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Distribution of TT virus (TTV), TTV-like minivirus, and related viruses in humans and nonhuman primates

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Abstract

TT virus (TTV) and TTV-like minivirus (TLMV) are small DNA viruses with single-stranded, closed circular, antisense genomes infecting man. Despite their extreme sequence heterogeneity (>50%), a highly conserved region in the untranslated region (UTR) allows both viruses to be amplified by polymerase chain reaction (PCR). TTV/TLMV infection was detected in 88 of 100 human plasma samples; amplified sequences were differentiated into TTV and TLMV by analysis of melting profiles, showing that both viruses were similarly prevalent. PCR with UTR primers also detected frequent infection with TTV/TLMV-related viruses in a wide range of apes (chimpanzees, gorillas, orangutans, gibbons) and African monkey species (mangabeys, drills, mandrills). These findings support the hypothesis for the co-evolution of TTV-like viruses with their hosts over the period of primate speciation, potentially analogous to the evolution of primate herpesviruses.

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Keywords: TT virus; TTV-like minivirus; Circovirus; Untranslated region; Primate; Ape; Old World monkey

Introduction

TT virus (TTV) is a newly discovered nonenveloped virus with a small, covalently closed circular genome of single-stranded DNA. TTV was originally classified as a member of the family *Circoviridae*; this included a number of small DNA viruses with circular, single-stranded genomes that infected several domesticated animal and bird species [chicken anaemia virus (CAV), porcine circovirus (PCV), psittacine beak and feather disease virus (BFDV)]. Alternative proposals for its classification include the designation of TTV as the type member of new virus family, the *Circinoviridae* (Mushahwar et al., 1999). More recently, TTV along with TLMV and CAV have been proposed as members of a new family, the *Paracircoviridae* (Takahashi et al., 2000), as it is now clear that CAV shows greater similarity to TTV and TLMV than it does to other members

of the original circovirus family. For example, PCV and BFDV have ambisense genomes, a different virion size and appearance from CAV and TTV, and major differences in genome organisation.

TTV shows considerable genetic diversity, with at least 28 genotypes with nucleotide sequences differing from each other by least 30% (Heller et al., 2001; Okamoto et al., 2001a; Hijikata et al., 1999). These genotypes can be further classified into at least four genetic groups that differ from each other by at least 50% in nucleotide sequence (Hijikata et al., 1999). However, despite this extensive sequence diversity, all variants of TTV share a common genome organisation, with three predicted encoded proteins of similar length and likely function. The great genetic diversity of TTV complicates the interpretation of previous investigations of the prevalence of infection in different human populations, risk groups, and patient cohorts. A polymerase chain reaction (PCR) based method originally used to detect TTV DNA sequences in plasma samples used primers from a region in ORF1 that shows considerable sequence diver-

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sity between TTV genotypes (Okamoto et al., 1998; Simmonds et al., 1998, 1999; Itoh et al., 1999). Detected frequencies of active TTV infection based on this method were therefore invariably underestimates, as TTV variants showing substantial sequence divergence from the prototype strain (TA278) (Nishizawa et al., 1997; Okamoto et al., 1998) were not efficiently amplified (measurements of levels of viraemia in plasma samples were similarly flawed). Combining the results from several subsequent studies that used primers from the more conserved untranslated region (UTR), it appears that TTV infection is universal (or nearly universal) in humans (Okamoto et al., 2001a; Simmonds et al., 1999; Itoh et al., 1999).

Recently, another small DNA virus distantly related to TTV was described (Takahashi et al., 2000). Its name as TTV-like minivirus (TLMV) reflected the fact that its genome was smaller than that of TTV (2915 bases compared with 3852 of the prototype TTV sequence). The difference in size results from a shorter ORF1 encoding the capsid protein and shorter untranslated region. TTV and TLMV show even greater sequence divergence from each other than between genotypes of TTV; however, the precise degree of divergence is difficult to determine as neither the UTR nor the coding sequences from the two viruses can be easily aligned.

Infection with both TTV and TLMV has been detected in a range of nonhuman primates (Verschoor et al., 1999; Abe et al., 2000; Okamoto et al., 2000a,b; Romeo et al., 2000). However, complicating the interpretation of many of the primate studies is the occurrence of cross-species transmission of TTV genotypes. For example, it is known that human TTV variants can infect chimpanzees and macaques (Luo et al., 2000; Tawara et al., 2000). Cross-species transmission may occur in captivity, or through the administration of human plasma-derived blood products containing infectious TTV and TLMV that frequently occur in experimental animals. Beyond primates, the host range of TTV and TLMV is uncertain. One study describes frequent TTV infection of domestic animals, such as cows, pigs, sheep, and chickens. This study used a PCR-based method with primers from the N22 region, implying the presence of genetic variants closely related to the prototype TTV isolate, TA278 (Leary et al., 1999). Whether TTV infection in these species was acquired through contact with humans or vice versa was not determined. More recently, highly divergent TTV-like viruses were detected in pigs, cats, and dogs, distinct from those found using the N22 primers (Okamoto et al., 2002), suggesting that this virus family may indeed be widely or ubiquitously distributed in the mammalian order.

