Use of a TT virus ORF1 recombinant protein to detect anti-TT virus antibodies in human sera

Catherine Ott,1 Laurent Duret,2 Isabelle Chemin,3 Christian Trépo,3 Bernard Mandrand1 and Florence Komurian-Pradel1

1 Unité Mixte de Recherche 2142 CNRS-biomerieux, Ecole Normale Supérieure de Lyon, 46 Allée d’Italie, 69364 Lyon Cedex 07, France
2 Laboratoire de Biométrie Génétique et Biologie des Populations, UMR CNRS 5558, Université Claude Bernard-Lyon 1, 69622 Villeurbanne Cedex, France
3 INSERM Unité 271, 151 Cours Albert Thomas, 69424 Lyon Cedex 03, France

TT virus (TTV), isolated initially from a Japanese patient with hepatitis of unknown aetiology, has since been found to infect both healthy and diseased individuals and numerous prevalence studies have raised questions about its role in unexplained hepatitis. In order to determine the prevalence of ongoing TTV infection as well as resolved infection, a serological study was performed with a recombinant protein generated from the open reading frame 1 (ORF1) sequence isolated from a French patient infected by TTV. The C-terminal end of the ORF1 protein, containing a particularly hydrophilic region, was retained to be used as antigen to detect the presence of anti-TTV antibodies in serum samples by a Western blot analysis. For this purpose, the C-terminal ORF1 region was expressed in fusion with a hexahistidine tail in E. coli and purified by metal-chelate affinity chromatography. The serological screening of 70 human sera, including 30 patients with hepatitis of unknown aetiology, 30 blood donors and 10 healthy children, allowed the immune response of infected hosts to be identified by the detection of TTV-specific antibodies, with a very high prevalence of 98.6% in the human sera tested. In contrast, TTV DNA was detected by PCR in only 76.1% of the tested sera. The use of the ORF1 C-terminal recombinant protein thereby provided a diagnostic tool to follow the immune response of the host against TTV.

Introduction

In the context of the discovery of new agents responsible for hepatitis with unknown aetiology, TT virus (TTV) was identified from the serum of a Japanese patient with post-transfusion non-A–E hepatitis by representational difference analysis (Nishizawa et al., 1997). This novel agent is a non-enveloped, circular single-stranded DNA virus, belonging to the family Circoviridae (Mushahwar et al., 1999; Miyata et al., 1999). Its genome of negative polarity and 3852 nucleotides contains two open reading frames (ORFs) named ORF1 and ORF2, encoding the putative coat and non-structural proteins, respectively, and shows high variability (Mushahwar et al., 1999), ranging up to 52%, which allows its classification in at least 16 different genotypes (Okamoto et al., 1999b).

In initial studies, the prevalence of TTV infection was determined by nested PCR amplification with different sets of primers defined from the N22 clone (Nishizawa et al., 1997). Although the first results revealed a higher frequency of TTV infection in patients with fulminant hepatitis, chronic liver disease of unknown aetiology or in haemophiliac patients than in blood donors (Okamoto et al., 1998a), further studies have shown no significant differences among each group (Fukuda et al., 1999), suggesting that TTV is probably not the main causative agent of acute sporadic hepatitis of unknown origin.

The differences observed in these epidemiological data could be explained by the lack of detection of some TTV isolates due to nucleotide sequence divergence and the use of suboptimal pairs of primers (Desai et al., 1999; Irving et al., 1999; Okamoto et al., 1999b). Moreover, the low virus load in human sera, which does not exceed 104 copies/ml (Nishizawa et al., 1999), requires the use of sensitive and reliable PCR protocols that could differ among laboratories.
Until now, few reports have described the detection of anti-TTV antibodies. An indirect method, consisting of the precipitation of TTV particles with serum followed by the PCR amplification of TTV DNA sequences from the immune complex, has been reported (Tsuda et al., 1999). However, this technique is not adaptable to a large-scale and/or routine survey and it still relies on the PCR technique, with its limitations.

In this study, we describe the expression in E. coli of a recombinant protein encoded by the 3’ region of the ORF1 sequence isolated from a French patient, which we used to detect antibodies directed against TTV by Western blots. A serological survey of a panel of French human sera from different groups (non-A–G hepatitis patients, healthy blood donors, healthy children) and also of animal sera gives a new estimate of the prevalence of TTV infection.

