infected and noninfected individuals. Previous studies verified mainly ALT and AST levels, but in general, although these observations were contrary to initial reports (Nishizawa et al., 1997; Okamoto et al., 1998a), they are in agreement with more recent reports on TTV (Ikeda et al., 1999; Yang et al., 2000; Okamoto 1999; Ding et al., 1999; Das et al., 2004; Michitaka et al., 2000). Therefore, no clear evidence of hepatic pathogenicity for TTV was found, as is the case in many other reports. Since TTV has frequently been described among individuals with other pathologies, such as gastrointestinal disease (Dai et al., 2003), diabetes (Iriyama et al., 1999), thalassemia (Gallian et al., 2000), hemophilia (Kondili et al., 2001) and especially in kidney disease (Yokozaki et al., 1999; Forns et al., 1999) clearly TTV does not only infect the liver, but also extrahepatic tissues, such as the kidney, spleen and heart. In this study, urea, creatinine, sodium and potassium levels were also analyzed, but the differences between TTV-positive and negative individuals were not significant.

TTV was originally thought to be a candidate for a new hepatitis virus and although its role in liver disease remains controversial, until its pathogenic potential is fully characterized it will remain relevant to investigate its implications in individuals presenting confections with other viruses and other diseases with biochemical alterations.

The present study found no clear evidence of TTV pathogenicity. This led us to question the role of TTV and doubt remains as to whether or not it is just a commensal virus. Further studies are necessary to confirm and extend these findings.

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