In the current study, we have developed a highly sensitive PCR-based method to detect both TTV and TLMV sequences in plasma and serum using primers from a highly conserved region in the UTR. Detection of amplified DNA sequences in the Roche Lightcycler provided a semiquantitative estimate of the degree of TTV/TLMV viraemia in plasma, while measurement of melting profiles of ampli-

cons allowed TTV sequences to be reliably distinguished from TLMV. The PCR was used to determine frequencies of TTV/TLMV infection in a wide range of wild-caught apes and African monkeys to establish whether infection was indigenous to these species. Finally, using large panels of samples from cows, sheep, goats, and chickens, we determined whether TTV infection was present in domestic animals.

Results

Sequence conservation of TTV and TLMV in the UTR

Despite the extensive sequence divergence of TTV and TLMV in coding regions of the genome, three areas of remarkable conservation have been identified in the part of the UTR that contains transcriptional promoters and splice sites (Kamahora et al., 2000; Okamoto et al., 2000c) (Fig. 1). Sequence conservation is found among all known human isolates of TTV and TLMV, as well as those recovered to date from a range of nonhuman primates. One region is additionally conserved with the very distantly related bird virus, CAV. Based on this sequence alignment, we developed a heminested PCR using primers matching the three conserved regions in the UTR and used them to screen a low-risk, adult human population (blood donors; Table 1). For comparison, each sample was also tested by previously published PCR methods (Okamoto et al., 1998; Leary et al., 1999) using primers from a region of ORF1 corresponding to the originally described N22 clone of TTV (Okamoto et al., 1998) (Table 1). A total of 88 from 100 samples were positive using the UTR primers, but only 5 of 100 were positive using primers from the N22 region (Table 1).

Differentiation of TTV and TLMV sequences

Because of the sequence conservation between TLMV and TTV in the region targeted by the UTR primers, DNA amplified by the UTR primers from the human samples may correspond to either virus or potentially mixtures of both TTV and TLMV genotypes. To investigate the diversity of the detected viruses, we analysed amplified DNA in the Roche Lightcycler. This analysis revealed that melting temperature (T_m) values, as defined by maxima in rate of change of fluorescence with temperature [$d(F1)/dT$], fell into two separate distributions, while some samples contained apparently heterogeneous DNA sequences with maxima in both distributions (examples of melting curves are shown in Fig. 2A). An analysis of T_m values for the 88 PCR-positive blood donors indicated that 34 contained sequences in the high T_m distribution (median value 90.7°C; range 89.0–92.7°C), 18 in the low distribution (median 86.3°C; range 84.5–87.8°C), and 36 with heterogeneous sequences with both T_m s (Fig. 3A).

Sequence composition differences in TTV and TLMV in the amplified region may influence T_m values. TTV se-

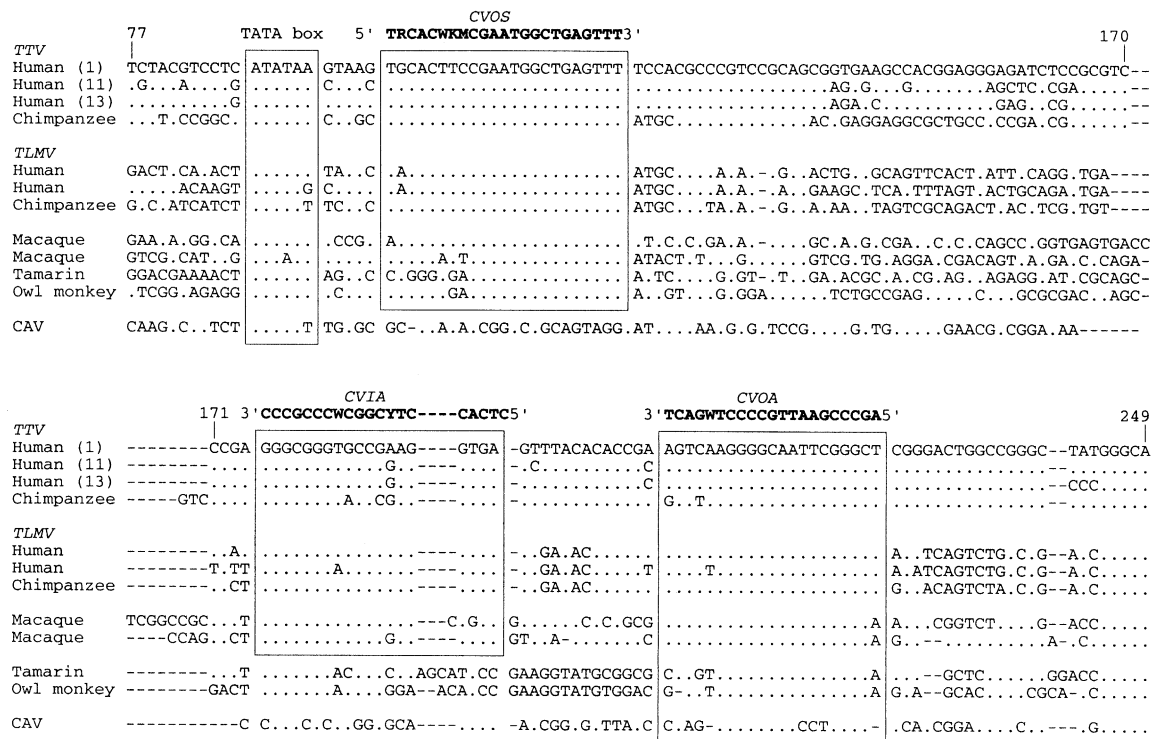


Fig. 1. Alignment of nucleotide sequences from the TTV/TLMV UTR showing the highly conserved region between genotypes 1, 11, and 13 (NC_002076, AB017613, and AB025946), TLMV (NC_002195, AB038629), and homologous sequences of TTV and TLMV from nonhuman primates [chimpanzee (AB041957, AB041963), macaque (AB041958, AB041959), and the new world primates tamarins (AB041960), and owl monkey (AB041961)], and CAV (NC_001427). Conserved regions used to specify primers indicated in boxes. Sequences numbered according to the TTV sequence NC_002195. Symbols: :, sequence identity with prototype TTV sequence; -, gap introduced to preserve alignment of homologous nucleotide sites.

