High prevalence of TT virus (TTV) in naive chimpanzees and in hepatitis C virus-infected humans: frequent mixed infections and identification of new TTV genotypes in chimpanzees

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A recently discovered DNA virus, TT virus (TTV), is prevalent in humans. In the present study, the genetic heterogeneity of TTV was evaluated in hepatitis C virus (HCV)-infected patients and in chimpanzees. TTV DNA was detected by PCR in serum samples from all ten HCV-infected patients studied; at least five major TTV genotypes, all previously identified in humans, were recovered. Eight patients were infected with multiple variants of TTV. TTV DNA was detected by PCR in serum samples from 11 (65%) of 17 naive chimpanzees bred in captivity; a persistent infection was present in three of six animals. At least five chimpanzees were infected with more than one TTV variant. Detection of TTV DNA in chimpanzee faecal samples suggests the possibility of faecal–oral transmission. Phylogenetic analysis of ORF1 sequences amplified from chimpanzees identified three major genotypes which had not previously been recognized in humans.

Recently, TT virus (TTV) was isolated from serum of a patient with post-transfusion non-A–G hepatitis (Nishizawa et al., 1997). This DNA virus is ubiquitous among humans with a prevalence of up to 83% in rural populations of developing countries (Prescott & Simmonds, 1998). Additionally, 34% of US blood donors (Leary et al., 1999) and 92% of healthy Japanese adults (Takahashi et al., 1998a) were infected with TTV. It is not known, however, whether TTV causes disease in humans (Charlton et al., 1998; Naoumov et al., 1998; Okamoto et al., 1999b; Simmonds et al., 1998).

TTV transmission can occur by the parenteral route (Nishizawa et al., 1997; Simmonds et al., 1998) and the relatively high prevalence of TTV among drug addicts, haemodialysis patients and haemophilia patients suggests that this is an important transmission route (Desai et al., 1999; Forns et al., 1999; Leary et al., 1999). Shedding of TTV in bile and faeces has been demonstrated, suggesting that faecal–oral transmission may also occur (Okamoto et al., 1998a; Ukita et al., 1999).

TTV has a negative-sense, single-stranded circular DNA genome of approximately 3-8 kb (Hijikata et al., 1999; Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 1998b, 1999a). Because of the lack of significant sequence similarity between TTV and circoviruses, it has been proposed that TTV belongs to a new virus family tentatively designated Circoviridae (Mushahwar et al., 1999). Three putative ORFs were recognized (Erker et al., 1999). The sequence between the end of ORF3 and the beginning of ORF2 is defined as the UTR.

TTV is characterized by extreme genetic heterogeneity: currently >15 major genetic groups have been identified in humans (Okamoto et al., 1998b, 1999a, b; Prescott et al., 1999; Tanaka et al., 1998). The primary aim of the present study was to determine whether naive chimpanzees bred in captivity were infected with TTV. The genetic heterogeneity of TTV in chimpanzees as well as in hepatitis C virus (HCV)-infected humans was studied by analysis of two independent genome regions.

For TTV DNA detection, 100 µl serum was diluted 1:5 with 50 mM Tris–HCl (pH 8), 1 mM EDTA, 100 mM NaCl and 0.5% SDS and digested with 1 µg/ml proteinase K, at 65 °C for 1 h. DNA was extracted by 500 µl phenol : chloroform : isoamyl alcohol (25:24:1) (Gibco/BRL) and precipitated overnight in 1/10 vol. 3 M sodium acetate, 2 vol. ice-cold ethanol and 1 µl glycogen (Boehringer Mannheim). After centrifugation at 4 °C for 20 min, the pellet was washed with 1 ml 70% ethanol. DNA amplification was
performed by PCR as described previously, using two different sets of primers located within ORF1 (Forns et al., 1999) and the UTR (Takahashi et al., 1998a), respectively. The positive control was 100 µl of a 10⁻² dilution of a serum sample from a TTV-positive patient with a titre of 10⁶ genome equivalents (GE)/ml, as determined by hemi-nested PCR (Okamoto et al., 1998b). This sample had a titre of 10⁻¹–10⁻² GE/ml in both ORF1-PCR and UTR-PCR. At least three negative controls were included in each experiment.

A 10% (w/v) faecal suspension in PBS (pH 7.4) (10 ml) was centrifuged in an IEC Centra-8R refrigerated centrifuge at 3000 r.p.m. for 30 min. Supernatant was recovered and centrifuged in an Eppendorf Refrigerated Microcentrifuge at 7500 r.p.m. for 10 min. DNA was extracted from 200 µl aliquots of supernatant as described above. TTV DNA was amplified by UTR-PCR.

