Analysis of the entire genomes of torque teno midi virus variants in chimpanzees: infrequent cross-species infection between humans and chimpanzees

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Humans are frequently infected with three anelloviruses which have circular DNA genomes of 3.6–3.9 kb [Torque teno virus (TTV)], 2.8–2.9 kb [Torque teno mini virus (TTMV)] and 3.2 kb [a recently discovered anellovirus named Torque teno midi virus (TTMDV)]. Unexpectedly, human TTMDV DNA was not detectable in any of 74 chimpanzees tested, although all but one tested positive for both human TTV and TTMV DNA. Using universal primers for anelloviruses, novel variants of TTMDV that are phylogenetically clearly separate from human TTMDV were identified from chimpanzees, and over the entire genome, three chimpanzee TTMDV variants differed by 17.9–20.3 % from each other and by 40.4–43.6 % from all 18 reported human TTMDVs. A newly developed PCR assay that uses chimpanzee TTMDV-specific primers revealed the high prevalence of chimpanzee TTMDV in chimpanzees (63/74, 85 %) but low prevalence in humans (1/100). While variants of TTV and TTMV from chimpanzees and humans were phylogenetically interspersed, those of TTMDV were monophyletic for each species, with sequence diversity of <33 and <20 % within the 18 human and three chimpanzee TTMDV variants, respectively. Maximum within-group divergence values for TTV and TTMV were 51 and 57 %, respectively; both of these values were substantially greater than the maximum divergence among TTMDV variants (44 %), consistent with a later evolutionary emergence of TTMDV. However, substantiation of this hypothesis will require further analysis of genetic diversity using an expanded dataset of TTMDV variants in humans and chimpanzees. Similarly, the underlying mechanism of observed infrequent cross-species infection of TTMDV between humans and chimpanzees deserves further analysis.

INTRODUCTION

Torque teno virus (TTV) was first discovered in the serum of a patient with cryptogenic hepatitis following transfusion in 1997 (Nishizawa et al., 1997; Okamoto et al., 1998). In 2000, torque teno mini virus (TTMV) was accidentally identified by PCR using TTV-specific primers that partially matched homologous sequences but generated unexpectedly shorter amplicons (Takahashi et al., 2000). TTV and TTMV are both small, unenveloped spherical viruses with circular single-stranded DNA genomes of 3.6–3.9 and 2.8–2.9 kb, respectively (Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 1998, 1999, 2000b; Takahashi et al., 2000; Peng et al., 2002). The International Committee on Taxonomy of Viruses officially classified TTV and TTMV into the new floating genus Anellovirus (Biagini et al., 2005).

Recently, a third group of TTV-like viruses, termed small anellovirus (SAV) types 1 and 2 (SAV-1 and SAV-2) were cloned from plasma samples of individuals at high risk for human immunodeficiency virus (HIV) infection (Jones et al., 2005). Further characterization revealed that these viruses have a circular DNA genome of 3.2 kb, intermediate in size between TTV and TTMV, but with an otherwise similar genomic organization (Ninomiya et al., 2000).
2007a). We have provisionally designated this new virus as torque teno midi virus (TTMDV), in preference to their earlier designation as 'small anellovirus', since the original sequences of SAV-1 and SAV-2 are likely to be incomplete.