quences have a higher G+C content than TLMV sequences (mean values of 65.4 and 58.6%, respectively), and TTV sequences are generally 2-4 bases longer. It is therefore possible that the upper distribution of T_m values corresponds to TTV sequences, and the lower distribution of T_m values corresponds to TLMV as both G+C content and amplicon length will increase binding strength. To investigate this, we

developed primers that were specific for TTV or TLMV sequences (TTV-OS and TLMV-OS) that bind in the variable region upstream of the TATA box (Fig. 1). A total of 20 samples were amplified using TTV-OS or TLMV-OS with CVOA in the first-round PCR, followed by a nested PCR with CVOS and CVIA. The T_m s of the amplified DNA sequences were compared (Fig. 3B).

From the 20 samples, 7 were PCR-positive using the TTV-specific primer, 1 with the TLMV-specific primer, and 6 with both primers (total 14). The T_m values of the amplified DNA showed a strict dependence on the primers used; all 13 samples amplified using the TTV-OS primers showed high T_m values (mean 91°C), while those amplified with TLMV-OS produced DNA with T_m values exclusively in the low distribution (mean 86.8°C). To confirm that the sequences amplified with the virus-specific primers differentiated between TTV and TLMV, amplified DNA from four samples positive with TTV-OS and 4 with TLMV-OS were cloned and sequenced (Fig. 4). In each case, nucleotide sequences corresponding closely to either TTV or TLMV were found in DNA amplified with homologous type primers.

Finally, the cloned sequences were reamplified with CVOS and CVIA, and T_m s determined (Fig. 2B). For each clone, sequences identified by nucleotide sequencing as

Table 1
Detection of TTV and related viruses by PCR

Sample	Number tested	Number positive (%)		
		UTR	N22	N22/2
Apes				
Human	100	88 (88%)	5 (5%)	n.d.
Chimpanzee	5	4 (80%)	0	1 (20%)
Orangutans	14	14 (100%)	0	0
Gibbon	19	12 (63%)	0	0
African monkeys				
Drill	13	12 (92%)	0	0
Mandrill	2	2 (100%)	0	0
Mangabey	4	4 (100%)	0	0
Nonprimates				
Cows	20	0	0	0
Sheep	20	0	0	0
Goats	20	0	0	0
Chickens	29	0	0	0

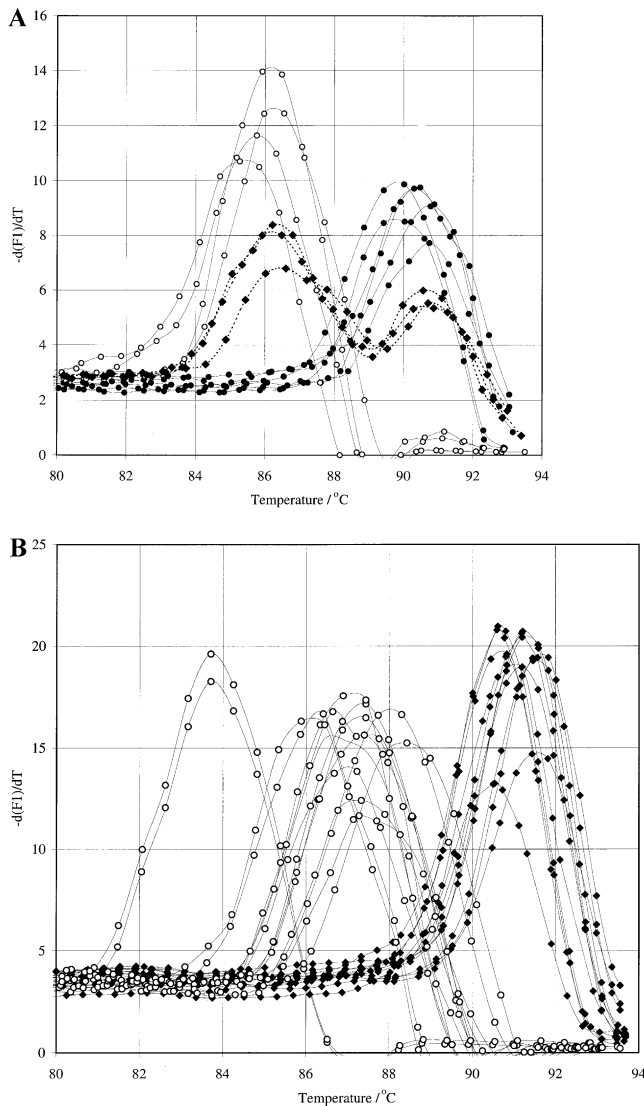


Fig. 2. Melting curve profiles of TTV/TLMV sequences amplified using the UTR primers and analysed in the Roche Lightcycler, plotted as temperature (x -axis) vs rate of change in fluorescent intensity (F1) over temperature [$d(F1)/dT$]. (A) Amplified DNA sequences from a random selection of TTV/TLMV PCR-positive blood donors. Symbols: ●, samples showing high T_m ; ○, low T_m ; ◆, evidence for mixed virus population with high and low T_m TTV/TLMV variants. (B) Amplified DNA sequences from molecular clones identified as TTV-like (●) or TLMV-like (○) from sequence comparisons with standard isolates (Fig. 4). The two clones with T_m s of 83.7°C correspond to TLMV1/1 and 1/2 (see text).

