

Isolation of Multiple TT Virus Genotypes from Spleen Biopsy Tissue from a Hodgkin's Disease Patient: Genome Reorganization and Diversity in the Hypervariable Region

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Received 3 February 2004/Accepted 1 March 2004

We report the isolation of 24 novel genotypes of TT viruses from a surgically removed spleen of a patient with Hodgkin's disease. The sequence analysis of our 24 isolates revealed the remarkable heterogeneity of TT virus isolates not only from the same patient but also from the same biopsy material. These isolates belong to four phylogenetic groups of TT viruses. Nucleotide sequence analyses revealed five distinct genotypes (tth3, tth4, tth5, tth6, and tth7). The limited variation in sequence identity of the other isolates defines the latter as variants of four of these genotypes. A group of 6 isolates (the tth7 group) revealed a reorganization of open reading frame 1 (ORF1) leading to one larger and a varying number of smaller ORFs. The nucleotide difference of the full-length genomes was less than 1%. A variation of 69 to 97% in amino acids of a second group of 8 isolates (the tth3 group) was restricted to the hypervariable region of ORF1, indicating the existence of a quasi-species. These isolates differed by less than 2% in the remainder of their nucleotide sequences. An alignment of these isolates with 79 previously reported TT virus genotypes permits the proposal of TT virus genera and species within the family *Anelloviridae* in analogy to a previous proposal for the papillomaviruses (family *Papillomaviridae*).

TT viruses (TTV) are ubiquitous in nature and have been demonstrated in more than 90% of serum samples from healthy individuals where they persist over time (14, 16, 27, 41). Viral particles have been purified from feces (17, 34) and, in addition, are excreted in saliva, breast milk, and bile juice (9, 38, 47, 49). Transplacental transmission from mother to child has been controversial, but postnatal transmission has been confirmed repeatedly (15, 20, 24, 30, 33, 37, 48, 51, 52, 57). Replicative intermediate forms of the viral genome have been isolated from bone marrow cells (42) and demonstrated in a number of other organs (39, 44). Peripheral blood mononuclear cells act as a reservoir for TTV (41), but the highest viral load is found in the granulocytes (54). Viral transcription naturally occurs in bone marrow cells and not in peripheral blood mononuclear cells (40), although transcription *in vitro* has been achieved by DNA transfection into stimulated peripheral blood monocytes (29) and in a monkey cell line (19).

A large number of full-length or near full-length genomes of TTV has been isolated from humans and primates. They have also been identified in farm animals (23), although the genomes of recent isolates from pigs, dogs, and cats proved to be considerably smaller in size than the TTV found in humans and primates (43). The highly conserved noncoding region constitutes about one-third of the genome. The coding region consists of a large open reading frame (ORF), ORF1, coding for the viral capsid protein as well as the smaller ORF2, ORF3, and ORF4. Additional mRNAs result from splicing events (19,

40). Many different genotypes have been isolated, and an extremely wide range of DNA sequence divergence has been demonstrated (45). The steadily increasing number of new genotypes and the high sequence variability point to the need for a uniform and defined classification of this virus family.

Several attempts have been made to link TTV to the etiology of a specific disease. TTV were originally isolated from a patient suffering from posttransfusion hepatitis (35), but a direct role in the pathogenesis of this disease or in hepatocellular carcinoma has not been established (31, 32, 46). Several studies have failed to demonstrate a direct association of TTV with lymphomas and myelomas (2, 8, 60). Conditions of immune suppression may, however, lead to increased virus levels in the blood (39, 60). Our laboratory recently reported the presence of TTV DNA in specific human cancers, most notably in cancers of the gastrointestinal tract, lung, and breast and in a number of multiple myelomas (6). PCR amplification of fragments of the genome was used to detect a series of newly identified TTV-related sequences.

The present study describes the isolation of 24 full-length genomes and three 2-kb fragments from the spleen of a patient with Hodgkin's disease. These isolates belong to four different genotypic groups of TTV. The sequences of 8 clones from one TTV type were nearly identical to each other over the length of the complete genome, except for a high variation between clones in the highly variable region. In a second set, 6 isolates of the same TTV type varied in as little as 1% of nucleotides over the full genome length, but this variation induced stop codons within the ORF1, generating smaller individual ORFs in this region. We propose a more-defined classification system resulting from comparisons of our TTV types to 79 previously reported TTV genomes. The genomic diversity of the TTV

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TABLE 3. Pairwise percent homology comparisons of full-length nucleotide sequences of tth clones

Clone	% Homology with:		
	tth14	tth29	tth5
tth14	100.0	99.7	99.7
tth29		100.0	99.7
tth5			100.0

AJ620220; tth17, AJ620221; tth25, AJ620222; tth26, AJ620223; tth27, AJ620224; tth31, AJ620225; tth4, AJ620226; tth5, AJ620227; tth14, AJ620228; tth29, AJ620229; tth7, AJ620230; tth8, AJ620231; tth13, AJ620232; tth19, AJ620233; tth22g4, AJ620234; tth23, AJ620235; N57.72, AJ620236; N58.46, AJ620237; N58.22, AJ620238; N58.15, AJ620239; N58.11, AJ620240; N58.5, AJ620241; L2T1.57, AJ620242.