Although TTV, TTMDV and TTMV have a presumed common genomic organization with four open reading frames (ORFs) (ORF1–ORF4) and a short region of high GC content (approx. 90 %), they are substantially dissimilar in genomic length and genetic identity (Biagini et al., 2001a, 2005; Hino & Miyata, 2007; Ninomiya et al., 2007a; Okamoto et al., 1999, 2004; Simmonds, 2002; Takahashi et al., 2000; Thom et al., 2003). TTV shows considerable genetic diversity and is classified into at least 39 genotypes with nucleotide divergence of >30% from one another, or into five major genetic groups (groups 1–5) with a sequence divergence of >50% from one another (Hijikata et al., 1999; Okamoto et al., 2004; Peng et al., 2002). In the case of TTMV, full-length genomic sequences of 17 isolates have been determined thus far, and four highly divergent genetic groups have been proposed (Biagini et al., 2001b, 2007; Okamoto et al., 2000b; Takahashi et al., 2000). Upon analysing more TTMDV sequences, we noticed that they formed a large swarm of isolates differing in length (3175–3230 nt) and in sequence (33% divergence over the entire genomic sequence) (Ninomiya et al., 2007b). Our previous study using a newly developed PCR method revealed high frequencies of vireaemia with TTV (99.2 %), TTMDV (82.4 %) and TTMV (89.7 %) among apparently healthy subjects of 1–81 years of age. Additionally, dual or triple infection of these three anelloviruses was seen frequently, even among infants (Ninomiya et al., 2008).

Infection with TTV is not restricted to humans; various animal species including non-human primates, tupaias, livestock and some companion animals carry a wide range of highly divergent TTV-like viruses (Inami et al., 2000; Okamoto et al., 2000a, b, 2001, 2002; Thom et al., 2003). Notably, and in contrast with other non-human primates and other animal species, chimpanzees are also infected with TTMVs (Okamoto et al., 2000b). Chimpanzees have been experimentally infected with human TTVs and a simian TTV was detected in a human host (Iwaki et al., 2003; Luo et al., 2000; Mushahwar et al., 1999; Okamoto et al., 2000a, b; Tawara et al., 2000), suggesting the occurrence of cross-species transmission of TTVs between humans and chimpanzees. Phylogenetic analysis revealed that both chimpanzee TTVs and TTMVs were interspersed with human TTVs and TTMVs, respectively (Ninomiya et al., 2007a; Thom et al., 2003).

These observations suggest that chimpanzees might also be infected with TTMDV, contributing to dual or triple infection of TTV, TTMDV and TTMV as in humans, but this hypothesis currently remains undetermined. In the present study, we aimed to investigate the presence of TTMDV in chimpanzees and to clarify whether dual or triple infection of the three described anelloviruses is prevalent in chimpanzees, similar to humans. Contrary to our initial expectations, human TTMDV was not detectable in any of 74 chimpanzees that had been raised in the Primates Park in Japan. However, novel variants of TTMDV that were phylogenetically clearly separate from human TTMDV were detected in chimpanzees and the entire genomic sequence was determined for three chimpanzee TTMDV isolates. To further elucidate the possibility of cross-species infection of TTMDV, the prevalence rates of chimpanzee TTMDV and human TTMDV were examined among chimpanzee and human populations by using PCR with species-specific primers for TTMDV. These new investigations into the host range and specificity of TTV-like viruses contribute to a better understanding of the evolution and pathogenesis of anelloviruses.

METHODS

Serum samples. Serum samples obtained between 1991 and 1997 from 74 chimpanzees that had been kept at Kumamoto Primates Park (Sanwa Kagaku Kenkyusho Co. Ltd, Kumamoto, Japan) (Okamoto et al., 2000a) were used in the present study. Of these, 34 were wild-caught and the remaining 40 were bred at the Primates Park. The animals studied were maintained and monitored under conditions that met all relevant requirements for the humane care and ethical use of primates in an approved facility. Eight of 74 chimpanzees were positive for antibodies to hepatitis C virus (HCV) (anti-HCV) (Abbott Japan) and none of them tested positive for hepatitis B surface antigen (HBsAg) after testing with the MyCell kit (Institute of Immunology Co.). Thirty-six chimpanzees had undergone transmission experiments. In addition, serum samples that had been collected between 2001 and 2003 from a total of 100 apparently healthy Japanese adults (mean age 54.9 ± 11.9 years; 57 males and 43 females) were used in the present study. These samples were also tested for HBsAg and anti-HCV, and additionally for antibodies to HIV type 1 tested using the SERODIA-HIV kit (Fujirebio); all samples were negative in all three tests. All serum samples were stored at −80 °C until testing.

