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TT virus (TTV) genotyping in blood donors and multiple transfused patients in Brazil

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Abstract TT virus (TTV) is widely distributed in the general population. The objective of the present study was to investigate the prevalence and distribution of TTV genotypes among blood donor candidates and multiple transfused patients in the Southeast region of the state of São Paulo, Brazil. TTV-DNA detection by amplification of a segment of the ORF-1 region, presented a prevalence of 11.9% in 270 serum samples from blood donors, of 46.2% in 18 samples from patients with coagulopathies, and of 31.8% in 15 samples from patients with hemoglobinopathies. When specific primers for the non-coding (UTR) region of the TTV genome were used the prevalences were 50.5%, 95.0%, and 82.0% for blood donors, patients with coagulopathies and patients with hemoglobinopathies, respectively. Positive samples from 49 individuals were sequenced and partial segments of 230 base pairs referring to the ORF-1 region of the TTV genome were used for the determination of their genotypes with the aid of phylogenetic analysis. The most frequent genotype was 1 (74.0%), followed by genotype 2 (26.0%). These data indicate a high prevalence of this virus in the populations of blood donors and transfused patients, providing further evidence for the role of transfusions as an efficient pathway in the transmission chain.

Keywords TT virus · *Anellovirus* · Prevalence · Phylogenetic analysis · Genotypes · Blood donors · Multiple transfused patients

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Introduction

TT virus (TTV) was isolated in 1997 from the serum of a patient (T.T.) with post-transfusional hepatitis of unknown etiology (non-A-G) [1]. TTV was first classified as a member of the family *Circoviridae* [2] and more recently denoted *Torque teno virus* and classified as a member of the genus *Anellovirus* together with TTMV (*Torque teno mini virus*) [3]. TTV has no envelope and its genome is composed of a single-stranded circular DNA of approximately 3,800 nucleotides. The genomic sequences of TTV present wide variability both in nucleotide and amino acid sequences, with identity ranging from 59% to 71% [4]. The marked divergence among the TTV sequences is reflected on the identification of more than 30 genotypes, most of which have been identified on the basis of the phylogenetic study of the N22 region, located in the open reading frame 1 (ORF-1) region. These genotypes are classified into five groups: group 1 consists of genotypes 1–6, group 2 of genotypes 7, 8, 17, 22 and 23, group 3 of 11 genotypes (9–16 and 18–20), group 4 of 9 genotypes, and group 5 of 3 genotypes [5].

TTV is globally distributed and is detected in healthy individuals [6–13] and in multiple transfused patients such as patients with thalassemia major, sickle-cell anemia, and hemophilia [14–19]. The prevalence of TTV reported for these groups varies according to the genomic segment studied. The use of the N22 region permits the detection of

genotypes 1–6 of group 1, while amplification of the segments located in the non-coding region (UTR) permits the detection of practically all the genotypes of the five groups [5, 20]. Most genotyping studies were conducted on sequences of the N22 region, demonstrating that genotypes 1, 2, and 3 are highly distributed worldwide [21–25]. Little is known about the nature and pathogenicity of this virus. Histopathological studies have suggested that TTV may be responsible for a mild form of hepatitis [26]. One study reported a 45% prevalence of TTV in patients with non-A-E acute hepatitis, while the prevalence detected among healthy individuals was only 10% [27]. In addition, TTV-DNA was detected in the liver at levels 10–100-fold higher than in the serum of the same patient [9]. Despite this evidence, the relation between TTV and hepatitis has not been fully clarified.

The objectives of the present study were to determine the prevalence of TTV-DNA in blood donors population and multiple transfused patients of the North and Northeast region of the State of São Paulo, Brazil, to genotype the Brazilian isolates for phylogenetic analysis, and to investigate the possible association between number of blood transfusions and TTV prevalence.

Material and methods

Study population

Blood donors

A total of 270 serum samples were obtained from blood donors of the Regional Blood Center of Ribeirão Preto, São Paulo, Brazil. The population consisted of 18.5% (50) females and 81.5% (220) males with a mean age of 35.7 years (19–59 years). The majority blood donors were in the 19–48 years age range, corresponding to 90.7% of the study group.

Multiple transfused patients

A total of 94 blood samples were collected from multiple transfused patients who regularly receive blood transfusions at the Regional Blood center of Ribeirão Preto. The samples were divided into two groups according to disease: group 1, 32 patients with coagulopathies (hemophilias A and B and von Willebrand disease) and group 2, 43 patients with hemoglobinopathies (thalassemia and sickle cell anemia).