Methods

**Subject.** A French patient, X94 (male, 30 years old), who presented high levels of alanine aminotransferase without detectable hepatitis B virus (HBV) or hepatitis C virus (HCV) viremia and did not show an immune response against these viruses after heart–lung transplant, was diagnosed with hepatitis of unknown aetiology. Serum of this patient was selected for our study because its HBV/HCV negativity was confirmed by PCR and Southern hybridization and because it was found to be positive for the presence of TTV DNA by a nested PCR assay based on the method described by Simmonds et al. (1998). Serum was obtained from the Hôpital Hôtel-Dieu in Lyon.

**Serum samples for serological study.** Thirty serum samples from blood donors were provided by the Etablissement de Transfusion Sanguine in Lyon, 30 serum samples from patients diagnosed with hepatitis of unknown aetiology were obtained from the Hôpital Hôtel-Dieu and 10 serum samples from healthy children were obtained from Unité 271 INSERM in Lyon. Serum samples from several animal species (dog, cat, goat, sheep and rabbit) were provided by the Ecole Vétérinaire in Lyon. All sera used in this study were obtained with the statement of the Ethics Committee of Lyon (identification number 98013/52A).

**Amplification of TTV ORF1 coding sequences.** Total nucleic acids were extracted from 140 µl of serum from patient X94 with the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer’s instructions and subjected to PCR for amplification of the ORF1 coding region of TTV in two overlapping fragments. The ORF1 N-terminal region was obtained by nested PCR with sense oligonucleotides CO6+ and CO14 + D, deduced from well-conserved regions in TTV isolates of different genotypes and subtypes available in the GenBank database, and the antisense oligonucleotides A5427 and A5432, published by Simmonds et al. (1998) (Table 1). The amplification of the ORF1 C-terminal sequence from the same virus subtype was achieved by semi-nested PCR with sense primers CO15 + and CO16 +, designed from the C-terminal end of the sequence previously obtained, and antisense primer CO12— (Table 1).

The first amplification was performed with the Expand High Fidelity PCR system (Roche Diagnostics) on the total extracted nucleic acids as follows: 94 °C for 10 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 68 °C for 2 min; final extension at 68 °C for 7 min. The second round was performed under the same conditions with 10 µl of the first round. PCR products were analysed on 0.8 % agarose gels and detected by exposure to UV light after ethidium bromide staining.

**Cloning and sequence analysis.** Amplified fragments of the N- and C-terminal parts of ORF1 were cloned separately into pCR2.1-TOPO by using the TOPO TA Cloning kit (Invitrogen). These two fragments were sequenced in both directions by using the Prism Ready Reaction Dye Deoxy terminator cycle sequencing kit (Applied Biosystems) with Applied Biosystems 377 and 373A automated DNA sequencers using custom oligonucleotides.

Sequence analysis was carried out with the GeneWorks (IntelliGenetics) and MacVector 4.5 (Kodak) software packages. Database searches were performed by BLAST (http://www.ncbi.nlm.nih.gov/blast) on all major sequence databases (GenBank, EMBL, PIR, SWISS-PROT, Dbest) to generate multiple alignments at both the nucleotide and protein levels.

**Phylogenetic analysis.** The X94-TTV ORF1 protein was compared with BLASTP (Altschul et al., 1997) to all sequences available in the NCBI BLAST databases (non-redundant compilation of DNA and protein sequence databases; update November 26, 1999). We found 26 complete TTV ORF1 sequences. These proteins were aligned with CLUSTAL W (Thompson et al., 1994). This protein alignment was used as a template to compute the DNA alignment of ORF1 coding sequences. A