PCR products corresponding to positions 26–184 (159 nt) and 1939–2163 (225 nt) of isolate TA278 (GenBank accession number AB008394) (Okamoto et al., 1998b), excluding primer sequences, were purified by the Wizard PCR purification kit (Promega). When necessary, PCR products were cloned into vector pCR2.1 with the original TA cloning kit (Invitrogen). Both DNA strands were sequenced. Relevant TTV sequences published up to August 1999 were analysed in the present study. Sequences were analysed with GeneWorks and MacVector (Oxford Molecular Group), as well as CLUSTALW (version 1.6) and PAUP (version 4.0), both from the GCG Sequence Analysis Package (version 10).

Serum samples were collected from nine Italian patients with chronic hepatitis C and a plasma sample from patient H obtained during the acute phase of post-transfusion hepatitis C (Feinstone et al., 1981) were studied. Eight patients (80%) were positive for TTV DNA by ORF1-PCR. The ORF1-PCR product, which was sequenced directly, was 225 nt in each case (Fig. 1). Phylogenetic relationships of these ORF1 sequences to those of published TTV strains representing recognized genotypes were determined (Fig. 2a). Genotypes 1, 2, 3 and 6 were recovered from Italian patients, whereas genotype 4 was found in the US patient.

All ten HCV patients were positive for TTV DNA by UTR-PCR. Direct amplicon sequencing suggested that eight patients had mixed TTV infections. Therefore, we cloned the PCR products and sequenced 6–19 clones from each sample. Although the majority of these clones contained a TTV sequence of 159 nt (range 158–161 nt), many clones had nucleotide insertions or deletions compared with the prototype strain of TTV. Sequence analysis confirmed that each of these eight patients was infected with multiple variants (between 3 and 7) of TTV (Fig. 2b; Table 1). Published reference variants within the same genotype (genotype 1 or 2) have identities of 83–96% within this region. Therefore, different variants were defined as having identities of < 97% (Table 1). Phylogenetic analysis of the UTR genomic sequences from our patients and from published reference strains of genotypes 1, 2, 11 or 13 is shown in Fig. 2(b). Although ORF1 analysis indicated that seven major TTV genotypes (1, 2, 3, 4, 6, 11 and 13) were present in this same group of patients, this division was not apparent in phylogenetic analysis of UTR sequences.

Serum samples were collected from 17 chimpanzees (Pan troglodytes) (< 3 years of age) shortly after their arrival in our animal facility. These chimpanzees were all bred in captivity in the USA and had never been inoculated with human faecal samples, blood samples or blood-derived products. TTV DNA was detected by UTR-PCR in 11 (65%) of 17 chimpanzees. Direct amplicon sequencing confirmed their identity as TTV sequences. Infected animals all had normal liver enzyme values. Three of six positive animals subsequently retested were TTV DNA-positive 22–54 months later; the sequences recovered from the follow-up sample were all closely related (≥ 98% identity) to sequences present in their respective previous samples, suggesting persistent infection. Five chimpanzees were apparently infected with a mixture of TTV. We cloned PCR products from three of these and sequenced 10–12 clones for each. These three chimpanzees were infected with two or
TTV infection in non-human primates

Fig. 2. (a) Genetic analysis of TTV ORF1 sequences. The sequences of 222 nt fragments of TTV (corresponding to nt 1939–2160 of strain TA278; Okamoto et al., 1998) from previously published reference strains (Forns et al., 1999; Erker et al., 1999; Goto et al., 1999; Hijikata et al., 1999; Hohee et al., 1998; Mushahwar et al., 1999; Okamoto et al., 1998a, b, 1999a, b; Takahashi et al., 1999; Tanaka et al., 1998; Takayama et al., 1999; Tanaka et al., 1999; Viazov et al., 1998), from HCV-infected patients (isolates VT, AA, DM, TD, BG, IE, H77, DP; marked with **) and from chimpanzees [isolates CH1304, CH1545, J4-91 (Inoculum CH1410), CH1410 (week 12 post-inoculation) and CH1410 (weeks 281 and 301 post-inoculation); marked with *] were analysed. For previously published reference strains, the GenBank accession numbers are indicated. Phylogenetic trees were constructed with the aid of PAUPSEARCH and PAUPDISPLAY from the PAUP software package (version 4.0). A 50% majority-rule consensus tree (midpoint rooting) was obtained by performing heuristic search (optimality criterion, maximum parsimony; all characters, equal weight; 100 replicates) and displayed by aid of TREEVIEW (version 1.5) (Page, 1996). A 70% majority-rule consensus tree (not shown) indicated the same classification of TTV isolates. Genotype designations and bootstrap values are indicated on the branches. The genotype designations used in the present study reflect the priority date of previous publications (Okamoto et al., 1998b, 1999a, b; Simmonds et al., 1998; Tanaka et al., 1998) since, in some cases, different genotype designations had been assigned in the literature to isolates of the same genotype. (b) Genetic analysis of TTV UTR sequences. The sequences of fragments of 159 nt (corresponding to nt 26–184 of strain TA278; Okamoto et al., 1998b) from 10 HCV-infected patients (multiple clones were sequenced for eight of these patients) and from nine chimpanzees (multiple clones were sequenced for three of these chimpanzees) were analysed. Clones from patient VT, patient H77 and chimpanzee 1313, respectively, with identical sequences are boxed in order to condense the figure. Phylogenetic trees were constructed as in (a).
Table 1. Mixed TTV infection in HCV-infected patients