The study was approved by the Institutional Ethics Committee of the University Hospital of Ribeirão Preto, University of São Paulo (process number 5137/2001).

Serologic analysis

All samples were tested for anti-HBc antibodies (HBc ELISA test system, ORTHO, Raritan, NJ, USA) and anti-HCV antibodies (HCV 3.0 ELISA test system with Enhanced Save, ORTHO), and HbsAg (Hepatonostika-HbsAg Uni-Form II, Organon, RM Boxtel, Netherlands). Alanine aminotransferase (ALT) levels were also determined using the activated SGpT kit and ALT Alcyon (Abbott, Abbott Park, IL, USA). Only the samples from blood donors were tested for anti-HIV1/2 antibodies (Vironosticka HIV Uni-Form II plus O, Organon), HIV1/HIV2 (Ab-Capture ELISA test system, ORTHO), anti-HTLV-I/II antibodies (Vironosticka HTLVI/II, Organon) and HTLVI/HTLVII (Ab Capture ELISA, ORTHO), and for Chagas' disease (Chagatek, Organon) and syphilis (Hemacruzi, Biolab, Rio de Janeiro, RJ, Brazil).

DNA preparation

DNA was extracted from 50 µl of serum previously treated with 150 µl of TNE extraction buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl), 20 µl 10% SDS (final concentration of 0.8%), and 10 µl proteinase K (10 mg/ml) (final concentration 0.4 mg/ml). The material was kept at 56°C for 40 min and DNA was then extracted by the phenol/chloroform method and precipitated with ethanol in the presence of glycogen (20 mg/ml). After drying, the DNA was dissolved in 40 µl sterile water and denatured at 95°C for 15 min.

Detection of TTV-DNA by nested-PCR

The PCR was carried out with 0.1 pmol of each primer, 0.05 mM dNTPs, and 1.5 U Taq polymerase in a total volume of 50 µl. About 10 µl of the denatured DNA sample were used for nested-PCR. The first round was conducted with the primers A5427 and A5430 [11] which amplify a 278 bp fragment of the ORF-1 region. The second round was conducted with 5 µl of the first round using the primers A5432 and A8761, which amplify a fragment of 271 bp. After an initial time of 10 min at 55°C and 10 min at 72°C, the first round of the PCR assay was carried out for 30 cycles of 20 s at 94°C, 30 s at 55°C and 90 s at 72°C, with a final extension of 10 min. The second round was performed on the same way as the first round except the initial time that was not carried out.

The PCR amplification of a UTR fragment (222 bp) from the TTV genome was carried out using the Set B primers previously described by Leary et al. [7]. The first round was conducted under the following conditions: 0.15 mM buffer containing 100 mM Tris-HCl, pH 8.3,

500 mM KCl, 15 mM $MgCl_2$ and 0.01% (m/V) gelatin, 0.5 pmol of each primer, 0.18 mM dNTPs and 1.5 U *Taq* polymerase. Nested-PCR was carried out under the same conditions as described above.

Sequencing reaction

The amplification products of the ORF-1 region were purified with the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA) according to manufacturer specifications. The purified samples were submitted to sequencing reaction with the A5432 and A8761 primers using the ABI Prism Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Norwalk, CT, USA). Electrophoresis was carried out using an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems).

Phylogenetic analysis

The TTV sequences obtained in the present study were initially submitted to analysis of similarity and identity matrices were constructed using BioEdit program, version 5.0.9. The nucleotide sequences were aligned with the reference sequences extracted from GenBank (NCBI, Bethesda, MA, USA) with the aid of the ClustalW tool (BioEdit version 5.0.9). The phylogenetic trees were constructed by phylogenetic analysis using parsimony (PAUP) program, version 4.0 beta. The Neighbor-Joining (NJ) method was used and the Kimura 3 parameter (K3p) substitution model was applied. The reliability of the tree was evaluated by the analysis of 1000 replicates and the tree was visualized using the Treeview 1.4 program.

Statistical analysis

Data were statistically analyzed by the Fisher exact test with the aid of the Intercooled STATA 8.0 program (College Station, TX, USA).

Results

Serologic tests

Out of the total 270 serum samples from blood donors analyzed, five were positive for anti-HBc antibodies (1.9%), one was positive for anti-HCV antibodies (0.4%), one showed reactivity for *Trypanosoma cruzi*, and four presented elevated ALT levels. All samples were negative for anti-HIV and anti-HTLV antibodies, for HBsAg and for non-treponemal antibodies.