Table 1. Primers used for amplification of TTV ORF1

<table>
<thead>
<tr>
<th>Primer region</th>
<th>Primer</th>
<th>Name</th>
<th>Nucleotide sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1 N-terminal region</td>
<td>Outer sense</td>
<td>CO6 +</td>
<td>5’ AGCGAAAAACCTGCCCTCCG 3’</td>
<td>519–537</td>
</tr>
<tr>
<td>Inner sense</td>
<td>CO14 + D</td>
<td>5’ GAGCACCATTGCGCTATGGSSTGG 3’</td>
<td>582–603</td>
<td></td>
</tr>
<tr>
<td>Outer antisense</td>
<td>A5427*</td>
<td>5’ TACCAYTTAGCTCTACATTCTWA 3’</td>
<td>2228–2207</td>
<td></td>
</tr>
<tr>
<td>Inner antisense</td>
<td>A5432*</td>
<td>5’ CTACCCTCCTGCGATTATACCA 3’</td>
<td>2192–2172</td>
<td></td>
</tr>
<tr>
<td>ORF1 C-terminal region</td>
<td>Outer sense</td>
<td>CO15 +</td>
<td>5’ TACACATGAATGGCAGACTAC 3’</td>
<td>2063–2083</td>
</tr>
<tr>
<td>Inner sense</td>
<td>CO16 +</td>
<td>5’ TACAGACCCCAATAGTAC 3’</td>
<td>2100–2122</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>CO12—</td>
<td>5’ GTAACAAGTGGTGATAC 3’</td>
<td>2857–2836</td>
<td></td>
</tr>
</tbody>
</table>

* Primers published by Simmonds et al. (1998).
Anti-TTV antibody detection in human sera

Fig. 1. Nucleotide sequences of the two overlapping fragments from the X94-TTV ORF1 with the deduced amino acid sequence underneath. The arginine-rich region is underlined, putative sites for asparagine-linked glycosylation motifs are underlined in bold and conserved Rep protein motifs proposed by Niagro et al. (1998) are boxed. The C-terminal ORF1 region subcloned in the expression vector pET21b is flanked by arrows.

Phylogenetic tree was derived from this multiple alignment (727 codons, gaps excluded) by using the neighbour-joining method (Saitou & Nei, 1987) with synonymous (Ks) and non-synonymous (Ka) distances (Li, 1993).

Prokaryotic expression of the ORF1 C-terminal region and purification. The whole C-terminal region of the ORF1 protein cloned in pCR2.1 TOPO was re-amplified with the synthetic oligonucleotides 5′-TCTCTCGATCTGGACAGACAGACAGACACTTG-3′ and 5′-TCTCTCGATCTGGACAGACAGACAGACACTTG-3′.
CGAGCTCGGGTGATATCTTGATTTTG 3′ in order to introduce BamHI and ScaI restriction sites (underlined) at the 5′ and 3′ ends, respectively. These oligonucleotides overlapped the CO16+ and CO12— cloning primers used previously and spanned nt 2101–2118 and nt 2840–2827 of the TTV prototype TA278, respectively. The BamHI/ScaI-digested PCR product was ligated into the BamHI and ScaI sites of the expression vector pET21b (Novagen) upstream of a hexahistidine tail. Transformation of the ligation mixture was achieved in E. coli strain JM109 and DNA of selected recombinant clones was extracted by using the QiAfilter Plasmid Midi kit (Qiagen). Expression of the recombinant protein was subsequently performed in the host E. coli strain BL21 (DE3). After an overnight culture at 37 °C in LB medium containing 100 µg/ml ampicillin, a 1:50 dilution was grown until the OD₆₅₀ reached 0.6–1. Cultures were then induced for 3 h by adding IPTG (Gibco BRL) to a final concentration of 1 mM. Bacteria were pelleted at 3000 r.p.m. at 4 °C for 20 min, resuspended in 0·1 vol. PBS and lysed by sonication on ice until the supernatant was cleared.

Inclusion bodies were treated with 1% Sarkosyl in STE buffer (10 mM Tris–HCl, pH 8·0, 100 mM NaCl, 1 mM EDTA) to obtain the C-terminal ORF1 protein in the soluble fraction. Alternatively, the recombinant protein was solubilized with 8 M urea in 0·1 M Na₂HPO₄, 0·01 M Tris–HCl, 0·05% Tween 20 buffer, pH 8·0, and purified under denaturing conditions on Ni-NTA magnetic agarose beads (Qiagen). The His-tagged protein was then eluted from the beads by reducing the pH to 4·5 and analysed by Coomassie blue staining after electrophoresis on 10% SDS–PAGE under reducing conditions (Laemmli, 1970).