being TTV (and originally derived from PCR with the TTV-specific primer) showed high T_m values, while TLMV clones showed T_m values corresponding to the low distribution, as predicted. Interestingly, two sequences (TLMV1/1 and TLMV1/2) that have an additional deletion of five nucleotides in the amplified region (Fig. 4) showed T_m values even lower than observed for other TLMV sequences (83.7°C; Fig. 2B); this observation further indicates the dependence of T_m on sequence length.

Returning to the T_m analysis presented in Fig. 3, it therefore appears that either TTV or TLMV or mixtures of both may predominate in the plasma of individual blood donors.

Analysis of TTV/TLMV variants infecting nonhuman primates

To investigate the frequency of infection of viruses related to TTV and TLMV in other mammals, we screened several species of ape [*Pan troglodytes troglodytes* and *Pan troglodytes vellerosos* (chimpanzees), *Gorilla gorilla gorilla* (western lowland gorilla), *Pongo pygmaeus pygmaeus* (Bornean orangutan) and *Hylobates agilis*, *Hylobates lar*, *Hylobates muelleri*, *Hylobates moloch*, and *Hylobates gabriellae* (gibbons)] and African monkey species [*Mandrillus leucophaeus leucophaeus* (drill), *Mandrillus sphinx* (mandrill), and *Cercocebus torquatus* (cherry-capped mangabey)] by PCR using UTR primers and those based on the N22 gene (Table 1). To investigate the claim that nonprimates may also be TTV positive (Leary et al., 1999), we additionally tested samples from domestic animals (cows, sheep, goats, and chickens) using a PCR protocol and primers identical to those described in the original study (N22/2). High frequencies of viraemia were detected in each of the nonhuman primate species tested using the UTR primers (63–100%), while only one chimpanzee from the 57 nonhuman primates tested was positive using N22-based primers. A total of 89 samples from domestic animals was also tested with each of the three sets of primers. All samples were PCR negative, even using primers and PCR conditions exactly as described previously (Leary et al., 1999).

The distribution of T_m values of DNA amplified from the primate samples differed considerably from those of human samples (Fig. 3C). Amplified sequences from each species showed considerable heterogeneity, and in some cases, heterogeneous sequences with two T_m maxima were observed within a sample (as with TTV/TLMV coinfection in humans). The distribution of T_m values observed in ape samples differed from African monkey species, the former showing a much greater range in values, which, in the case of the chimpanzee samples, corresponded approximately to the range in values observed between human TTV and TLMV variants. To confirm the sequence heterogeneity of the amplified sequences, and to investigate the sequence relatedness of viruses infecting nonhuman primates to human TTV and TLMV, amplified DNA from a selection of chimpanzee, gorilla, orangutan, gibbon, drill, mandrill, and cherry-capped mangabey samples was cloned and sequenced (Fig. 5). The sequences obtained were compared with human TTV and TLMV and previously published sequences from the chimpanzee, two sequences from long-tailed macaques (*Maccaca fascicularis*), two sequences from New World primates [*Aotus trivirgatus* (owl monkey) and *Sanguinis oedipus* (cotton-topped tamarin)], and a sequence from the tree shrew (*Tupaia belangeri chinensis*).

Sequence comparison and phylogenetic analysis was

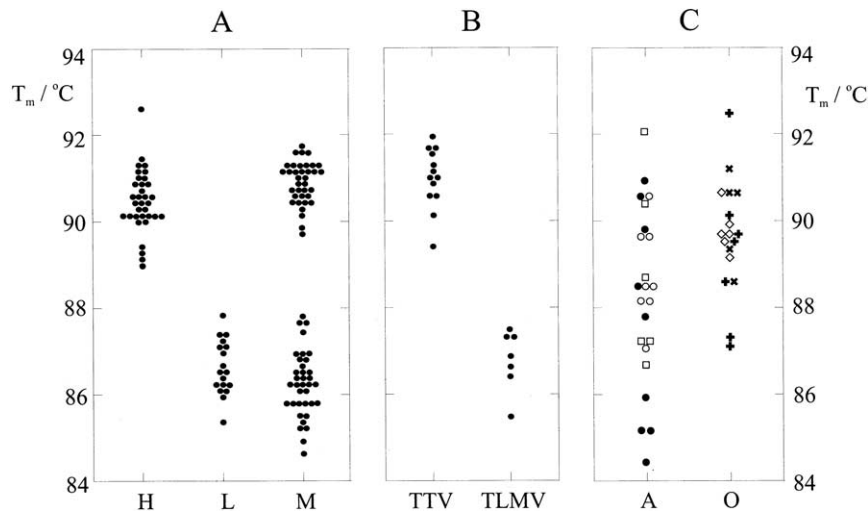


Fig. 3. Distribution of T_m values (corresponding to $d(F1)/dT$ maxima by melting curve analysis; Fig. 2). (A) Samples from the 88 TTV/TLMV-positive blood donors, categorised in high (H), low (L), and mixed (M) distributions. (B) Distribution of T_m values for samples amplified with TTV-specific or TLMV-specific primers (TTV-OS and TLMV-OS). (C) Comparison of T_m values of TTV/TLMV-related viruses directly amplified from serum samples of apes (A) and Old World monkeys (O). Symbols: ●, chimpanzee; ○, orangutan; □, gibbon; ◇, cherry-capped mangabey; +, mandrill; X, drill.

