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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.**

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

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At present stringent donor selection and blood unit screening procedures have reduced the risk of transmission by blood and plasma products of transfusion-related viruses such as hepatitis B and C viruses (HBV and HCV) and the human immunodeficiency viruses 1 and 2 (HIV 1-2). However, a small but significant risk remains due to the serological window period and to donations from carriers of low level infectivity. Moreover, non-A to -G hepatitis and CMV infection, probably the most common infectious complication in transfusion medicine, appear to account significantly for transfusion-associated events.[1,2]

In 1997, some Japanese authors described the molecular cloning and characterization of a non-enveloped, DNA virus isolated from the serum of a Japanese patient with post-transfusion hepatitis of unknown etiology. This virus was designated TT virus (TTV).[3,4] Recently, molecular and biophysical characterization of TTV (GH1 isolate) demonstrated a circular structure and negative polarity of the genome, a single-stranded DNA 3,852 nt long.[5] Moreover, the buoyant density in CsCl (1.31-1.34 g/cm^3), the particle size as determined by filtration (30-50 nm) and the absence of sequence similarities between TTV and Circoviruses suggested that TTV might be part of a new virus family, tentatively named *Circinoviridae*.[5]

At the beginning, the clinical significance of TTV infection remained uncertain. Some contradictory reports were published on the role of TTV in the development of acute and chronic liver disease of unknown etiology.[6,7] However, there is now a general agreement based on very recently published studies, that there is not an evident link between TTV and acute and chronic liver disease.[8,9]

As far as the route of transmission of TTV is concerned, the high prevalence of TTV found in groups at high risk of contracting blood-borne viruses such as hemophiliac and thalassemic patients suggests a parenteral mode of transmission.[10-12] On the other hand, the high prevalence of TTV in healthy blood donors suggests that there are also other routes of transmission.[13] Phylogenetic analysis provides evidence for TTV genome heterogeneity and indicates the existence of several viral types and subtypes, some of which have different geographic distributions.[14]

In this study, we present the results on the prevalence of TTV DNA in Italian volunteer blood donors with and without raised levels of ALT. Furthermore, we also analyzed TTV DNA reactive samples by sequencing in order to determine the TTV genotypes circulating in Italy.

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.[3] Primer set 1 is a combination of Okamoto's primer NG059[13] with Simmond's primers A 5432 and A 8761.[12] The efficiency and amplification conditions of this set of primers have already been described.[15] The primer set 2, (NG059, NG063 and NG 061) 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.*,[16] were taken to avoid false-positive results.

PCR product purification and sequence analysis

Amplicons 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.

			Mse I	Nde I	Nla III	EcoRI	PstI	PstI
G1	G1a	**N22**	+	+	+	-	-	-
		G1a	+	+	+	-	-	-
		Cri 200	+	+	+	-	-	-
		Cri 489	+	+	+	-	-	-
		Alt-1	+	+	+	-	-	-
		Cri 310	+	+	+	-	-	-
	G1b	Cri 222	-	+	+	-	-	-
		Cri 240	-	+	+	+	-	-
		Cri 221	-	+	+	+	-	-
		Cri 12	-	+	+	+	-	-
		G1b	-	+	+	+	-	-
		Cri 9	-	+	+	+	-	-
		Cri 152	-	+	+	+	-	-
		Alt-2	-	+	+	+	-	-
		Alt-3	-	+	+	+	-	-
		Cri 307	-	+	+	+	-	-
		Cri 232	-	+	+	+	-	-
		Cri 389	-	+	+	+	-	-
		Cri 249	-	+	+	+	-	-
		Cri 129	-	+	+	+	-	-
G2		**G2a**	-	-	-	-	+	-
		G2b	-	-	-	-	+	-
		Cri 246	-	-	-	-	+	-
		Cri 29	-	-	-	-	+	-
		Cri 455	-	-	-	-	-	+
		Cri 8	-	-	-	-	-	-
		Cri 270	-	-	-	-	+	-
		Cri 385	-	-	-	-	+	-
		Cri 44	-	-	-	-	+	-
		Cri 170	-	-	-	-	+	-
		Cri 202	-	-	-	-	+	+
		Cri 183	-	-	-	-	+	+
		Cri 226	-	-	-	-	+	+

Restriction sites recognized (+) or not (–) by MseI (pos. 1985), NdeI (pos. 2016), NlaIII (pos. 2068), EcoRI (pos. 2030) and PstI (pos. 2035 and 2074). Shaded areas show identical restriction site pattern indicating potential TTV isolates grouping.

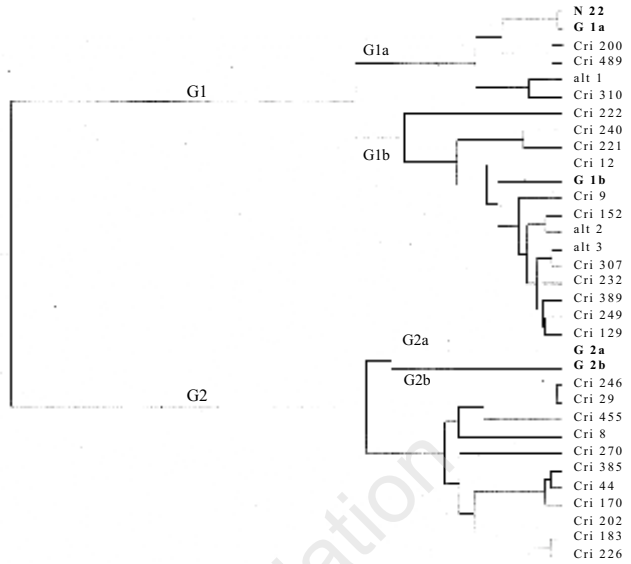

Figure 1. A phylogenetic tree drawn from a phylogenetic analysis (by unweighted pair group method with arithmetic mean – UPGMA) of 28 TTV isolates. The original clone N22 and G1a, G1b, G2a and G2b prototype isolates of TTV were taken from Nishizawa *et al.*[3] and Okamoto *et al.*[4]

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 compared to each other and with five different TTV isolates (N22 clone and the representatives of the putative genotypes 1a, 1b, 2a and 2b deposited in the GenBank) by the Pileup Program. Two major clusters were identified: one with 17 isolates and homology of 75-96%, the other with 11 isolates and homology of 59-63%, which corresponded respectively to groups G1 and G2, according to Okamoto's classification.[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.[4] In contrast, the second major group showed an asymmetric branching pattern in which subgroups G2a and G2b were excluded. Bootstrap analysis (1,000 replicates) confirmed the reliability of these groupings. The corresponding values for G1 and G2 groups and for subtypes 1a and 1b were higher than the arbitrary cut-off value of 75%.

A further genotype distribution was obtained on the basis of restriction fragment length polymorphism (RFLP) analysis described by Tanaka[17] (Table 1). Four out of 17 isolates of group 1, having the highest homology (90-96%), showed *MseI*, *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 present 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 infections by blood and blood derivates, such as the introduction of new serologic assays for the screening 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

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.[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.[13,21] Moreover the presence of TTV in patients with a recent history of blood or blood products transfusion[11] does not allow the relevant role of parenteral transmission of TTV to be excluded.

Very recently Itoh *et al.*[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.[8,9]

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.*[22] and widely reported by most workers.[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.[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.[17] 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. MW 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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The authors' order was chosen according to their individual contribution to the whole work, excepted the last name which indicates the group's leader.

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Disclosures

Conflict of interest: none.

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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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