Coinfection with Multiple TT Virus Strains Belonging to Different Genotypes Is a Common Event in Healthy Brazilian Adults

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TT virus (TTV) is a newly discovered human virus that was first detected in the serum of a Japanese patient (initials, T.T.) with posttransfusion hepatitis (12, 13). The TTV genome is constituted by a single-stranded, circular DNA of negative polarity (9, 13). The TTV nucleotide sequence (3,818 to 3,853 nucleotides) does not show a significantly high homology to the sequence of any other virus. Several TTV isolates have been entirely sequenced (4, 6, 8, 9, 14), revealing a high degree of divergence among strains and the existence of at least 16 genotypes, which are separated by an evolutionary distance greater than 0.30 (15).

Although TTV DNA titers closely correlated with amino-transferase levels in the sera of some patients during posttransfusion hepatitis (12), no clear association between TTV infection and human liver disease has been established at this time. Very high prevalences (62 to 96%) of TTV infection have been found in healthy populations of Japan (15, 20) as well as in developing Asian, African, and South American countries (1, 11, 17).

As was initially demonstrated, TTV transmission occurs through the parenteral route (12). However, very high prevalences in healthy populations indicate the existence of other routes of transmission.

Coinfection with multiple TT virus strains has been described for people exposed to blood and blood products (3, 5, 21), as well as for patients with liver disease (2, 7, 22). Here we show that such a coinfection is a common event in Brazilian health care workers and that a healthy person can be coinfected by at least seven strains.

MATERIALS AND METHODS

Population studied. In 1999, all persons working in a public hospital of the city of Rio de Janeiro, Brazil, were invited to receive immunization against hepatitis B. At the occasion of the first-dose injection, blood samples from 1,104 people were collected. Testing of the DNA of TTV was done with serum samples.

RESULTS

Sero-prevalence of TTV DNA. One hundred ninety-one persons working in a public hospital of the city of Rio de Janeiro, Brazil, were enrolled in this study. Serum samples were analyzed for the presence of TTV DNA. One hundred twenty-five samples were positive, corresponding to a prevalence of 65.4%.

Phylogenetic analysis. PCR products were cloned, and sequences of 159 bases (nucleotides 26 to 184) surrounding the TATA signal region were determined for 100 clones derived from 31 individuals. One clone from each of 23 subjects was sequenced, while 7 to 19 clones from eight individuals were sequenced. None of the sera contained a viral sequence identical to that of any other individual. Phylogenetic analysis revealed the existence of a divergent TTV genotype possessing a single-base deletion at position 140. Among the eight persons for whom various sequences were analyzed, six were coinfected with between two and seven TTV strains belonging to different genotypes. The results suggest that coinfection with multiple TTV strains belonging to different genotypes is a common event in healthy Brazilian adults.

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quences were identical when derived from different persons. Evolutionary distances between our sequences were up to 0.49. A group of 14 sequences constituted a separate branch (Fig. 1). These had in common a 1-nucleotide deletion at position 140. The genetic distances between strains of this cluster were less than 0.30.

Despite the large genetic diversity observed here, some stretches of the genome were perfectly conserved in all sequences. This was notably the case for the TATAA motif at nucleotides 86 to 90 and an ATG codon (position 107) which had been initially proposed to be the translation initiation codon of ORF2 (13).

**Coinfection with multiple TTV isolates.** Several clones derived from the same serum were sequenced to determine if...
The prevalence (65.4%) obtained now in a group of 191 health blood donors living in the city of Rio de Janeiro, Brazil (11), was not significantly different from that determined in a previous study (5). The TTV genome, which is 417 nucleotides long, encodes two open reading frames (ORFs) (6, 15, 18, 23). The PCR assays developed soon after the discovery of this virus (4, 6, 14) and are based on NG059, NG061, and NG063, but which were degenerate to allow the detection of a higher number of TTV variants. We recently reported a TTV seroprevalence of 62% in 110 unrelated Chinese individuals (15, 16). Here we show that such a mixed infection (or superinfection) with multiple TTV strains occurred. This was performed for eight individuals whose demographic, professional, serological, and clinical data are shown in Table 1.