problematic for this region of genome because of the sequence diversity between variants amplified from different primate species, and between TTV and TLMV, which is so extreme that attempts to align the sequences are necessarily arbitrary and therefore somewhat subjective. For consistency, we have allowed only one gap to be inserted between position 161 and 162 to allow alignment of the downstream primer. This data presentation reveals clearly the length polymorphism of this region, with one particularly long

sequence recovered from an orangutan (Pp-E2/2; 18 bases longer than the prototype TTV sequence), and short sequences from the gibbon (Hl-E2) and the tree shrew (Tbc-TTV14; 6–8 bases shorter than the prototype). Because of the alignment problems, we believe that phylogenetic analysis is inappropriate for the UTR sequences, as the method assumes that homologous bases are aligned.

Combined with published sequences, variants infecting chimpanzees fell into two groups, those resembling TTV

	99	CVOS							CVIA	193		
NC_002076	TGCACTTCCG	AATGGCTGAG	TTT	TCCACGC	CCGTCCGCAG	CGGTGAAGCC	ACGGAGGGAG	ATCTCCGCGT	CCCGA	GGGCG	GGTGCCGAAG	GTGAG
AB017613AGA.C...GA	G..CG.....G..
AB025946AG.G...GAGC	TC.CGA...G..
TTV1/1	.A.....A..AGA.CA..TC	-G.....-G..
TTV1/2	.TA.....A..AGATC.GAGA	TCG-A.....G..
TTV1/3	.A.....A..AGATC.GAGC	GA..GA.....G..
TTV3/1	.A.....A..AGA.C...CT.....G..
TTV3/2	.A.....A..AGA.C...CT.....G..
TTV3/3	.A.....A..AGA.C...CT.....A..
TTV4/1	.A.....A..AGATC.GAGC	GATCGA.....A..
TTV4/2	.A.....A..AGATC.GAGC	GATCGA.....A..
TTV4/3	.A.....A..	A.....G..AGC	GATCGA.....A..
TTV5/1	.A.....A..AGATC.GAGC	GATCGA..CG	T.....A..
TTV5/2	.A.....A..AGATC.GAGC	GATCGA..CG	T.....A..
NC_002195	.A.....	ATGC...	A.A.G-G..	ACTG..GCAG	TTCAGT.ATT	CA-G..TG	A..A..
AB038627	.A.....	ATGC...	A.A.G-A..	ACAG..TCA	TTCAGT.ACT	CCA-G..TG	A..A..
TLMV1/1	.A.....A..	ATGC...	TA.A.G-G..	GAC...TA.	ATTCA..C	TAA-----	-ACA..
TLMV1/2	.A.....A..	ATGC...	TA.A.G-G..	GAC...TA.	ATTCA..C	TAA-----	-ACA..
TLMV1/3	.A.....A..	A.GC...	TA.A.G-G..	AACCGGTGAG	TTCAGT.ACT	CGG--A.TG	.ATCA..
TLMV1/4	.A.....A..	ATGCT..	A.A.G-A..	GCTGC.CCA	TTCAGT.ACT	GCA-G..TG	A..A..A..
TLMV2/1	.A.....A..	ATGC...	A.A.G-G..	AC.G.TCA	TTCAGT.ACT	CCA-G..TG	AA.TTA..
TLMV2/2	.A.....A..	A.GC...	A.A.G-G..	GC.G.TCA	TTCAGT.ACT	CCA-G..TG	A..A..A..
TLMV2/3	.A.....A..	ATGC...	A.A.G-G..	AC.G.TCA	TTCAGT.ACT	CCA-G..TG	AA.TTA..
TLMV3/1	.A.....GA..	ATGC...	A.A.G-G..	AC.G.GCAG	TTCAGT.ACT	CCA-G..TG	A..A..A..
TLMV3/2	.A.....A..	ATGC...	A.A.G-G..	AC.G.GCAG	TTCAGT.ACT	CCA-G..TG	A..A..A..
TLMV3/3	ATT.....A..	A.....	A.A.G-G..	TC.GC.TCA	AAAGT.C..	GC--GA.TG	AT..TTA..
TLMV3/4	.A.....A..	ATGC...	A.A.G-G..	AC.G.TCA	TTCAGT.ACT	CCA-G..TG	AA.TTA..
TLMV4/1	.A.....A..	ATGC...	A.A.G-G..	ACAAC.CCAA	GTTA..A..CT	G.T--G..TG	A..A..G..
TLMV4/2	.A.....A..	ATGC...	A.A.G-G..	ACAAC.CCAA	GTTA..A..CT	G.T--G..TG	A..A..A..
TLMV4/3	.A.....A..	ATGC...	A.A.G-G..	ACAAC.CCAA	GTTA..A..CT	G.T--G..TG	A..A..A..
TLMV4/4	.A.....A..	ATGC...	A.A.G-G..	ACAAC.CCAA	GTTA..A..CT	G.T--G..TG	A..A..A..

