Prevalence of TT viral DNA in Italian blood donors with and without elevated serum ALT levels: molecular characterization of viral DNA isolates

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ABSTRACT

Background and Objectives. A novel non-enveloped DNA virus, called TT virus (TTV), has been reported to be associated with post-transfusion hepatitis of unknown etiology. Although its clinical role still remains obscure, its presence in blood donations might cause problems. It, therefore, appeared of interest to investigate TTV prevalence in voluntary blood donors.

Design and Methods. A total of 595 Italian blood donors with and without elevated serum alanine aminotransferase (ALT) levels were tested by polymerase chain reaction using two sets of semi-nested primers that amplify the well-known region in the N22 clone. The amplified products were then sequenced to assess the genotype by phylogenetic and restriction fragment length polymorphism analyses.

Results. The prevalence of TTV in blood donors was 5±1.9% (25 out of 500) with a 95% confidence limit. A similar prevalence was found in 95 selected blood donors with increased ALT levels. A viral load of $10^3$–$10^4$ viral DNA molecules/mL was found, thus indicating a rather narrow range of variability. A phylogenetic tree built up on the basis of 210 base sequences of ORF1 allowed isolates to be classified into 2 groups corresponding, at least, to two of the putatives TTV genotypes, group 1 and group 2 of Okamoto’s classification. A similar classification was also obtained by site restriction enzyme analysis.

Interpretation and Conclusions. The results show that TTV infection is present among Italian blood donors. No significant difference in prevalence of TTV infection was found between patients with normal and increased ALT, making the association between TTV infection and human hepatitis questionable.

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Key words: TTV DNA; blood donors; ALT; genotypes; restriction fragment polymorphism; hepatitis viruses

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Design and Methods

Samples
Samples were randomly collected from 500 voluntary healthy blood donors who donated at the National Blood Transfusion Center of the Italian Red Cross (Rome, Italy). The study group included 426 males and 74 females aged from 19 to 65 years old (median age: 38 years). An additional group of 95 selected blood donors, 89 male and 6 female (median age: 36 years) with elevated ALT levels (mean±SD 60.2±25 U/L, range 41-212) were included in the study. These donors, as the other 500 with normal ALT levels, were recruited by the above Transfusion Center. All the 595 blood donations were collected in the same period (December 1998) and were found to be negative for HbsAg, anti-HCV and anti-HIV1-2 markers.

The serum samples were pooled in a 2-dimensional matrix obtaining one 10-sample pool for each dimension. Pools and the remaining individual samples were stored at –30°C until testing. Aliquots of each sample were also frozen at –30°C for further investigation.

Sample preparation and PCR-based amplification of TTV DNA

TTV DNA was isolated and purified from 200 μL serum (pooled or single samples) using silica columns provided with the QiaAmp™ kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Ten microliters of purified nucleic acid were used for TTV detection by polymerase chain reaction (PCR) using two sets of semi-nested primers recognizing an internal region of the N22 clone.1 Primer set 1 is a combination of Okamoto’s primer NG05913 with Simmond’s primers A 5432 and A 8761.12 The efficiency and amplification conditions of this set of primers have already been described.14 The primer set 2, (NG059, NG063 and NG061) was that used by Okamoto.13 PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Extracts of positive samples were serially diluted in half log intervals prior to amplification. The highest dilution that gave a positive result, taken as end-point dilution, was assumed to contain at least one viral DNA molecule.

Appropriate precautions, as described by Kwok et al.,15 were taken to avoid false-positive results.

PCR product purification and sequence analysis

Amplons were purified from residual primers, nucleotides and enzymes by spin columns provided with the QIAquick PCR Purification kit (Qiagen) and then concentrated using Centricon-100 membrane (AMICON, Beverly, USA). The PCR purified products were directly sequenced using the DNA sequence kit (Perkin Elmer) and the ABI 373A DNA Sequencer (Applied Biosystem, Foster City, CA, USA).

Partial ORF1 sequences (210 bp excluding the primer sequence) from positive samples were compared to each other and with five sequences of prototype TTV strains and subjected to molecular evolutionary analysis using the computer software programs Pileup (Wisconsin Sequence Analysis package, GCG, Madison, WI, USA). A phylogenetic tree was constructed using the unweighted pair-group method with arithmetic average (UPGMA).