Based on analysis of molecularly cloned PCR-amplified UTR sequences (corresponding to nt 26–184 of isolate TA278; Okamoto et al., 1998). See Fig. 2(b). Single variant, > 97% nucleotide identity; different variants, 68–96% nucleotide identity.

<table>
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<th>Patient</th>
<th>No. of clones (identity)</th>
<th>No. of variants (identity)</th>
<th>1st variant</th>
<th>2nd variant</th>
<th>3rd variant</th>
<th>4th variant</th>
<th>5th variant</th>
<th>6th variant</th>
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<td>5 (99–100%)</td>
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* Variants are given in descending order of prevalence. Identities are given in parentheses for variants with more than one clone.

more TTV variants (Fig. 2b). The length of the different UTR sequences was 156–166 nt. Phylogenetic analysis showed that all but one of the chimpanzee TTV sequences clustered separately from the human TTV sequences analysed (Fig. 2b). There was, however, bootstrap support for a closer evolutionary relationship of these chimpanzee sequences with clonal UTR sequences recovered from three of our patients [H77 (ORF1, genotype 4); DP (ORF1, genotype 6); VT (ORF1, genotype 1)] than with other sequences. These human sequences would have to be extended to other genomic regions in order to determine whether they represent any of the recognized TTV genotypes.

To genotype the chimpanzee strains, we tried to amplify the ORF1 region used to genotype the human strains. However, ORF1 DNA was amplified from only two (CH1304 and CH1545) of eight naive chimpanzees that tested TTV DNA-positive by UTR-PCR (three positive animals were not tested by ORF1-PCR). The ORF1 sequence recovered from chimpanzee 1545 in nested PCR was 222 nt long with a single amino acid deletion compared with the prototype (Fig. 1). It shared approximately 40–55% nucleotide and 25–50% amino acid identity with the corresponding sequences from humans. The corresponding ORF1 sequence from chimpanzee CH1304, which was positive after single-round PCR, but negative in nested PCR, was 231 nt with two amino acid insertions compared with the TTV prototype sequence (Fig. 1). It shared approximately 40–55% nucleotide and 25–50% amino acid identity with the corresponding sequences from humans. Phylogenetic analysis of the ORF1 nucleotide consensus sequences suggested that TTV recovered from these two chimpanzees represented two new genotypes (Fig. 2a).

Sera from a chimpanzee (CH1410) that had been inoculated with plasma containing both TTV and HCV were retrospectively analysed to determine if the chimpanzee became infected with TTV. Chimpanzee CH1410 was inoculated intravenously with 1 ml plasma HC-J4/91 from a chimpanzee chronically infected with HCV (Okamoto et al., 1992) after inoculation with plasma pooled from several chimpanzees infected with a Japanese blood donor-derived agent. ORF1-PCR products amplified from the inoculum and from chimpanzee CH1410 at week 12 post-inoculation had 100% sequence similarity, indicating that TTV in the inoculum had infected the chimpanzee (Fig. 1; Fig. 2a). This sequence belonged to the same TTV genotype as the sequence recovered from naive chimpanzee CH1545 (see above). The ORF1 sequence recovered during late follow-up in this experimentally infected chimpanzee belonged to a third new TTV genotype (Fig. 1; Fig. 2a); it had approximately 45–60% nucleotide and 30–50% amino acid identity to the corresponding human sequences.

Since TTV has been reported in human faeces, we examined faeces from infected chimpanzees for TTV. TTV DNA was detected by UTR-PCR in faeces from two of five animals tested. Both animals were repeatedly positive for TTV DNA in faecal samples taken during the chronic phase of infection. In each case, TTV sequences recovered from faeces had a high similarity (> 98%) with sequences recovered from serum of the same animal.

We also tested non-human primates other than chimpanzees for TTV. Serum collected from 40 tamarins (Saguinus mystax), four owl monkeys (Aotus trivirgatus) and six thesus monkeys (Macaca mulatta) were all TTV DNA-negative by UTR-PCR.

In the present study, we found a high prevalence of TTV in HCV-infected humans and in naive chimpanzees bred in captivity. All TTV sequences recovered from patients be-
longed to genotypes already recognized among humans. In contrast, TTV sequences recovered from chimpanzees could not be classified within the genotypes currently recognized.