Fig. 4. Comparison of nucleotide sequences of molecular clones obtained from DNA amplified with TTV-specific (TTV1–TTV5; clone number added as suffix) or TLMV (TLMV1–TLMV4) with previously published sequences of TTV and TLMV (indicated by accession numbers). Sequences numbered according to the TTV sequence NC_002195. Symbols: -, sequence identity with prototype TTV sequence; -, gap introduced to preserve alignment of homologous nucleotide sites.

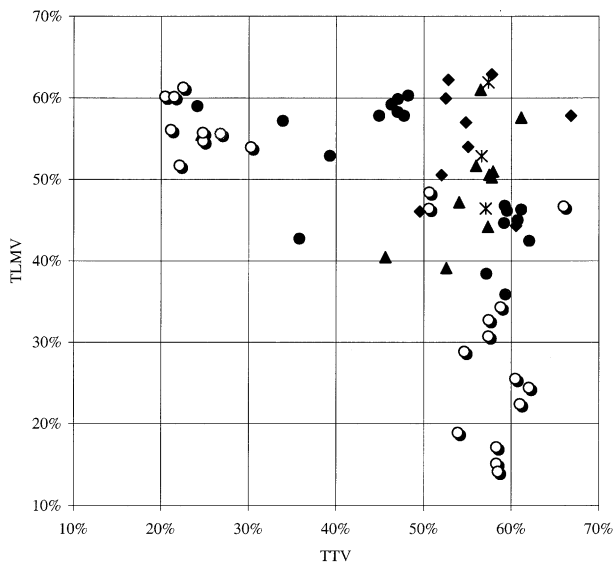


Fig. 6. Sequence divergence of human (this study; Fig. 4) and primate (this study and previously published; Fig. 5) TTV-related UTR sequences from representative sequences of TTV ($n = 58$) and TLMV ($n = 50$) (the full list of reference sequences used is available from the authors on request). Distances from TTV (x-axis) and TLMV (y-axis) sequences shown as uncorrected percentage divergence. Sequence comparisons used the minimal alignment shown in Fig. 5. Symbols were used to indicate different species of origin of UTR sequences; ○, human; ●, chimpanzee; ▲, other apes; ◆, Old World monkey species; *, New World primates and tree shrew.

some species, the genetic diversity within each of the primate species matches the diversity of TTV and TLMV variants found in human samples.

Discussion

Amplification of TTV and TLMV UTR sequences

Despite the great sequence diversity of human TTV and TLMV genotypes, and homologues in nonhuman primates, it is fortunate that conserved regions exist in the UTR to allow a common set of heminested primers to detect a wide range of TTV/TLMV genetic variants. The conserved regions are close to regions involved in transcription, such as the TATA box, and their lack of sequence divergence presumably reflects functional constraints, imposed by their interaction with host replicative machinery (Hijikata et al., 1999). The sequence conservation in this region allowed the development of a heminested PCR capable of amplifying TTV and TLMV viruses in human samples; this method uses primers that match the known variants of TTV and TLMV equally well and is therefore predicted to be equally effective at amplifying each virus from a heterogeneous mixture in a sample. It was therefore possible to carry out a meaningful comparison of their relative frequencies and abundance in human samples.

Among the 88 PCR-positive samples using the UTR primers, approximately one-third contained exclusively TTV sequences, one-third contained TLMV, and one-third contained mixed infection of TTV and TLMV based upon the T_m s of the amplified DNA measured on the Roche Lightcycler. These results do not rule out the absence of infection with both viruses in any of the samples, as amplified sequences with a low relative abundance may not be detectable by the melting curve analysis. To determine the frequency of infection with each, virus-specific primers would be required. However, our findings indicate that TLMV infection is equally prevalent to TTV in humans and that both viruses can coexist and replicate to similar titres in a substantial proportion of individuals. These findings are consistent with previous observations of the presence of multiple TTV genotypes in human plasma and tissues (Ball et al., 1999; Okamoto et al., 1999; Forns et al., 1999; Niel et al., 2000). The finding that plasma itself is not representative of the range of greater genetic variants that may be sequestered in tissues such as bone marrow, lymphoid tissue, and liver (Okamoto et al., 2001a) suggests that infection with both TTV and TLMV may be extremely widely distributed or possibly universal in the human population.

Detection of TTV/TLMV homologues in nonhuman primates

The sequence conservation of the UTR primers allowed an investigation of the distribution of TTV/TLMV-related viruses in a wider range of nonhuman primates than previously attempted. The primer sequences matched previously published sequences from chimpanzees and the macaque (an Old World monkey) (Fig. 1) (Inami et al., 2000; Okamoto et al., 2000b). There was also reasonable sequence conservation with TTV/TLMV sequences recovered from New World primates and the tree shrew (Okamoto et al., 2001b), although the sequence differences with the inner antisense primer would undoubtedly compromise the efficiency of the UTR PCR for these more divergent viruses.