RESULTS

Initial amplification of a short conserved region of the TTV genome led to the identification of 15 individual TTV sequences present in the spleen of a patient with Hodgkin's disease. These isolates were divided into 6 groups according to sequence homology to facilitate the selection of primers for amplifying the remaining part of the full-length genome of each type (data not shown). Amplification of the tumor DNA with one of these primer sets resulted in the isolation of 29 fragments larger than 3 kb and 2 fragments of 2 kb in size. A total number of 128 clones were grouped after restriction fragment length polymorphism analyses. Of these, 30 clones were sequenced and arranged into 5 groups according to sequence homology. The tth3 group consisted of 12 isolates, 2 of which were identical to two other clones (tth16 to tth12 and tth28 to tth26). Two fragments (tth1 and tth31) represented only partial TTV genomes. Summaries of all isolates and their DNA sequence homologies are presented in Tables 2, 3, 4, and 5.

The nearly full-length genomes of 79 TTV isolates were aligned to the 24 isolates described here. These included all isolates of at least 3,200 bp. The genome of isolate tth4 falls into group 3 of the TTV (45) and is most closely related to senv-e (55), sharing 70% identity. In addition, tth4 shares 60 to 70% identity with SANBAN (11), tjn02 (58), tchn-g2 (26), tvsan-ir1031, and tvsan-s039 (53) but less than 60% identity with all other tth and TTV isolates. Isolate tth5 shares 99% identity with isolates tth14 and tth29. It also shares 91% identity to jt33f (45) and 92% identity to l01 (25). The isolates tth7, tth8, tth13, tth19, tth22g4, and tth23 shared 99% identity with each other and 71% identity with the next closest jt33f, l01, and tth5. The group of tth6, tth10, tth11g2, tth18, tth20, and tth21

TABLE 4. Pairwise percent homology comparisons of full-length nucleotide sequences of tth clones

Clone	% Homology with:					
	tth10	tth11g2	tth18	tth20	tth21	tth6
tth10	100.0	99.4	99.6	98.0	99.4	98.2
tth11g2		100.0	99.3	97.8	99.3	98.0
tth18			100.0	97.9	99.3	98.1
tth20				100.0	97.9	99.2
tth21					100.0	98.2
tth6						100.0

TABLE 5. Pairwise percent homology comparisons of full-length nucleotide sequences of tth clones

Clone	% Homology with:					
	tth13	tth19	tth22g4	tth23	tth7	tth8
tth13	100.0	99.1	99.1	99.2	99.1	99.3
tth19		100.0	99.3	99.5	99.5	99.6
tth22g4			100.0	99.4	99.4	99.5
tth23				100.0	99.6	99.7
tth7					100.0	99.7
tth8						100.0

shared 97 to 99% identity with each other and 66% identity with the closest related KAV (10). The tth3 group comprises 8 full-length isolates, namely tth3, tth9, tth16, tth17, tth25, tth26, tth27, and tth31. The isolates tth16 and tth31 share 99% identity, and tth25, tth26, and tth27 share 99% identity with each other, forming 2 subgroups which, with tth3, tth9, tth17, share 97 to 98% identity with each other. The closest known TTV is P1C1 (13), sharing 85% identity. The next related is HEL32/6a (18), sharing 70% identity. These TTV are members of the group 1 of TTV (45).

The new isolates all constituted a genome organization characteristic of TTV (1, 45). By comparing the full-length sequences between isolates of the tth7 group on the one hand and the tth3 group on the other, two distinct observations were made. The 8 full-length isolates of tth3 group may be considered variants of one type, as measured by a sequence difference of less than 2% between isolates. The only region in which a high nucleotide sequence variability (80 to 99%) (Table 6 and Fig. 1) was observed was restricted to the highly variable region (HVR) within the ORF1 (36), whereas only single nucleotide differences were present in the remaining part of the genome. This HVR, spanning nt 1425 to 1693 of our isolates, varied not only in nucleotide sequence identity but also substantially in amino acid homology (between 69 and 97%). Several of the amino acid substitutions were nonconservative, which suggests a possible role of these changes in immune modulation of the ORF1 protein. It is obviously not possible to determine which isolate represented the prototype responsible for the initial infection or which isolates developed over time within this individual patient.