Detection of TTV, TTMDV and TTMV DNA in serum. The presence of TTV, TTMDV and TTMV DNA was determined by using a previously described PCR assay for differential detection of three human anelloviruses (Ninomiya et al., 2008). The expected sizes of the amplification products were 112–117 bp (TTV DNA), 88 bp (TTMDV DNA) and 70–72 bp (TTMV DNA).

Molecular cloning of partial TTV, TTMDV and TTMV sequences. The presence of chimpanzee-specific TTMDV DNA that could not be amplified by the previously described second round PCR using primers NG795 and NG796 (Ninomiya et al., 2008) was assessed by preparing recombinant DNA clones containing a DNA fragment of TTV, TTMDV or TTMV. Briefly, of the chimpanzees that tested positive for TTV and TTMDV DNAs, but negative for TTMV DNA, using the method described previously (Ninomiya et al., 2008), three chimpanzees were randomly selected and their serum samples were amplified with the mixed primers NG779/NG780 and NG781/NG782 (Fig. 2b; Supplementary Table S1, available in JGV Online). These first-round amplicons (approx. 130 bp) were ligated into p17 Blue T-Vector (Novagen). Independent clones obtained using this method were directly sequenced by using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).
Detection of chimpanzee TTMDV DNA in serum. Based on a partial TTMDV DNA sequence obtained in the present study from one chimpanze (no. 210), we designed inverted-nested primers for specific PCR amplification of the nearly full-length circular chimpanzee TTMDV genomes. Briefly, long-distance PCR was carried out using *TakaRa* LA Taq polymerase with GC buffer I (*TakaRa* Bio). The first PCR round consisted of 35 cycles of 94 °C for 45 s, with an additional 3 min in the first cycle, 60 °C for 45 s and 72 °C for 3 min, with an additional 7 min in the last cycle) and used primers NG797 and NG798. The second round consisted of 25 cycles of 94 °C for 45 s, with an additional 3 min in the first cycle, 60 °C for 45 s and 72 °C for 2 min, with an additional 7 min in the last cycle) using primers NG799 and NG800 (Supplementary Table S1). The approximately 3.2 kb amplification products were used for determination of the full-length genomic sequences of TTMDV as described below.

**Determination of the entire genomic sequence of chimpanzee TTMDV DNA.** To determine the full-length nucleotide sequence of three TTMDV isolates, the 1.0 kb genomic region including the GC-rich area that overlapped with the previously amplified region at both ends was amplified by PCR using nested primers that were specific for each isolate (Supplementary Table S1) and *TakaRa* LA Taq polymerase with GC buffer I (*TakaRa* Bio). In brief, the first PCR round consisted of 35 cycles of 94 °C for 30 s, with an additional 3 min in the first cycle, 60 °C for 30 s and 72 °C for 60 s, with an additional 7 min in the last cycle. This was followed by a second round consisting of 25 cycles using the same conditions as in the first round. The 1.0 kb amplicon obtained from this PCR or the 3.2 kb amplicon (above) were cloned into pT7BlueT-Vector (Novagen). Both strands of TTMDV DNA were sequenced by using recombinant double-stranded DNA as templates and the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI 3100 Genetic Analyzer (Applied Biosystems). Because of the difficulty in sequencing GC-rich regions with stem and loop structures, when necessary, the dGTP BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems) and DYEEnamic ET Terminator Cycle Sequencing kit (GE Healthcare) were also used.

**Prevalence rates of Pan troglodytes (Pt)-TTMDV DNA in chimpanzees and humans.** In order to examine further the prevalence of Pt-TTMDV infection in chimpanzees or in humans, nested primers that were derived from a highly conserved area among Pt-TTMDVs but not among TTVs, TTMDVs and TTMVs were designed to specifically amplify a 121 bp product. *Platinum Taq* DNA polymerase (Invitrogen) was used to amplify DNA in the first round for 35 cycles (94 °C for 30 s, with an additional 2 min in the first cycle, 59 °C for 30 s and 72 °C for 30 s, with an additional 7 min in the last cycle) with primers NG824 and NG825, followed by 25 cycles of the second round PCR (conditions the same as in the first round) with primers NG826 and NG825 (Supplementary Table S1).