■ Immunoblot. After migration on preparative 10% SDS–PAGE, the Sarkosyl-solubilized or purified recombinant protein was transferred electrophoretically to PVDF membranes (Millipore) according to the method of Towbin et al. (1979). Blots were blocked for 30 min at room temperature with 5% non-fat milk powder in TBS (20 mM Tris–HCl, pH 7·5, 500 mM NaCl) before an overnight incubation at 4 °C with human or animal serum diluted 1:100 in TBS containing 0·05% (v/v) Tween 20 (TBS-T). For human serum tests, the membranes were washed three times with TBS-T and incubated at room temperature for 1 h with alkaline phosphatase-conjugated goat anti-human IgG + IgM + IgA (Jackson ImmunoResearch) or alkaline phosphatase-conjugated mouse anti-human IgM (bioMérieux) monoclonal antibodies at a 1:2000 dilution in TBS-T. For animal serum assays, incubation of the membranes was achieved under the conditions described above with the use of alkaline phosphatase-conjugated anti-IgG species (dog, cat, goat, sheep or rabbit). The membranes were subsequently washed twice with TBS-T, once with borate buffer (31·5 mM boric acid–NaOH, pH 9·7, 10 mM MgSO₄) and incubated with substrate solution [0·05% (w/v) tetrazotized o-dianisidine and 0·05% (w/v) β-naphthyl acetate phosphatase] in borate buffer. Alternatively, blots were revealed by using either Ni²⁺–NTA–alkaline phosphatase conjugate in a one-step procedure (Qiagen) or a penta-histidine monoclonal antibody in a two-step procedure (Qiagen) according to the manufacturer’s protocols.

■ Determination of TTV viraemia in serum samples. Nucleic acids were extracted from 140 µl of serum by the method described previously and subjected to PCR in a 100 µl reaction with Takara Taq (Takara Shuzo) in the presence of primers T801 (5′-GCTACGTCAG-TAACACCGTG 3′) and T935 (5′-CTCGGGTGATATCTTGATTTTG 3′) as published by Takahashi et al. (1998b). A human serum known to be positive for TTV DNA was included in each test as a positive control. The amplified products of 199 bp were analysed by electrophoresis on 1·5% agarose gels, stained with ethidium bromide.

Results

Nucleotide sequence of X94-TTV ORF1

The ORF1 nucleotide sequence from a French patient (X94) infected with TTV was determined. The ORF1 region was obtained in two parts, as initial attempts to amplify it in one fragment were unsuccessful (data not shown). A first clone (7-94X), spanning nt 582–2192 of the prototype sequence TA278 (Okamoto et al., 1998a) and encompassing the amino terminus of TTV ORF1, was obtained. Specific primers designed according to the C terminus of this sequence were used to generate a second fragment spanning nt 2100–2857 of the prototype sequence TA278, which presented an overlapping region (92 nt) with clone 7-94X and encompassed the C-terminal region of TTV ORF1. This fragment was cloned and named 11-94X. Sequence analysis of clones 7-94X and 11-94X revealed 100% identity across the region of overlap. By combining the overlapping parts, the nearly full-length X94-TTV ORF1 sequence was reconstructed and found to contain an ORF encoding a protein of 756 amino acids (Fig. 1). Alignment analysis of the X94 and TA278 nucleotide sequences revealed high similarity, with 95% identity (data not shown).

Characterization of the ORF1 protein

The predicted amino acid sequence encoded by the X94-TTV ORF1 sequence was analysed. As shown in Fig. 1, it contains a highly arginine-rich domain at its N terminus, as has been previously identified for circovirus capsid proteins (Niagro et al., 1998; Takahashi et al., 1998a; Mushahwar et al., 1999), and four asparagine-linked glycosylation sites clustered in its central region. Additionally, three of the four conserved Rep protein motifs (FTL and YXXK) described previously by Niagro et al. (1998) in circoviruses were found in the X94-TTV
Anti-TTV antibody detection in human sera

Fig. 3. Unrooted phylogenetic tree generated by comparison of the X94-TTV ORF1 protein with 26 TTV ORF1 amino acid sequences. Branch lengths are proportional to amino acid divergence and bootstrap analysis was applied with 1000 bootstrap values. TTV genotypes 1, 2 and 3 are indicated, with subtypes 1a and 1b as described by Erker et al. (1999), but with a divergence from their phylogenetic analysis, as isolates JA1, JA2B and US35 grouped together whereas JA4 and JA10 formed another cluster, segregating from the first one. The human TTV isolates are identified by GenBank database accession numbers or by isolate definition with the corresponding accession numbers: GH1 (AF122913); JA9 (AF122915); BDH1 (AF116842); TA278 (AB008394); JA20 (AF122914); TX011 (AB011493); JA1 (AF122916); JA4 (AF122917); US35 (AF122920); JA2B (AF122918); JA10 (AF122919); TTVCHN1 (AF079173); and TTVCHN2 (AF129887).