Table 1 Prevalence of TTV according to age group

Age group	No of donors	PCR positive for TTV
19–28	85	10 (11.8%)
29–38	91	14 (15.4%)
39–48	69	6 (8.7%)
49–59	25	2 (8%)
Total	270	32 (11.9%)

For patients with coagulopathies, the reactivity for Hbs-Ag and anti-HBc and anti-HCV antibodies was 2.6%, 20.5%, and 36.0%, respectively. In the group of patients with hemoglobinopathies, reactivity was 20% for anti-HBc and 44.4% for anti-HCV, and HBs-Ag was not detected in any sample from this group.

Prevalence of TTV-DNA—ORF-1 region

When the coding region (ORF-1) for the TTV genome was analyzed, the prevalence of TTV-DNA was found to be 11.9% for blood donors ($n = 270$). About 75% of the positive individuals were concentrated in the age range of 19–38 years (Table 1). The prevalence detected in multiple transfused patients was 46.2% for group 1 ($n = 32$) and 31.8% for group 2 ($n = 43$) and differed significantly from the prevalence detected in blood donors ($P = 0.001$).

Alanine aminotransferase (ALT) activity was normal in TTV-DNA positive samples. They were also non reactive for HCV or HBc antibodies for blood donors and in patients with coagulopathies. Only one sample presented elevated ALT activity in the group of patients with hemoglobinopathies.

There was no correlation between the prevalence of TTV-DNA and the severity of hemophilia, with 42.3% of the patients with severe hemophilia A and 33.3% of those with moderate and mild hemophilia, presenting the viral DNA.

Prevalence of TTV—UTR region

The prevalence of TTV determined by the analysis of the UTR region was 50.5% for blood donors ($n = 91$), 95% for group 1 patients ($n = 32$), and 82% for group 2 patients ($n = 43$). The increased number of samples positive for TTV-DNA when the non-coding region was analyzed was significant ($P = 0.001$). About 63% of the positive individuals were concentrated in the 19–38 year age range (Table 1).

There was no correlation between the prevalence of TTV-DNA in the UTR region and the severity of hemophilia, with 100% of the patients with severe hemophilia A and 92% of those with mild to moderate hemophilia being positive for viral DNA.

Correlation between the number of transfusions received by patients with coagulopathies/ hemoglobinopathies and TTV infection

No correlation was observed between the prevalence of viral DNA and the quantity of factor VIII received in the last 6 months by the patients with hemophilia A or the quantity of red cell concentrate received by the patients with hemoglobinopathies in the last 12 months before the collection of a blood sample for the present study (data not shown).

Phylogenetic analysis

A total of 49 sequences (~230 bp belonging to the ORF-1 region) were obtained from 22 blood donor samples, from 12 samples from patients with coagulopathies, and from 15 samples from patients with hemoglobinopathies. Using multiple alignment, the TTV sequences obtained in the present investigation were studied for similarity, with the results showing that divergence among them in the segment studied (ORF-1) ranged from 39 (blood donors) to 43% (multiple transfused patients).

Phylogenetic analysis was carried out with 35 TTV prototypes including genotypes 1–6, and the SENV prototype which was used as an outgroup. The phylogenetic tree constructed with the prototypes demonstrated that 28.5% of the Brazilian isolates were classified as genotype 1a, 45% as genotype 1b, and 26.5% as genotype 2. Most of the TTV-DNA sequences in samples from Brazilian blood donors were classified as genotype 1b, whereas the sequences isolated from multiple transfused patients were preferentially grouped with genotypes 1b and 2 (Table 2). The reliability of this classification was supported by bootstrap values higher than 50%. These values are arranged in the knots of the phylogenetic tree (Fig. 1).

Discussion

The present results demonstrate that TTV viremia is frequently detected in the blood donors and multiple transfused populations of Ribeirão Preto and region. The presence of the virus in the population of healthy blood donors suggests other routes of transmission in addition to the parenteral one. Fecal–oral transmission has been suggested due to the presence of the virus in feces and saliva. Other routes have also been cited since TTV has also been detected in other body fluids such as semen, tears and maternal milk [28, 29].