**Analysis of nucleotide and amino acid sequences.** Sequence analysis was performed using Genetyx ver. 8 (Genetyx Corp.) and ODEN (version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) (Ina, 1994). Sequence alignments were generated by CLUSTAL W (version 1.8) (Thompson et al., 1994). Phylogenetic trees were constructed by the neighbour-joining method (*Saitou & Nei, 1987*). Phylogenetic analysis of sequences (Fig. 1; Supplementary Figs S1a, b and S2, available in JGV Online) used uncorrected p-distances between nucleotide sequences. The reliability of the phylogenetic results was assessed using 1000 bootstrap replicates (*Felsenstein, 1985*). The final tree was obtained with TreeView program (version 1.6.6) (Page, 1996).

**RESULTS**

**Detection of TTV, TTMDV and TTMV DNAs in serum samples of chimpanzees**

The PCR assays for differential detection of three human anelloviruses (*Ninomiya et al., 2008*) detected TTV and TTMDV DNA in serum samples from 73 of 74 chimpanzees (98.6%). This is similar to detection in humans, in which the prevalence rates of TTV and TTMDV DNA were 100 and 82%, respectively. There was no discernible difference in the prevalence of TTV and TTMV between wild-caught and bred chimpanzees. However, TTMDV DNA was not detectable in any of the 74 chimpanzees tested, despite a high prevalence rate (75%) of TTMDV DNA in humans (Table 1).

**Analysis of molecular clones of partial TTV, TTMDV and TTMV sequences in chimpanzees**

Anellovirus DNA from three randomly selected chimpanzees (nos. 108, 206 and 210) was amplified by using the universal primers NG779/NG870 and NG781/NG782 in the first round (*Ninomiya et al., 2008*); clones from the Pt-TTMDV isolates were identified and characterized phylogenetically. The phylogenetic tree constructed based on the 78–92 nt sequence (primer sequences at both ends excluded) of the 72 clones obtained from chimpanzee 108 revealed the distribution of only TTV (*n*=66) and TTMV (*n*=6) (Supplementary Fig. S1a). Similarly, 76 DNA clones obtained from chimpanzee 206 were classified as TTV (*n*=62) and TTMV (*n*=14) (Supplementary Fig. S1b). However, one TTMDV-like clone that is remotely related to all reported human SAV/TTMDV isolates was identified from chimpanzee 210 (Fig. 1) and provisionally designated Pt-Anello210-35.