ORF1 protein, suggesting the possibility of virus replication by a rolling circle mechanism (Mushahwar et al., 1999).

Further characterization of the ORF1 protein by hydrophilicity analysis, using the method of Hopp & Woods (1981), allowed two major hydrophilic regions to be located (Fig. 2). The first hydrophilic region was found at the N-terminal part of the amino acid sequence and contained the previously defined basic, arginine-rich domain, while the second one was located close to the C-terminus of the ORF1 sequence. As some hydrophilic regions are predicted to have potential antigenic properties (Hopp & Woods, 1981), these two domains are of a major interest for serological analysis.

Phylogenetic analysis

A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) after alignment of the X94-TTV ORF1 protein with 26 complete TTV ORF1 sequences available in protein sequence databases (Fig. 3). Three major branches were observed, with significant bootstrap support of 100%, corresponding to genotypes 1, 2 and 3 as defined previously (Mushahwar et al., 1999; Erker et al., 1999). Similar results were observed after construction of an unrooted phylogenetic tree based on the nucleotide sequences (data not shown). The X94-TTV sequence was determined to be most closely related to the genotype 1 sequences and deduced to belong to subtype 1a (Fig. 3).

Expression and purification of the recombinant C-terminal ORF1 protein

Because hydrophilic domains have been described as important for the antigenic properties of various proteins (Hopp & Woods, 1981), we chose to express the C-terminal region of the X94-TTV ORF1 protein from amino acids 504 to 752 containing the distal hydrophilic domain mentioned above to be used as antigen for further detection of TTV-specific antibodies in human sera. An amplicon was therefore generated from the clone 11-94X (Fig. 1) and subcloned in the pET vector (Novagen), allowing the expression of the recombinant protein in fusion with a hexahistidine tail. The efficient translation of the C-terminal ORF1 protein of theoretical molecular mass 32-12 kDa was verified by SDS–PAGE analysis of the crude extract (Fig. 4a). After bacterial lysis by sonication, overexpressed recombinant protein was retrieved in the insoluble fraction of the cell lysate, suggesting protein aggregation in insoluble inclusion bodies or co-precipitation with insoluble bacterial cell debris. Protein aggregates were solubilized with Sarkosyl detergent or a strong denaturing agent such as 8 M urea. This last condition allowed us to purify protein on Ni-NTA magnetic agarose beads. As shown in Fig. 4(b), the histidine residues of the recombinant protein bound efficiently by Ni²⁺-chelating affinity under denaturing conditions, as only a small amount of protein was detected in the flow-through fraction after contact with the beads. The degree of purity of the eluted fraction was found to be > 95% as assessed by Coomassie brilliant blue-stained SDS–PAGE (Fig. 4b). A Western blot analysis of the purified protein with an anti-
histidine antibody confirmed the expected molecular mass of the C-terminal ORF1 protein, with the detection of a positive signal around 32 kDa.

Detection of anti-TTV antibodies in human sera

The immunoreactivity of the C-terminal ORF1 protein against human sera was assessed by Western blot analysis. Immunoblots of the Sarkosyl-solubilized C-terminal ORF1 protein incubated with sera from adults and children showed the presence of antibodies to TTV (anti-TTV) directed against the recombinant protein of 32 kDa (Fig. 5a). Further analysis with the purified recombinant protein led us to identical results without the previously observed cross-reactivity of E. coli proteins with sera, which confirmed the immunogenic character of the native protein (Fig. 5b). The specificity of the anti-TTV response was controlled by the absence of reactivity of positive sera towards a mock E. coli extract, purified under the same conditions as the recombinant protein (Fig. 5b, lane 11). Moreover, successive dilutions of three positive sera gave a positive signal until the 1:1000 dilution in each case (data not shown). Additionally, the reactivity of two anti-TTV sera was reduced significantly when these serum samples were previously pre-incubated with captured C-terminal ORF1 protein on Ni-NTA agarose beads (data not shown).