Table 2 Genotypes distribution of TTV according to studies populations

TTV-DNA positive	Genotype 1a	Genotype 1b	Genotype 2
Blood donors (n = 22)	8 (36.4%)	11 (50%)	3 (13.6%)
Multiple-transfused patients (n = 27)	6 (22.3%)	11 (40.7%)	10 (37%)

The 11.9% prevalence detected among Brazilian blood donors by analysis of the ORF-1 region of the TTV genome was similar to that observed among German and Japanese blood donors [30, 31]. Several studies have demonstrated that the detection of TTV-DNA-positive samples can vary within a single population according to the gene region amplified. In Italy, the study of TTV prevalence using different regions of the genome demonstrated different values ranging from 5 to 22% [10]. The same was observed among Brazilian blood donors, for whom prevalences of 5.5 and 85% were detected [32]. In the present study, the number of TTV-DNA-positive samples increased significantly (50.5%) when the UTR region of the viral genome was analyzed. The different methodological approaches adopted directly affect the variation in TTV prevalence detected in the population of blood donors from Brazil and from other countries.

The higher prevalence of TTV detected among blood donors aged from 19 to 38 years did not differ from the prevalence observed in other age ranges ($P > 0.05$), demonstrating that in the population studied here, the prevalence of TTV was not correlated with age. In contrast to these data, other studies have demonstrated that the prevalence of TTV increases with age [33–35]. However, the intrinsic characteristics of the population and the number of individuals studied represent important factors for data correlation.

The present study demonstrated a significant increase in the prevalence of TTV-DNA among multiple transfused patients compared to blood donors. The high frequency of TTV-DNA in the population of patients who receive repeated transfusions of blood and blood products (hemophiliacs and thalassemics) has also been observed in other studies [14, 16, 34, 36].

Some studies have suggested that the prevalence of TTV in multiple transfused patients is related to the number of transfusions received, with a significant increase in positivity for TTV-DNA being observed among patients receiving a larger number of transfusions [11, 15]. Despite this initial evidence, our study did not detect a correlation between positivity for TTV-DNA and the number of transfusions. The same occurred regarding the severity of hemophilia A, as also observed by Yokosaki et al. [37].

The sequencing of the ORF-1 segment revealed a high-genomic variability of this region. In agreement with literature data, the TTV sequences obtained demonstrated a divergence of about 41%. The origin of the high divergence detected among the TTV sequences is unknown.