**Determination of the full-length nucleotide sequence of TTMDV-like isolates from chimpanzees**

Inverted-nested primers that have nucleotide sequences unique to the Pt-Anello210-35 clone (Fig. 2a), but are markedly different from known human and chimpanzee TTV isolates, human TTMDV isolates and human and chimpanzee TTMV isolates within the corresponding sequences, were designed for specific amplification of the full-length circular Pt-Anello210-35 genome (Supplementary Table S1). When the long-distance inverted PCR was performed in serum samples from seven chimpanzees including chimpanzee 210, 3.2 kb amplicons were detected in two chimpanzees (nos. 210 and 225). Five DNA clones, each of 3.2 kb, were identified from the two PCR-positive chimpanzees and were subjected to analysis by partial nucleotide sequencing. Chimpanzee 225 had two distinct groups of clones (Pt-TTMDV225-1 and Pt-TTMDV225-2) that, in the partial 0.5 kb sequence, differed by 25%. The entire nucleotide sequence for a representative clone(s)
**Fig. 1.** Phylogenetic tree constructed by the neighbour-joining method based on the partial nucleotide sequences (78–92 nt) of TTV, TTMDV and TTMV isolates obtained from chimpanzee 210, using a macaque TTV-like variant (Mf-TTV3) as an outgroup. Isolates are all Pt-Anello210 strains, which are indicated as, for example, ‘35’ for Pt-Anello210-35 for simplicity. The representative TTV and TTMV isolates, as well as all 22 reported SAV/TTMDV isolates whose entire or near-entire sequence is known, are indicated in bold type; *, isolates of chimpanzee origin. Branch lengths are proportional to uncorrected p-distances between sequences (see scale bar). Bootstrap values are indicated for the major nodes as a percentage obtained from 1 000 resamplings of the data. GenBank accession numbers are given in parentheses.
from each of the two samples, and their 5' and 3' terminal sequences corresponding to the primers that were used for the 3.2 kb long-distance PCR, was confirmed by sequencing the 0.8–1.2 kb product amplified by the inverted PCR with isolate-specific nested primers (Supplementary Table S1). Consequently, the complete genomic sequence was determined for the three TTMDV-like isolates in the present study, named Pt-TTMDV210, Pt-TTMDV225-1 and Pt-TTMDV225-2, respectively. These isolates had circular genomes of 3257, 3256 and 3269 nt, respectively. These are similar in size to human TTMDV, longer than human and chimpanzee TTMVs and shorter than human and chimpanzee TTVs. Each chimpanzee TTMDV isolate possessed four major ORFs, regions with high GC content, a coding region defined as the sequence between the beginning of ORF2 and the end of ORF4 that has a high degree of genetic divergence and a non-coding region between the end of ORF4 and the beginning of ORF2, with a relatively conserved area (Fig. 3a).

Although the TTMDV-specific mRNAs have not yet been analysed, the consensus motifs of donor and acceptor sites (Breathnach et al., 1978; Mount, 1982) for three splicings (Fig. 3b) were found in three chimpanzee TTMDV isolates, similar to those in TTVs which are known to have three distinct species of TTV mRNAs (2.9–3, 1.2 and 1 kb) with three different splicings (Kamahora et al., 2000; Okamoto et al., 2000c). The short splicing of 110–111 nt (Splice 1 in Fig. 3b) was assumed to be present in the three mRNAs of chimpanzee TTMDV. The two longer splicings of 1386–1404 nt (Splice 2 in Fig. 3) and 1558–1576 nt (Splice 3 in Fig. 3b) were hypothesized to exist in the two short mRNAs.

In the deduced amino acid sequences encoded by ORF1 and ORF2, several motifs known to be characteristic of human and chimpanzee TTVs and TTMVs as well as human TTMDVs were also preserved in the three Pt-TTMDV isolates. ORF1 encoded a sequence of 658–663 aa that is rich in arginine at its N terminus and ORF2 encoded the conserved motif W-X7-H-X5-C-X1-C-X5-H (Hijikata et al., 1999; Okamoto et al., 2000b; Takahashi et al., 2000; Ninomiya et al., 2007a).

Table 1. Detection of human anelloviruses (TTV, TTMDV and TTMV) and chimpanzee-specific TTMDV in serum samples obtained from humans and chimpanzees

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total no.</th>
<th>Human- TTV PCR</th>
<th>Human-TTMDV PCR</th>
<th>Human-TTMV PCR</th>
<th>Pt-TTMDV-specific PCR</th>
</tr>
</thead>
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<td>Chimpanzees</td>
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<td></td>
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<tr>
<td>Experiment (−)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>31 (82)</td>
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<td>82 (82)*</td>
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</table>

*Data from Ninomiya et al., (2008).

Comparison of the three Pt-TTMDV isolates with each other and the reported anelloviruses

The three Pt-TTMDV isolates obtained in the present study were 79.7–82.9 % similar to each other over the entire genome. They showed only 56.4–59.6 % identity with 18 human TTMDV isolates whose entire genomic sequence has been determined (Ninomiya et al., 2007a, b). A phylogenetic tree was constructed based on the entire nucleotide sequence of the three Pt-TTMDV and 18 human TTMDV isolates, which revealed that Pt-TTMDV isolates segregate into the same cluster with a bootstrap value of 100 % and are clearly separate from human TTMDV (Fig. 4a).