Despite the sequence diversity of primate viruses and the potential problem this poses for PCR-based screening, close to universal infection was found in each ape and Old World monkey species screened (Table 1). Comparisons of amplified sequences from the UTR of each species confirmed it was improbable the viruses were human in origin; none corresponded to known human genotypes of TTV or TLMV (Fig. 5 and data not shown). Furthermore, amplified DNA from each of the primate species (particularly the African species) showed a distribution of T_m s quite distinct from the narrow ranges found among the TTV and TLMV sequences amplified from human subjects (Fig. 3C).

The great sequence diversity of the UTR sequences prevented any defensible strategy for the alignment of homologous bases. On this basis, it was felt unjustified to attempt to carry out formal phylogenetic analysis. However, inspection of the minimal alignment (Fig. 5) and sequence diver-

gence from representative data sets of human-derived TTV and TLMV sequences (Fig. 6) indicated that the division of the chimpanzee UTR sequences into TTV and TLMV groups was potentially justifiable, principally on the basis of the sequence at the 5' end of the amplicon (positions 115–122), although the “ATGC” motif found in human TLMV sequences at position 120 is also present in the TTV-like chimpanzee sequences. The assignment of chimpanzee sequences into TTV- and TLMV-related groups is also supported by their complete genome sequences; apart from their greater sequence similarities, Pt-TTV6 has a genome length of 3690 bases, comparable to that of the prototype human TTV sequence, NC_002076 (3853 bases), while Pt-TTV8/II is only 2785 bases, comparable to the TLMV variant NC_002195 (2860 bases) (Okamoto et al., 1998, 2000b). This finding suggests that the evolutionary split between TTV and TLMV therefore must have occurred before the speciation of humans and chimpanzees (7 Myr) if TTV/TLMV-related viruses coevolved with their hosts (see below).

In contrast to the chimpanzee-derived UTR sequences, sequences from the other apes and African monkey species analysed in this study showed no specific relationship to TTV or TLMV (Fig. 5), being equally dissimilar to human-derived TTV and TLMV sequences (Fig. 6). Supporting this conclusion, the complete genome sequences from macaques (Mf-TTV3 and -TTV9, also dissimilar to TTV or TLMV in the UTR; Fig. 5) are 3798 and 3763 bases in length, but show no close sequence relationship to either human or chimpanzee TTV sequences (Okamoto et al., 2000b).

Despite the difficulties associated with the comparison of highly divergent sequences in the UTR, and elsewhere in the genome (Okamoto et al., 2000b), none of the data obtained in the current study is inconsistent with the hypothesis of coevolution of TTV/TLMV-related viruses with primates over several tens of millions of years. Evidence for cospeciation includes the increasing divergence of Old and New World primate variants to human TTV/TLMV (Figs. 5 and 6) (Okamoto et al., 2000b), and the outlier phylogenetic position of the TTV/TLMV-related virus recovered from tree shrews (Okamoto et al., 2001b), reflecting the depth of the split of the host species from other species in this primate order. In many respects, this scheme corresponds closely to the proposed coevolution of herpesviruses in primates and other mammals (McGeoch et al., 2000). While the three groups of herpesviruses (α , β , and γ) are represented in each mammalian order investigated and presumably diverged before or during their original differentiation 90 million years ago, some γ -herpesviruses show evidence for more recent evolutionary splits and cocarriage during the period of primate evolution, analogous to the TTV/TLMV split suggested by the comparison of sequences from some ape species.

Detection of TTV/TLMV-related viruses in other mammals

The previous finding (Leary et al., 1999) of TTV infection in several domestic animals (pigs, cows, sheep, and chickens) is clearly inconsistent with the hypothesis of TTV/TLMV coevolution in primates, particularly as the isolates from farm animals were not apparently genetically distinct from those found in humans (Leary et al., 1999). To resolve this discrepancy, we attempted to reproduce the findings of the study using a nationally collected set of serum samples from cows, sheep, goats, and chickens. While this was by no means a comprehensive selection of farm animals, the high frequencies of TTV detection reported previously in a probably similarly geographically restricted sample collection suggested that the materials available for this study would suffice to reinvestigate the issue.

To detect TTV infection, we used PCR methods involving UTR primers (this study), N22 primers used extensively in previous surveys of human and primate TTV prevalences (Okamoto et al., 1998), and a PCR method that precisely reproduced the experimental conditions of the original study (Leary et al., 1999). All 89 samples tested were negative with each of set of primers and PCR conditions (Table 1). While we cannot rule out that domestic animals may vary in TTV prevalence between countries, another interpretation is that the previously reported findings are incorrect. Returning to the coevolution hypothesis, the increasing sequence divergence of TTV/TLMV-related viruses from human variants observed in primate screening, including the appearance of sequence differences in the primer sites (Fig. 1), suggests that TTV/TLMV-related viruses split from primate variants even earlier in mammalian evolution (90 Myr) and may therefore be refractory to amplification with even with heminested UTR primers. Very recently, evidence for the existence of the predicted highly divergent viruses in non-primates has indeed been obtained using nonnested primers for the UTR corresponding approximately in position to CVOS and CVOA (Okamoto et al., 2002). Complete genome sequences from cat-, dog-, and pig-derived TTV-related viruses were obtained by reverse PCR from the amplified UTR region and found to be even more divergent from the prototype human TTV than those from any of the primates or the tree shrew. None of the nonprimate viruses showed any significant sequence similarity to the N22 primers.