Results

TTV prevalence in blood donors

A preliminary TTV PCR-based screening was performed on 100 pools obtained according to the 2-D matrix described in the Design and Methods section. Forty-one of these pools were TTV positive. Since more than one sample in the same column and/or in the same row was identified as positive, 85 aliquots of single samples were re-tested. A total of twenty-five out of 500 samples were confirmed as being TTV positive, representing a prevalence rate of 5±1.9% (p <0.05%). A similar prevalence was found in donors with elevated ALT levels (3 out of 95). All positive

Table 1. RFLP analysis of TTV isolates.

<table>
<thead>
<tr>
<th>TTV Isolate</th>
<th>MseI</th>
<th>NdeI</th>
<th>NlaIII</th>
<th>EcoRI</th>
<th>PstI</th>
<th>PstII</th>
</tr>
</thead>
<tbody>
<tr>
<td>N22</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1a</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1b</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2a</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2b</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Restriction sites recognized (+) or not (−) by MseI (pos. 1985), NdeI (pos. 2074). Shaded areas show identical restriction site pattern indicating potential TTV isolates grouping.
TT Virus in Italian blood donors

and negative results obtained with the two sets of
primers were in perfect concordance between them.
As estimated by end-point dilution assay TTV viral
load ranged from $10^3$ to $10^4$ viral DNA molecules/mL.

Sequences and computer analysis
To determine the specificity of amplification and
the degree of sequence variability, all samples found
positive for TTV DNA were directly sequenced. The
nucleotide sequences of the 210 bp region spanning
the putative ORF1 sequence (1939–2149)  were com-
pared to each other and with five different TTV iso-
lates (N22 clone and the representatives of the puta-
tive genotypes 1a, 1b, 2a and 2b deposited in the
GenBank) by the Pileup Program. Two major clus-
ters were identified: one with 17 isolates and homol-
gy of 75-96%, the other with 11 isolates and homol-
gy of 59-63%, which corresponded respectively to
groups G1 and G2, according to Okamoto’s classifi-
cation.4

A phylogenetic tree was constructed for the 28 TTV
DNA isolates by the UPGMA program (Figure 1). All
TTV sequences were segregated similarly into 2 major
groups. Within the first group two additional clusters
of related variants were observed, corresponding to
the previously described genotypes 1a and 1b.6 In
contrast, the second major group showed an asym-
metric branching pattern in which subgroups G2a and
G2b were excluded. Bootstrap analysis (1,000
replicates) confirmed the reliability of these group-
ings. The corresponding values for G1 and G2 groups
and for subtypes 1a and 1b were higher than the arbi-
trary cut-off value of 75%.

A further genotype distribution was obtained on
the basis of restriction fragment length polymor-
phism (RFLP) analysis described by Tanaka17 (Table
1). Four out of 17 isolates of group 1, having the
highest homology (90-96%), showed MsèI, NdeI
and NlaIII restriction sites, as did the prototypes G1a and
N22, and therefore could be assigned to subgroup
G1a. The other 13 isolates contained restriction sites
recognized only by NdeI and NlaIII typical of the G1b
subgroup and hence should belong to this subgroup,
although three of them (isolates Cri221, Cri222 and
Cri240) showed a lower homology (75-82%) than
the other ten (85-88%). In addition, 12 out of 13
G1b isolates contained a restriction site recognized
by EcoRI. The remaining 11 isolates were neither
digested by NdeI nor by NlaIII and therefore can be
assigned to G2. Although PstI sites (positions 2038
and 2077) do not discriminate between G2a and
G2b, they allowed additional subtypes, which at pre-
cent cannot be given a designation, to be detected in
our isolates. The isolate Cri8 was not recognized by
any of the enzymes considered and showed the lowest
sequence homology (59%).

Most nucleotide changes occurred in the third
position of codons: of a total of 1,507 mutations
against the consensus sequence, 29% were at the first
position, 22% at the second and 49% at the third,
resulting in a 5-47% amino acid change rate.