To elucidate the genetic relatedness of Pt-TTMDV with other anelloviruses of human and non-human origin, a phylogenetic tree was constructed based on inferred amino acid sequences from ORF1 (Fig. 4b). To avoid measuring distances between mis-aligned sequences, we excluded the lysine/arginine-rich region at the start of the protein (codons 1–68 in the prototype TA278 sequence), as it was impossible to identify homologous amino acids in sequences largely restricted to these two residues. We similarly excluded the C-terminal region sequence beyond amino acid 288 as sequences of non-human primates could not be defensibly aligned with each other or with primate-derived TTV sequences. The tree showed that chimpanzee TTMDV is a virus species that is phylogenetically distinguishable from TTVs and TTMVs of human and chimpanzee origin as well as TTVs of macaque, douroucouli, tamarin, tupaia, dog, cat and pig, whose entire
genomic sequence has thus far been determined (Okamoto et al., 2000a, b, 2001, 2002). Of note, chimpanzee TTMDVs were clearly separate from human TTMDVs, while chimpanzee TTVs (Pt-TTV6, s-TTV.CH65-1, s-TTV.CH65-2 and s-TTV.CH71) and chimpanzee TTMV (Pt-TTMV8-II) were interspersed with human TTVs and human TTMVs, respectively.

Detection of Pt-TTMDV DNA in serum samples from chimpanzees and humans

In an attempt to examine more accurately the prevalence of Pt-TTMDV in chimpanzees, nested primers that are specific to Pt-TTMDV located in the highly conserved genomic region just downstream of the TATA box were designed for specific amplification of Pt-TTMDV DNA by
a PCR assay (Fig. 2b). By using this newly developed PCR assay (Pt-TTMDV-specific PCR), Pt-TTMDV DNA was detected in serum samples from 63 (85%) of 74 chimpanzees (Table 1). Sequence analysis of the 20 randomly selected amplicons demonstrated the specificity of the PCR assay (Supplementary Fig. S2): all 20 chimpanzee TTMDV isolates were grouped into the cluster comprising Pt-TTMDV210, Pt-TTMDV225-1 and Pt-TTMDV225-2 isolates. No significant difference was noted in the prevalence of chimpanzee TTMDV between wild-caught and bred chimpanzees or between chimpanzees that had or had not taken part in a transmission experiment in the past. Of interest, when the Pt-TTMDV-specific PCR was applied to 100 human sera, one subject who tested positive for human TTV and TTMDV DNA was found to be co-infected with chimpanzee TTMDV. Reproducibility of the PCR assay was verified by repeated assays and the specificity of the assay was confirmed by sequence analysis of the amplicon (Supplementary Fig. S2). The TTMDV isolate (Hu-TTMDV1075) obtained from this subject was segregated into the cluster consisting of three chimpanzee TTMDV isolates whose entire nucleotide sequence was determined and 20 other isolates with the prefix of Pt-TTMDV whose partial nucleotide sequence was determined.

**DISCUSSION**

**Detection and characterization of TTMDV**

In the present study, when the differential PCR assays for three human anelloviruses were applied to serum samples from chimpanzees, both TTV and TTMV DNA was detected in all but one of the 74 chimpanzees tested, indicating that chimpanzees are also frequently infected with human TTVs and TTMVs. Phylogenetic analysis indicated that chimpanzees are also infected with their own TTVs and TTMVs that are closer to those of chimpanzee origin (Fig. 1). Contrary to our expectations, DNA of...
human TTMDV was not detectable in any of the chimpanzees tested, regardless of the past history of transmission experiments with human viruses. However, taking into account that anelloviruses are markedly heterogeneous, which may cause false-negative detection of the viral genome due to mismatches of primer sequences, it seems likely that chimpanzees have their own TTMDVs. To search for TTMDV-like viruses in chimpanzees, a total of 70, 72 or 76 molecular clones of amplicons, obtained from DNA from sera of each of three randomly selected chimpanzees, were subjected to sequence analysis. One clone (Pt-Anello210-35) that was remotely related to all known human TTMDV isolates was found in one of the three chimpanzees. This finding led to the identification of chimpanzee-specific TTMDV, which was provisionally designated Pt-TTMDV, and resulted in the determination of the entire nucleotide sequence of three such isolates (Pt-TTMDV210, Pt-TTMDV225-1 and Pt-TTMDV225-2) each with a 3.2 kb genome, identical to that of human TTMDV isolates.