In summary, the use of direct nucleotide sequencing combined with melting curve analysis of amplified DNA sequences documents the extreme sequence diversity of TTV, TLMV, and related viruses in humans and nonhuman primates. With the limitations on primer design that results from this genetic variability, we have also shown that infection with this virus family is likely to be ubiquitous or near ubiquitous in primates at least down to the Old World monkey/ape split. In terms of their ubiquity and persistence, TTV and TLMV are comparable to the distributions of

herpesviruses, but differ uniquely in their high-replicative capacity that leads to life-long persistent viraemia in most individuals. Their genetic diversity greatly exceeds that of any other known virus, even that of the papillomaviruses infecting the skin. Investigating the mechanisms by which TTV, TLMV, and related viruses can establish multiple, largely, or entirely nonpathogenic persistent infections in primates is clearly a fertile area for fundamental virology and immunology research in the future.

Materials and methods

Samples

A total of 100 human plasma samples from Scottish blood donors were available for investigation of TTV/TLMV prevalence in a human population. All samples were negative for antibodies to human immunodeficiency virus type 1 and 2 and hepatitis C virus, and negative for hepatitis B virus surface antigen. Sera from 13 drills, 2 mandrills, 4 cherry-capped mangabeys, 5 chimpanzees, 14 orangutans, 19 gibbons, and 1 gorilla were available for investigation of TTV/TLMV infection in nonhuman primates. Sanctuaries in Nigeria, Cameroon, and Taiwan supplied these samples, with the exception of the gorilla sample supplied by Edinburgh Zoo, and all the primates used had originally been wild-caught. Each primate species was housed separately from other primates. Finally, we tested sera from 20 cows, 20 sheep, 20 goats, and 29 chickens collected from a variety of locations in England and Scotland.

Extraction of nucleic acid

Nucleic acid from 2 ml human plasma was extracted using a guanidinium thiocyanate/silica-based method (NucLisens, Organon-Teknika, Boxtel, Netherlands) and eluted in 50 μ l Tris–HCl buffer. Nucleic acid from all other samples was extracted from 100 μ l serum using a standard phenol–chloroform protocol and precipitated with ethanol. The resulting pellet was resuspended in 25 μ l nuclease-free water.

Amplification of TTV and TLMV

Three sets of primers were utilised during this study to allow a comparison of primer sensitivity and of virus prevalence in different host species. Heminested primers NG059, NG061, and NG063 (primer set N22), derived from the original N22 clone, have been used extensively in previous investigations of TTV epidemiology (Okamoto et al., 1998). Fully nested primers (primer set N22/2), originally published in conjunction with a study of TTV viraemia in farm animals, were also investigated (Leary et al., 1999). Conditions for PCR using these primers were as previously described (Okamoto et al., 1998; Leary et al., 1999). The

third primer set (primer set UTR) is heminested and spans a conserved region of the UTR. Ambiguities were included in the primer design to allow TLMV to be coamplified. In the first round of the PCR 5 μ l of extracted DNA was amplified using primers CVOA and CVOS (sequences 5'-AGC-CCGAATTGCCCTWGACT-3' and 5'-TRCACWKMC-GAATGGCTGAGTTT-3'). Two microliters of this reaction was carried over to a second round and amplified using primers CVOS and CVIA (sequence 5'-CTCACCTYCG-GCWCCCGCCC-3'), resulting in a 95-bp amplicon. Amplification conditions were 94°C/18 s, 55°C/21 s, and 72°C/90 s for 30 cycles, with an additional 6 min extension at 72°C to finish. PCR products were visualised on a 2% agarose gel stained with ethidium bromide.

Differentiation of TTV and TLMV using real-time PCR

The first-round PCR using UTR primers was carried out as above, and 2 μ l of the reaction product was used in a second round which was performed on a Lightcycler (Roche Diagnostics GmbH) using primers CVOS and CVIA and Lightcycler-DNA Master SYBR Green I (Roche Diagnostics GmbH). Amplification of the template (initial denaturation 95°C/30 s, then 30 cycles of 95°C/0 s, 55°C/5 s, and 72°C/10 s) was followed by melting curve analysis, during which the samples are heated from 60 to 95°C to allow the determination of the melting temperature (T_m) of the amplified DNA sequences. The analysis of this melting curve produced a peak with a virus-specific T_m , which we used to differentiate and characterise the products.

Molecular cloning and sequencing of TTV and TLMV DNA

Human samples positive with the UTR primers were subjected to a second nested PCR in which primer CVOS in the first round was replaced with a TTV- or TLMV-specific primer [TLMV specific primer 5'-GGAGGAGHHWMHM-HACTATATAA-3' (TLMV-OS) and TTV specific primer 5'-AACMKGGTCTACRTCCTSATATAA-3' (TTV-OS)]. The second round of the PCR and conditions are as described for UTR primers, and products were run on the Lightcycler to ensure the correct virus had been amplified. Primate samples were amplified with the UTR primers prior to cloning. PCR products were ligated into pGEM-T Easy Vector (Promega Corp., Madison, WI) and cloned according to manufacturer's instructions. Nucleic acid was purified using QIAprep Spin Miniprep kit (Qiagen).

Nucleotide sequencing and sequence analysis

Miniprep DNA was sequenced from both ends by manual cycle sequencing using a thermostable DNA polymerase (Promega) and primers specific to the M13 portion of the vector. Sequences were analysed using Simmonic 2000 sequence analysis package. Sequences obtained in this

study have been submitted to GenBank and have been the assigned the Accession Nos. AY187821–AY187866.

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