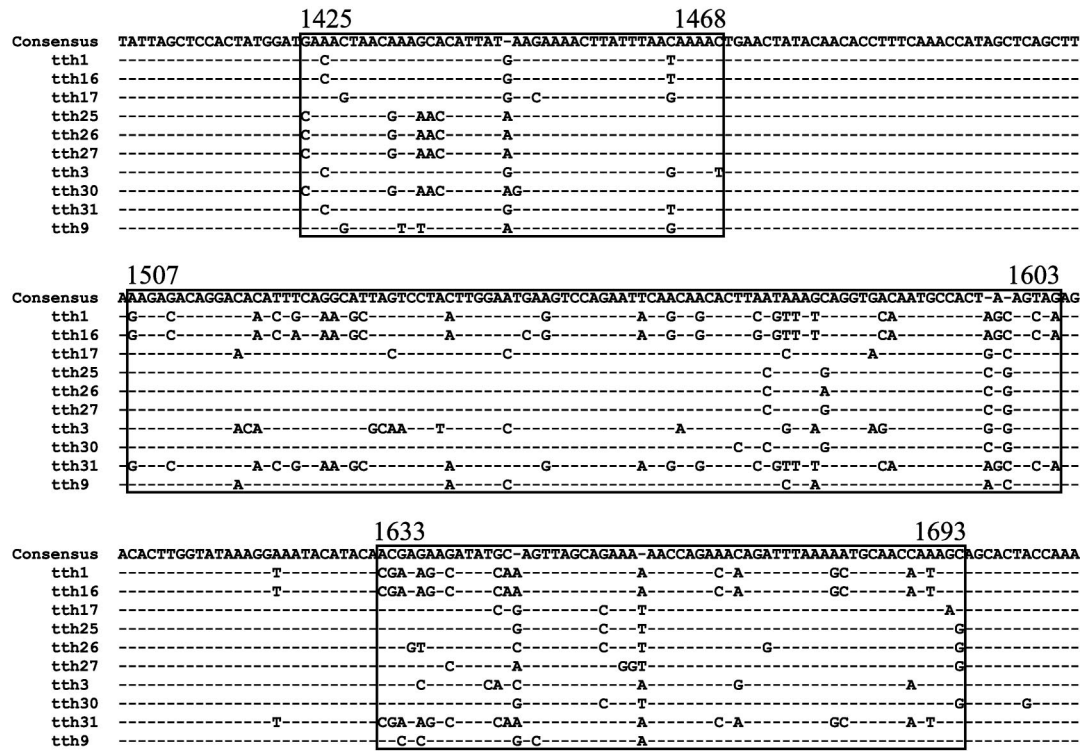
The second observation was the presence of premature stop codons within ORF1 in the isolates tth7 and tth22g4, falling

TABLE 6. Pairwise percent identity comparisons of nucleotide and amino acid sequences of the HVR of tth3 clones^a

Clone	% Identity with:							
	tth16	tth17	tth25	tth26	tth27	tth3	tth31	tth9
tth16		82.7	81.4	80.7	81.7	82.1	99.0	83.7
tth17	69.1		93.0	91.7	91.4	91.7	83.1	94.7
tth25	70.0	84.5		98.3	98.3	88.4	81.7	92.4
tth26	70.0	82.7	97.3		97.0	88.0	81.1	91.4
tth27	71.8	80.9	96.4	94.5		87.7	82.1	91.4
tth3	72.7	81.8	78.2	79.1	78.2		82.4	91.0
tth31	97.3	70.0	70.9	70.9	72.7	73.6		84.1
tth9	70.9	86.4	84.5	84.5	84.5	83.6	71.8	

^a Comparisons of nucleotide sequences are shown above the diagonal, and comparisons of amino acid sequences are shown below the diagonal.