Based on these results, the prevalence of the human immune response against TTV was investigated by testing 70
sera including samples from 30 voluntary blood donors, 30 patients with non-A–G hepatitis and 10 healthy children aged from 2 to 5 years. All but one human serum we tested showed immunoreactivity against the recombinant protein revealed by anti-human IgG + IgM + IgA and IgM responses. Undiluted sera were subjected to PCR amplification with primers described previously (Takahashi et al., 1998b) for the detection of TTV DNA. Values are no. positive/no. tested; values in parentheses are percentages.

Table 2. Anti-TTV response against the C-terminal ORF1 recombinant protein and detection of TTV DNA in human sera

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-TTV antibodies</th>
<th>Metal-chelate purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG + IgM + IgA</td>
<td>IgG + IgM + IgA</td>
</tr>
<tr>
<td>Blood donors</td>
<td>30/30 (100)</td>
<td>18/18 (100)</td>
</tr>
<tr>
<td>Hepatitis of unknown aetiology</td>
<td>29/30 (96.6%)</td>
<td>10/11 (90.9)</td>
</tr>
<tr>
<td>Children</td>
<td>10/10 (100)</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td></td>
<td>22/30 (73.3%)</td>
<td>25/30 (83.3%)</td>
</tr>
<tr>
<td></td>
<td>4/7 (57.1%)</td>
<td></td>
</tr>
</tbody>
</table>

Animal sera were also tested for the presence of TTV or TTV-like sequences with the primers used to amplify TTV DNA from human sera. No amplification could be obtained for the five samples tested (data not shown).

Discussion

Several reports have evaluated the prevalence of infection with TTV in blood donors and hepatitis patients by detection of TTV DNA by PCR. However, little is known about immune surveillance of the hosts and antibodies in the blood circulation. While anti-TTV antibodies have been detected previously in human serum by means of immune precipitation of TTV particles followed by PCR analysis (Tsuda et al., 1999), we report here the first serological study based on the use of a recombinant TTV protein from the C-terminal end of ORF1, allowing the detection of anti-TTV antibodies in serum samples by Western blot analysis. By means of a survey of 70 serum samples, we confirm the existence of an immune response against TTV in blood donors and patients with hepatitis of unknown aetiology, but also in children and animals.

The recombinant protein used in the serological screening assay was obtained from the ORF1 sequence isolated from a French patient infected with TTV. The N- and C-terminal ORF1 regions were obtained with an overlap of 92 nucleotides showing perfect similarity in a genomic region of the virus that has been described to contain a large number of nucleotide mismatches (Takahashi et al., 1998a). Furthermore, these sequences corresponding to the clones 7-94X and 11-94X presented identities of 94 and 99%, respectively, after alignment with the prototype sequence, TA278. These observations, together with the phylogenetic analysis of the X94 TTV sequence, suggested that the two partial ORF1 sequences 7-94X and 11-94X were amplified from the same virus subtype, namely subtype 1a, previously defined by
Okamoto et al. (1998a) and observed among European isolates (Biagini et al., 1999; Maggi et al., 1999; Höhne et al., 1998).