Altogether, 77 clones (7 to 19 from each person) were sequenced. Figure 2 shows the alignment of the nucleotide sequences, and Table 2 summarizes the characteristics of the sequences derived from each person. Surprisingly, in only one case (a young medical student, subject SC189), all the clones showed identical sequences. In another individual (attendant SC484), four closely related sequences (evolutionary distances less than or equal to 0.02) were found (Table 2). In the other subjects (five assistant nurses and one attendant), between two and eleven distinct sequences were found. The different TTV sequences deriving from single individuals could be genetically very close or very separated (evolutionary distances from 0.006 to 0.45). By using sequences of TTV strains of known genotypes and subtypes, genetic distances between two genotypes and between subtypes of the same genotype were calculated. All distances (calculated for the genome segment covering nucleotides 26 to 184) were greater than or equal to 0.11. On this basis, it could be concluded that six of the eight persons under study were coinfected with between two and seven different TTV genotypes or subtypes (Table 2, last column).

**DISCUSSION**

TTV is a virus with a wide nucleotide sequence divergence (6, 15, 18, 23). The PCR assays developed soon after the discovery of the virus were not able to amplify DNA of all genotypes, and the TTV prevalences in the populations of different countries have therefore been dramatically underestimated. Recently, improved PCR protocols and new sets of primers have led to increased rates of TTV DNA detection. The PCR method employed in this study has thus allowed the detection of TTV in 92% of the serum samples of a group of Japanese blood donors (20), a prevalence much higher than the 23% obtained with the same samples by using the previously designed primers NG059, NG061, and NG063 (13). Another independent study has shown that the method allows the detection of five- to sixfold more TTV-positive samples than another protocol (7). Using PCR primers whose design was based on NG059, NG061, and NG063, but which were degenerated to allow the detection of a higher number of TTV variants, we recently reported a TTV seroprevalence of 62% in blood donors living in the city of Rio de Janeiro, Brazil (11). The prevalence (65.4%) obtained now in a group of 191 health care workers living in the same city was not significantly different.

Although the DNA segment analyzed here is relatively short and is more conserved than other regions of the TTV genome (4, 14), no two sequences were identical when derived from different persons. Eighty-nine percent of the genetic distances between two of our strains were higher than 0.10 (17% higher than 0.30), with a maximum value at 0.49. These findings demonstrate the large genetic diversity of TTV strains circulating in Brazil. At the moment, few TTV genotypes have been sequenced in the genome region under study. This made it difficult to ascribe a genotype, from the 16 previously described (15), to each of our samples. However, it is interesting that 14 sequences, with a single-nucleotide deletion at position 140, constituted a separate genotype (Fig. 1). Examination of the nucleotide sequences deposited in Genbank by using the BLAST program revealed that two sequences (AF109811 and AF109812), from Chinese TTV isolates, presented the 1-nucleotide deletion at position 140. These two sequences presented an overlapping of only 72 bp with ours. On this small genome segment, a very high homology (93 to 98%) was observed between the Chinese sequences and the 14 Brazilian sequences belonging to the separate genotype. The ORF2 translation initiation codon was initially localized to position 107 (9, 13). However, further studies suggested that another ATG codon, at position 263, is preferentially recognized as the start codon (4, 6, 14). The existence of a single-nucleotide deletion at position 140 in 14 sequences, which would introduce a frameshift mutation, reinforced this hypothesis. However, it is noteworthy that there was no case in which all TTV clones derived from the same individual contained that deletion. Therefore, a complementation between wild-type and deletion mutant strains cannot be excluded.

Mixed infections of TTV have been reported in individuals at high risk for infection with parenterally transmitted viruses, such as intravenous drug users (9), hemophiliacs (9, 21), and hemodialysis patients (3, 5), as well as in patients with liver disease (2, 7, 9, 22). Recently, coinfections with two or three TTV strains have been reported to be in some healthy Japanese individuals (15, 16). Here we show that such a mixed infection is a common event in healthy Brazilian people, at least in health care workers. Our results confirm and extend previous observations showing that infection by a given genotype is not protective against the superinfection by another type (5, 15). Furthermore, we show that the number of TTV isolates infecting an individual can be high. For example, for subject SC894, the nucleotide sequences of 19 clones were determined and 11 distinct sequences were obtained (Table 2). Genetic distances between two TTV sequences present in the same serum could be very close or very divergent (up to 0.45 for subject SC314). Although the mutation rate of the TTV