Discussion
Much progress has been made in the last 15-20
years in reducing the risk of transmitting virus infec-
tions by blood and blood derivates, such as the
introduction of new serologic assays for the screen-
ing of viral markers, the adoption of stricter criteria
for selection of blood donors and the development of
several viral inactivation and removal procedures.18
However, transfusion-associated hepatitis is still the
most frequent infectious complication of transfusion
medicine.2

The recently discovered TTV is an example of
how many unidentified viruses may yet be detected in
blood donations. This non-enveloped ssDNA virus

Figure 1. A phylogenetic tree drawn from a
phylogenetic analysis (by unweighted pair
group method with arithmetic mean - UPG-
MA) of 28 TTV isolates. The original clone
N22 and G1a, G1b, G2a and G2b prototype
isolates of TTV were taken from Nishizawa
et al.3 and Okamoto et al.4

![Phylogenetic Tree](image-url)
has a world-wide distribution as reported by different workers who found different prevalences (from 1.9 to 64%) in volunteer blood donors from different countries.\textsuperscript{4,12,19,20} These high prevalences suggest that TTV can be transmitted in a non-parenteral or community-acquired fashion, such as via the fecal-oral route.\textsuperscript{11} Moreover, the presence of TTV in patients with a recent history of blood or blood products transfusion\textsuperscript{11} does not allow the relevant role of parenteral transmission of TTV to be excluded.

Very recently Itoh et al.\textsuperscript{22} reported a higher prevalence of TTV in blood donors with elevated ALT levels (22%) than in those with normal levels (16%). According to the authors, these findings strengthen the association of TT virus with non-A to -G hepatitis. In contrast, in the present study the prevalence was not significantly different between the two groups of blood donors (3% and 5% respectively; \( p < 0.56 \)), suggesting an asymptomatic carriage of TTV and making the association between TTV infection and human hepatitis questionable. This appears consistent with the point of view of several workers who very recently stressed the lack of evidence linking TTV with human hepatitis.\textsuperscript{1,9}

Moreover, the lower prevalence found in our study cannot be due to the sensitivity of the primer sets employed, since identical results were obtained with both sets and one of them (NG059, NG063, NG061) was that used by Itoh et al.\textsuperscript{22} and widely reported by most workers.\textsuperscript{17,19,21}

No mixed TTV genomes were observed in the sera tested, in contrast with the finding of the co-presence of different viral sequences in subjects at high risk of contracting blood-borne viruses.\textsuperscript{11} Further studies should be carried out to define whether donors from our study are persistently infected carriers or whether the viremia is detectable only for a short period corresponding to acute infection without significant clinical signs.

The prevalence of TTV reported here is much higher than that found during 1998 for the other hepatitis virus markers screened for in the blood donor cohort from which our samples came (0.075% for HBsAg and 0.093% for anti-HCV). This would indicate that TTV infection is generally spread in the Italian population and further studies, involving the same blood donor cohort in a different period, may be useful to assess whether TTV prevalence is influenced by seasonal changes.

Data on phylogenetic analysis of the 28 TTV isolates (Figure 1) are consistent with the reported genome heterogeneity of the virus. Moreover, they show that two viral types are present in Italy; these types correspond to Okamoto’s G1 (17 isolates) and G2 (11 isolates) groups. The same pattern of TTV isolates (Table 1) was obtained both with the computer programs and with the recently published RFLP analysis using four restriction enzymes.\textsuperscript{19} Moreover our results show that EcoRI may be included in the list of the restriction enzymes used to recognize genotype-specific sites since it could identify G1b isolates by itself with the exception of Cri222 which appears to be the most phylogenetically heterogeneous.

The marked heterogeneity of the nucleotide sequences of TTV isolates, particularly those included in the G2 group reported here confirming data from other workers, underscores the extreme difficulty of assessing the possible relationship between the biological characteristics of the virus and its clinical role if it has one. It should be noted, moreover, that exactly because of such a heterogeneity, it is not certain that the primers employed at present can detect all possible variants of the virus. Further research carried out on a much greater number of TTV isolates from widely spread geographic areas appears necessary to shed light on the genetic evolution of the virus.

Contributions and Acknowledgments

GP and GG formulated the design of the study and wrote the paper. PI and MM were responsible for the collection of plasma samples and biochemical/virologic tests. IA and GB pooled plasma samples and carried out TTV amplification. GP and GG carried out the sequencing and interpreted the data. M W and GB contributed to co-ordinating the project and writing the manuscript. IA, PI and MM helped to write the manuscript.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

- The present findings support the general agreement, matured on the basis of very recent studies, about the lack of an evident link between TTV and chronic and acute liver disease.

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