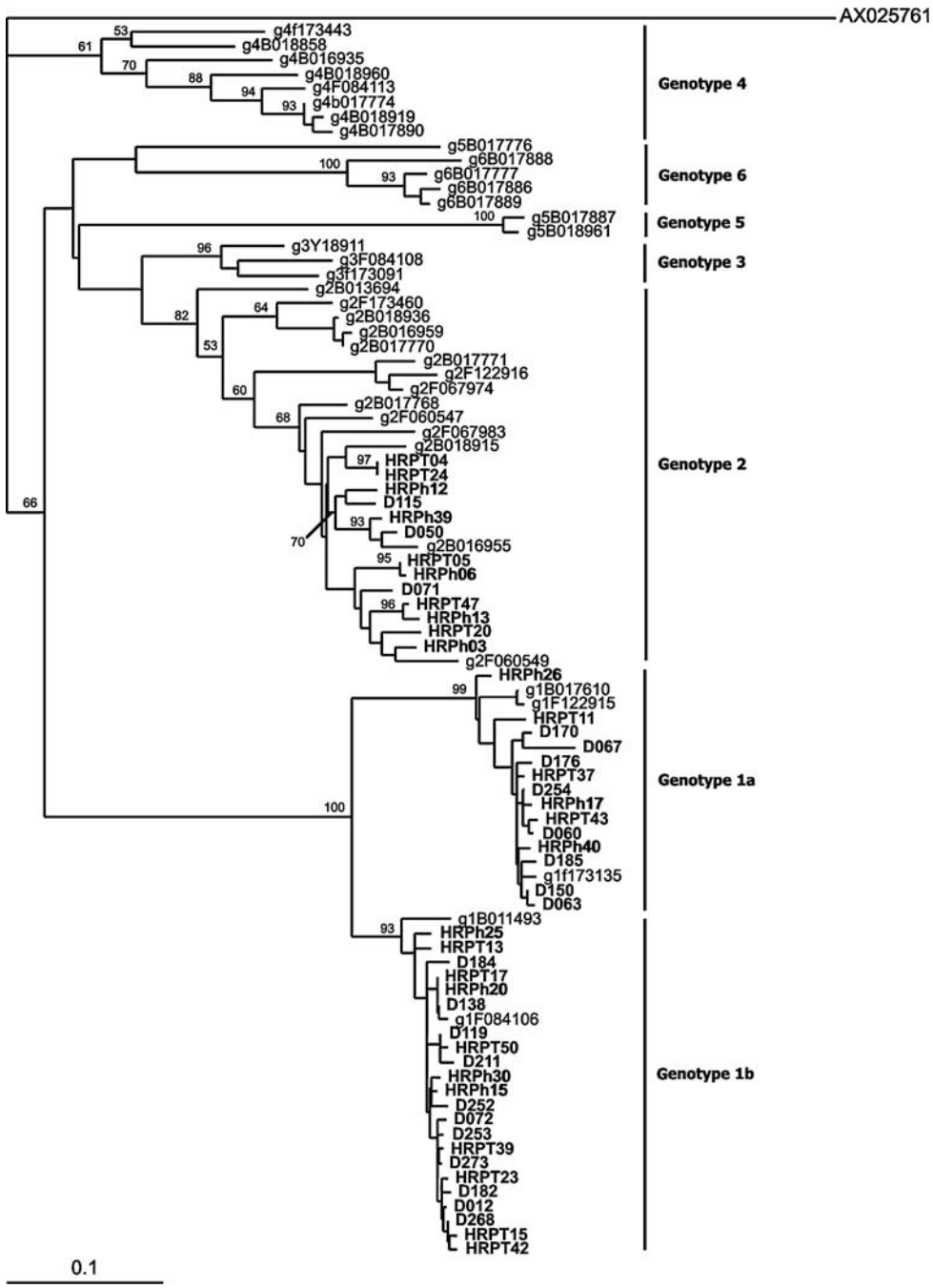

Fig. 1 Phylogenetic tree constructed by neighbour-joining method with nucleotide sequences of ORF-1 region of 47 isolated obtained from blood donors (D) and multiple-transfused patients (T/H). The prototypes are genotypes 1–6 according to classification of Okamoto et al. [20]. The prototype SENV (AX025761) was used as outgroup

However, the mechanisms of mutation such as substitution and recombination of viral genome segments may be responsible for the variability detected among the TTV-DNA sequences [38, 39]. The low similarity of the TTV sequences suggests that molecular studies should be conducted using more extensive and more conserved fragments of the TTV genome TTV [40].

In view of the high heterogeneity of the ORF-1 region, the TTV genotypes were determined in comparison to the prototypes that represent group 1 (genotypes 1–6) which can be detected by amplification of the N22 region. The SENV prototype (AX025761) was used as outgroup, because it belongs to the genus *Anellovirus* and presented high identity with TTV in the UTR region, showing 40–60% of divergence in the ORF-1 region [5].

The phylogenetic tree showed that genotypes 1a and 1b were the most frequent among blood donors (86.4%), as well as among multiple transfused patients (63.0%). Genotype 2 was more frequent (37.0%) among multiple transfused patients than among blood donors (13.6%), although the difference was not statistically significant (Table 2). Similarly, a study conducted in Turkey to

analyze the N22 region showed that genotypes 1 and 2 were the most frequent in the various groups investigated and the only ones detected in the blood donor group [24].

The present study detected the presence of genotypes belonging to group 1. However, it is necessary to evaluate the occurrence of other genotypes corresponding to groups 2–5. For this purpose, the detection of these genotypes requires the use of group-specific primers or of primers able to anneal to the different TTV genotypes (group independent) [3]. In a study using group-specific primers, the genotypes of groups 1 (8%), 3 (25%) and 5 (25%) predominated among 24 Brazilian blood donors [41]. These three groups were also detected more frequently among blood donors in France, although with a different distribution, being group 1 the most prevalent (34%) [3].

In conclusion, the current study represents an epidemiological molecular investigation evaluating a large number of Brazilian isolates. The high prevalence of TTV detected in the multiple transfused patient population shows the important role of transfusion in the transmission process, although no correlation was observed with the number of transfusion. Other studies involving molecular characterization of entire genome of *Annelovirus* genus should respond to questions related to TTV pathogenesis.

Accession numbers for the nucleotide sequences

All sequences obtained in the present study have been deposited in GenBank under the following accession numbers: Blood donors—AY147042, AY147043, AY147044, AY147045, AY147046, AY147047, AY147048, AY147049, AY147050, AY147051, AY147052, AY147053, AY147054, AY147055, AY147056, AY147057, AY147058, AY147059, AY147060, AY147061, AY147062, AY147063 and AY147064. Multiple transfused patients—DQ665270, DQ665271, DQ665272, DQ665273, DQ665274, DQ665275, DQ665276, DQ665277, DQ665278, DQ665279, DQ665280. DQ665281, DQ665282, DQ665283, DQ665284, DQ665285, DQ665286, DQ665287, DQ665288, DQ665289, DQ665290. DQ665291, DQ665292, DQ665293, DQ665294, DQ665295, DQ665296.

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