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Profession</th>
<th>Presence of protein in serum*</th>
<th>Clinical history</th>
<th>Blood transfusion</th>
<th>Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC098</td>
<td>F</td>
<td>44</td>
<td>Assistant nurse</td>
<td>Anti-HAV: Pos; HBsAg: Neg; Anti-HBs: Neg; Anti-HBC: Neg; Anti-HCV: Neg</td>
<td>ALT (IU/liter): 23; AS (IU/liter): 80</td>
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<td>No</td>
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<td>SC160</td>
<td>F</td>
<td>40</td>
<td>Assistant nurse</td>
<td>Anti-HAV: Pos; HBsAg: Neg; Anti-HBs: Neg; Anti-HBC: Neg; Anti-HCV: Neg</td>
<td>ALT (IU/liter): 15; AS (IU/liter): 34</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>SC189</td>
<td>F</td>
<td>22</td>
<td>Medical student</td>
<td>Anti-HAV: Neg; HBsAg: Neg; Anti-HBs: Neg; Anti-HBC: Neg; Anti-HCV: Neg</td>
<td>ALT (IU/liter): 16; AS (IU/liter): 16</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>SC314</td>
<td>F</td>
<td>43</td>
<td>Assistant nurse</td>
<td>Anti-HAV: Neg; HBsAg: Pos; Anti-HBs: Pos; Anti-HBC: Pos; Anti-HCV: Pos</td>
<td>ALT (IU/liter): 11; AS (IU/liter): 11</td>
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<td>Yes</td>
</tr>
<tr>
<td>SC319</td>
<td>M</td>
<td>50</td>
<td>Assistant nurse</td>
<td>Anti-HAV: Pos; HBsAg: Neg; Anti-HBs: Neg; Anti-HBC: Neg</td>
<td>ALT (IU/liter): 28; AS (IU/liter): 20</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SC484</td>
<td>M</td>
<td>42</td>
<td>Attendant</td>
<td>Anti-HAV: Neg; HBsAg: Neg; Anti-HBs: Neg; Anti-HBC: Neg</td>
<td>ALT (IU/liter): 36; AS (IU/liter): 34</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SC692</td>
<td>F</td>
<td>45</td>
<td>Attendant</td>
<td>Anti-HAV: Neg; HBsAg: Neg; Anti-HBs: Neg; Anti-HBC: Neg</td>
<td>ALT (IU/liter): 32; AS (IU/liter): 43</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>SC894</td>
<td>F</td>
<td>50</td>
<td>Assistant nurse</td>
<td>Anti-HAV: Pos; HBsAg: Neg; Anti-HBs: Neg; Anti-HBC: Neg</td>
<td>ALT (IU/liter): 24; AS (IU/liter): 28</td>
<td>No</td>
<td>No</td>
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</table>

* HAV, hepatitis A virus; HCV, hepatitis C virus; ALT, alanine aminotransferase; AS, aspartate aminotransferase. ** Hepatitis C virus RNA negative.
FIG. 2. Alignment of partial nucleotide sequences of 36 TTV variants found in eight health care workers. The number of clones showing identical sequences is given. The sequence of prototype isolate TA278 of genotype 1a (13) is indicated at the top. Dashes represent the same nucleotides as in the TA278 isolate. In two clones from subject SC319, an insertion of an A nucleotide occurs at position 45; slashes indicate the absence of this nucleotide in the other sequences.
genome as well as the duration of infection in the persons under study are unknown, it is likely that very close sequences (genetic distance < 0.02) derived from a single source of infection (quasispecies). On the contrary, there is no doubt that subject SC314 was infected from multiple sources. Using a cutoff value of 0.10, it was demonstrable that TTV strains belonging to at least seven genotypes or subtypes may infect the same healthy person (Table 2).

A recent study has demonstrated similar prevalences of TTV infection in medical workers and in age-matched controls (10). It remains to be determined if the phenomenon of multiple infections occurs during the life of healthy individuals or if such a tendency is restricted to health care workers.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to the GenBank database under accession no. AF216433 through AF216491.

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REFERENCES