Although chimpanzee TTMDV differs from human TTMDV by 40.4–43.6 % (mean=42 %) over the entire genome and by 60.6–68.9 % (mean=64.3 %) in the entire ORF1 amino acid sequence, it seems reasonable to classify chimpanzee TTMDV into the third group of the genus *Anellovirus* together with human TTMDV, based on the following reasons. Phylogenetic analysis revealed that chimpanzee TTMDV isolates segregate into an independent cluster supported by a high bootstrap value of 100 %, but they are closest to the cluster of human TTMDV isolates among all known anelloviruses in humans, non-human primates and non-primate animals whose ORF1 amino acid sequence has been determined (Fig. 4b). Human and chimpanzee TTMDV isolates have in common a circular 3.2 kb DNA genome, a genomic organization comprising four major ORFs with high diversity but of similar overall size, a well-conserved area within the non-coding region with GC-rich regions and several motifs of deduced amino acid sequences encoded by ORFs that are similar to TTVs and TTMVs, as well as a unique transcriptional profile that has been demonstrated for TTVs (Kamahora et al., 2000; Okamoto et al., 2000c).

A complication of primate studies is the cross-species transmission of TTVs and TTMVs. Human and chimpanzee TTVs do not cluster into species-specific monophyla. Rather, some genetic groups of human and chimpanzee TTVs cluster to make human/chimpanzee clades, as has been reported for T-cell leukemia virus type 1 and HIV type 1 (Gao et al., 1999; Korainik et al., 1994). Similarly, TTMVs of chimpanzee origin have genotypes distinct from, but interspersed between, human TTMV genotypes. Interestingly, it has been reported that simian TTV infections also occur in humans, indicating that TTV may be of zoonotic origin (Iwaki et al., 2003). However, it is also possible that some TTVs found in apes that are classified as simian TTVs are actually human TTVs, transmitted during capture, transportation or handling of the animals, as has been speculated for human TTV sequences that were detected in gibbons (Nopporpanth et al., 2001). Cross-species transmission may occur through the administration of human plasma-derived blood products containing infectious TTV or related viruses, which frequently occurs during animal experimentation. Both transient and persistent infections of human TTV in chimpanzees were observed in experimental transmission studies (Mushahwar et al., 1999; Tawara et al., 2000). The present study corroborates the previous studies because human TTV sequences have been identified in samples obtained from captive chimpanzees (Cong et al., 2000; Okamoto et al., 2000a; Romeo et al., 2000), supporting the transmissibility of human TTVs to chimpanzees. Therefore, to investigate further their species specificity, samples should ideally be collected from animals in the wild.

Although serum samples obtained from wild-caught and bred chimpanzees that had been raised in a Primates Park for more than 3 years were used in the present study, human TTMDV was not detected in any of the chimpanzees tested and chimpanzee TTMDV was detected in only one of 100 human subjects (Table 1). Phylogenetic analysis revealed that chimpanzee TTMDV isolates segregated into a cluster and were clearly separate from the human TTMDV isolates, suggesting that cross-species infection of TTMDV occurs rarely, if at all, between humans and chimpanzees. At present, serological methods to detect past infection and immunological responses to human TTMDV in chimpanzees are not available. The development of assays capable of detecting type-specific antibody to different anellovirus variants would be of use as a much more sensitive indicator of the frequency of exposure and infection of chimpanzees with human TTMDV.
Co-evolution of TTV-like viruses in primates

Observations of consistently congruent phylogenetic relationships between TTV-like viruses and their primate and non-primate hosts in which they are detected has widely suggested the possibility that these viruses have remained host-specific, and that their evolution has followed that of the animals they infect (Okamoto et al., 2000b, 2001, 2002; Thom et al., 2003; Verschoor et al., 1999). Although there are other potential explanations for these similarities in host and virus phylogenies, such as the effects of environmental overlap and of large-scale virus selection associated with adaptation for replication and persistence in a range of dissimilar mammalian species, the co-evolution hypothesis has several well-established precedents in other persistent mammalian DNA viruses, such as herpesviruses (McGeoch et al., 2006) and papillomaviruses (García-Vallvé et al., 2005).