The immuno-reactive value of the TTV coat protein was verified by the purification of the C-terminal ORF1 recombinant protein, which confirmed the specificity of the strong immunoreactivity observed with the protein solubilized in Sarkosyl. Furthermore, the absence of cross-reactivity between the purified mock E. coli extract and anti-TTV-positive human sera attested to the specificity of the signal. By testing 70 sera of French origin, we have observed a humoral response to the C-terminal ORF1 recombinant protein with 98.6% serum reactivity, indicating the very high prevalence of TTV infection in hepatitis patients with unknown aetiology as well as in blood donors. This rate appears to be much higher than that published by Tsuda et al. (1999), as their experiments to form immune complexes led to the detection of anti-TTV antibodies in only 27% of the tested blood donors. Whereas frequent TTV infection has already been reported in different population groups including blood donors by the detection of circulating viral DNA by PCR (Naoumov et al., 1998; Okamoto et al., 1998a; Prescott & Simmonds, 1998; Tanaka et al., 1998), a recent report with improved primers indicated an increased prevalence, with the detection of viral DNA in 92% of healthy Japanese subjects (Takahashi et al., 1998b). By using these primers, we determined a prevalence of 76.1% for the tested sera, which is lower than the positive serological response (98.6%) obtained from the same samples. The lack of detection of TTV DNA in 16 cases of 67 tested, despite the presence of an anti-TTV response, suggests the clearance of TTV from these sera or a virus load too low to be detectable. Another hypothesis is the presence of other subtypes of TTV, with sequences too divergent to be amplified by the primers used in this study. The one serum that was negative for anti-TTV IgG and IgM but positive for TTV DNA could represent a case of recent TTV infection without detectable antibody response. The possibility of the lack of an antibody response in this particular subject is not excluded.

It is now well established that, despite being a DNA virus, TTV is characterized by its high degree of genetic diversity (Okamoto et al., 1998a, 1999a, b; Hijikata et al., 1999). Most amino acid substitutions among distinct TTV strains have been located in the central portion of ORF1 within the glycosylated domain (Takahashi et al., 1998a), and the recent identification of three hypervariable regions (HVRs) of the capsid protein substantiates the high intra- and inter-patient variability of circulating TTV sequences. While the adaptability of the virus by changing its HVRs could reflect a strategy to escape immune surveillance of the hosts (Nishizawa et al., 1999), one can predict the existence of multiple serotypes depending on the genotype of the infectious strain(s). In such a context, and because of the high percentage of sera that reacted with the recombinant C-terminal ORF1 protein in our study, we can hypothesize that the expressed region is highly antigenic in vivo and that it might contain a major immunodominant epitope of TTV. Our results suggest that serological studies performed with the recombinant TTV protein may improve diagnostic efficacy, leading to the establishment of the true worldwide prevalence of TTV. In fact, it appears essential to take into consideration some real PCR limitations, as the choice of PCR methods may greatly affect the results of prevalence studies and the primer locations influence the sensitivity of virus detection, as described by recent reports (Okamoto et al., 1999b; Desai et al., 1999; Abe et al., 1999; Leary et al., 1999; Takahashi et al., 1998b). Another argument of serological interest is that antibody detection assays reflect recent or past infections of the host, whereas PCR screening only allows the detection of active viraemia. In our experiments, while the presence of TTV DNA was detected in 76.1% of cases, the absence of detectable anti-TTV IgM in all sera tested suggests that the initial infection was not recent. The observed TTV viraemia may reflect reinfection with another TTV strain or may be related to the level of TTV replication. Interestingly, by screening 10 sera from healthy children, we observed a negative IgM response, with the detection of anti-TTV IgG + IgA + IgM in all sera tested, suggesting an early acquisition of infection in childhood, with viraemia in four samples.

The high prevalence found in both healthy adults and children included in our studies suggests that the virus might be transmitted non-parenterally. Although TTV was first inferred to be blood-transmittable and was referred to as ‘transfusion-transmitted virus’ (Nishizawa et al., 1997; Charlton et al., 1998; Naoumov et al., 1998; Okamoto et al., 1998a; Simmonds et al., 1998), other potential modes of transmission have been identified, such as the faecal–oral route (Okamoto et al., 1998b) and intra- or extra-familial sources of exposure (Sugiyama et al., 1999; Saback et al., 1999; Hsieh et al., 1999). Another interesting observation is that we were able to detect anti-TTV antibodies in the sera of various animal species, whereas no TTV DNA could be detected in these sera, suggesting the lack of active viraemia or the possible presence of highly divergent TTV-like sequences. However, the possibility of cross-reactivity between the C-terminal ORF1 recombinant protein and antibodies raised against the ORF1 protein of animal circoviruses cannot be excluded.

The present study demonstrates the large spread of TTV infection. The reactivity of human sera against the recombinant protein implies the immunogenic nature of TTV ORF1 in humans. To date, the transmission routes of TTV and its causal role in human liver disease remain speculative and might be correlated to certain subsets of TTV genotypes/variants. Further clinical studies are therefore required to solve these multiple issues.

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