Clonal analysis demonstrated that eight of the ten TTV-infected patients were co-infected with multiple TTV variants; two patients were each infected with at least seven variants. The high degree of genetic heterogeneity suggested that most patients were co-infected with different TTV genotypes, although it must be acknowledged that the region analysed could not accurately predict TTV genotype distribution. In addition, we demonstrated that naive chimpanzees also had mixed TTV infections; this was previously reported for humans (Ball et al., 1999; Forns et al., 1999; Mushahwar et al., 1999; Takayama et al., 1999). Our data suggest mixed TTV infection is also common in chimpanzees. Therefore, infection with one TTV variant does not seem to generate protection against infection with other variants.

Based on our analysis of published ORF1 TTV sequences, at least 18 genotypes of TTV circulate among humans (Fig. 2a). We found genotypes 1, 2, 3 and 6 among Italian patients and genotype 4 in the US patient. Genotypes 1–4 have worldwide distribution, whereas genotype 6 has been identified only in Asia (Forns et al., 1999; Mushahwar et al., 1999; Tanaka et al., 1998). Unfortunately, different designations have been assigned by different groups to isolates of the same genotype. The genotype designations used in the present study are those used in the first published report of that genotype (Okamoto et al., 1998b, 1999a, 1999b; Simmonds et al., 1998; Tanaka et al., 1998).

We found that 65% of naive chimpanzees bred in captivity in the USA were infected with TTV. While we cannot rule out that these chimpanzees were infected by contact with humans, the more likely explanation for this high prevalence is that TTV was spread among the animals, most likely by faecal–oral transmission. TTV DNA was detected in faeces from two infected chimpanzees. In addition, the TTV genomic sequences recovered from the chimpanzees were significantly different from those recovered from humans; the ORF1 sequences recovered from a subset of the infected chimpanzees belonged to genotypes not previously recognized among humans and all but one of the UTR sequences recovered from these chimpanzees formed a separate cluster with 79% bootstrap support in the phylogenetic analysis. It is noteworthy, however, that we identified a subset of UTR sequences from three humans that were more closely related to these chimpanzee sequences (89% bootstrap support) than to the other TTV sequences analysed.

Recently, a similarly high TTV prevalence was reported for chimpanzees kept in captivity in the USA (Leary et al., 1999) and the Netherlands (Verschoor et al., 1999). Leary et al. (1999) reported that TTV sequences (corresponding to a different UTR region from the one we analysed) recovered from chimpanzees were similar to human sequences; however, this region might be too conserved to reveal species-specific differences. Verschoor et al. (1999) reported ORF1 sequences from chimpanzees; most of these formed a separate cluster from human sequences although there was some support for classifying these sequences with human genotype 5 isolates (bootstrap support 66%; in general, a bootstrap support of >70% is considered significant). These sequences were published following completion of our data analysis and therefore are not included in Fig. 2(a). However, subsequent phylogenetic analysis of selected isolates (data not shown) indicated that one chimpanzee isolate (Ch_Sy2) identified by Verschoor et al. (1999) belongs to the same genotype as that of naive chimpanzee 1545, as well as that of a Japanese chimpanzee (J4-91); identities among these three isolates were 85–91% and 93–96% at the nucleotide and deduced amino acid levels, respectively. In our analysis, we found no support for classifying our two isolates with isolates of genotype 5 or other human TTV isolates. We therefore believe that this genotype, infecting chimpanzees in three different animal facilities, represents a chimpanzee TTV. The two additional TTV variants identified in chimpanzees (CH1304 and CH1410) were not found by Verschoor et al. (1999). ORF1 sequences from CH1304 or CH1410 determined in the present study did not cluster with any human isolates but the CH1304 sequence clustered with a sequence determined for isolate Ch_Sy1 by Verschoor et al. (1999); however, the ORF1 identities between these two isolates were only 71% and 74% at the nucleotide and deduced amino acid levels, respectively.

TTV was not detected in other non-human primate species tested (tamarins, owl monkeys and rhesus monkeys). Leary et al. (1999) recently reported TTV in tamarins and owl monkeys, but not in common marmosets or rhesus monkeys, whereas Verschoor et al. (1999) did not detect TTV in tamarins, common marmosets or rhesus monkeys. Several studies have recently reported great variation in the detection of TTV DNA depending on the PCR primers used (Leary et al., 1999; Okamoto et al., 1999b; Takahashi et al., 1998a). Further studies using the most conserved primers in PCR assays and testing a larger number of naive animals are warranted to determine the prevalence of TTV in non-human primates other than chimpanzees. Interestingly, TTV was recently detected with a relatively high prevalence in chickens, pigs, cows and sheep, so TTV infection is apparently widespread (Leary et al., 1999).

In conclusion, our data indicate that TTV was present in chimpanzees, as in humans, with a high prevalence. Chimpanzees were naturally infected with TTV variants that have not been identified among humans.

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