TTV-like viruses infecting cats and dogs (two suborders in the order Carnivora) and pigs (order Artiodactyla) are highly divergent from each other and from those found in primates (Okamoto et al., 2002). The latter form a monophyletic group with variants recovered from the tupaia branching first followed by New World (owl monkey) and then Old World monkey species (macaque) viruses and finally a large group of viruses obtained from chimpanzees and humans. However, rather than being monophyletic, the three groups of ape-derived TTV-like viruses (TTV, TTMDV and TTMV) contain viruses from both humans and chimpanzees. The co-evolution hypothesis would, if confirmed, place their divergence from each other before the speciation of humans and chimpanzees, currently thought to have occurred approximately 5–7 million years ago (The Chimpanzee Sequencing and Analysis Consortium, 2005).

Although the co-evolution hypothesis suggests that the last common ancestor of human and chimpanzee must have been infected with progenitor strains of all three viruses, the current study identifies possible differences in their evolutionary timescales. As groups, TTV and TTMV are more diverse than TTMDV; the percentage identity over the entire genome among TTVs was recorded to be approximately 49 % (Peng et al., 2002) and approximately 43 % among TTMVs (Biagini et al., 2001b, 2007), while the lowest similarity among 21 human and chimpanzee TTMDV isolates was 56 % (this study; Ninomiya et al., 2007a, b). Furthermore, while variants of TTV and TTMV from chimpanzees and humans are interspersed, those of TTMDV are monophyletic for each species, with much more restricted sequence diversity within each; the lowest similarities were 67 and 80% within the 18 human and three chimpanzee TTMDV variants characterized to date. The available data therefore suggest that the TTMDV group originated at a later time than the time of chimpanzee/human speciation, rather than before it, as seems likely for TTV and TTMV. However, we fully acknowledge that at this early stage, the dataset of TTMDV sequences that is available may not encompass the full diversity of viruses within this group. Confirmation of the restricted sequence diversity of TTMDV and thus the later evolutionary origin for TTMDV that this would indicate will require further analysis of the genetic diversity of this virus group in humans, chimpanzees and, ideally, in other higher apes.

In conclusion, the present study revealed that wild-caught and bred chimpanzees that had been raised in a Primates Park for more than 3 years were frequently infected with human/chimpanzee TTV (viraemia rate 98.6 %) and human/chimpanzee TTMV (98.6 %), but not with human TTMDV. Instead, chimpanzees were found to be frequently infected with a distinct set of TTMDV variants (85.1 %), with a 3.2 kb-long genome, which is similar to the length of human TTMDV and between that of TTV and TTMV.

Chimpanzee (Pan troglodytes)-specific TTMDV was tentatively abbreviated Pt-TTMDV and designated as another member of the third group in the genus Anellovirus. As expected, frequent dual or triple infection of these three anelloviruses was observed in chimpanzees, similar to the infections observed in humans. Chimpanzees harbour a complex flora of TTV, TTMDV and TTMV that is similarly diverse to that found in humans (Simmonds et al., 1999). Unexpectedly, infrequent cross-species infection of TTMDV between humans and chimpanzees was noted in the present study (this is dissimilar to TTV and TTMV), the underlying mechanism of which warrants further analysis. Investigating the evolutionary mechanisms by which TTVs, TTMDVs and TTMVs can establish persistent infections in humans and chimpanzees would be a productive area for fundamental virology in the future.

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REFERENCES