Nucleotides:



Amino acids:

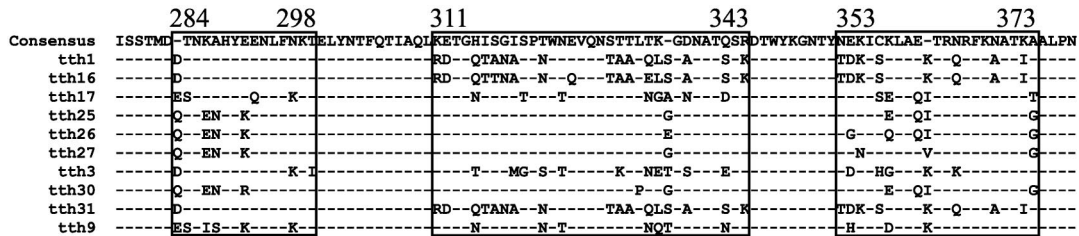


FIG. 1. Nucleotide and amino acid sequences of the HVRs (HVR1, HVR2, and HVR3) of the variants of tth3. Dashes indicate nucleotides or amino acids identical to those of the consensus sequence.

into group 5 (45) of TTV. Instead of the complete ORF1 constituting 743 amino acids (aa), one nucleotide change in the tth7 sequence resulted in a premature stop codon at position nt 1022, coding for a shortened ORF1 (ORF1a, 141 aa, 423 bp). The second ORF1b of the tth7 (nt 1331 to 2828) leads to a putative protein of 499 aa (1,497 bp) in length (Fig. 2). The ORF1 of isolate tth22g4 was interrupted by 2 stop codons, resulting in 3 putative ORFs: ORF1a (75 aa, nt 599 to 824, 225 bp), ORF1b (117 aa, nt 935 to 1286, 351 bp), and ORF1c (499 aa, nt 1331 to 2828, 1,497 bp). An alternative start codon (21) for ORF1 of isolate tth13 was GTG at nt 599, resulting in a putative protein of 743 aa. Single nucleotide changes occurred scattered throughout the rest of the genomes not leading to any obvious modifications. To verify whether the stop codons of isolates tth7, tth22g4, and tth13 resulted as PCR artifacts during amplification of the long fragments, we generated primers to amplify the region spanning the 5' end of ORF1 (nt 445

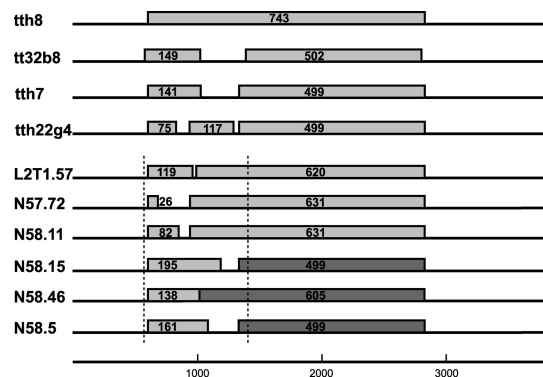
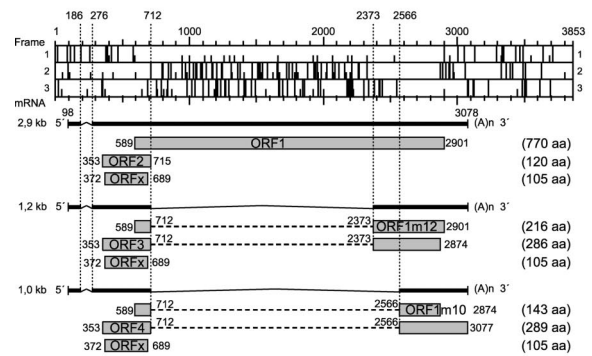


FIG. 2. Additional ORFs within ORF1 amplified from the spleen of a patient with Hodgkin's disease. The number of amino acids in each of the respective ORFs is indicated.

to 1506 of *tth8*). The original tumor DNA was used as a template, and an amplicon of 837 bp in size was generated by nested PCR with a low-error-rate proofreading enzyme. After cloning of the amplicon, the inserts of 244 resulting clones were sequenced on both strands, and the sequences were compared to the *tth8* isolate (with intact ORF1). Of these, 158 clones were identical to each other, but all varied from *tth8* at nt 794 of the complete genome sequence (G in *tth8* and A in the amplicon, leading to an amino acid modification of glycine [GGA] to arginine [AGA]). Seven additional clones contained premature stop codons, resulting in putative shorter ORF1 proteins. Two of these were identical to each other. The remaining 6 clones were fragment L2T1.57 with 2 ORFs, ORF1a (357 bp, 119 aa) and ORF1b (1,068 bp, 620 aa), fragment N57.72 with ORF1a (78 bp, 26 aa) and ORF1b (1,893 bp, 631 aa), fragment N58.5 with ORF1a (483 bp, 161 aa) and ORF1b (1,497 bp, 499 aa), fragment N58.11 with ORF1a (346 bp, 82 aa) and ORF1b (1,893 bp, 631 aa), fragment N58.15 with ORF1a (585 bp, 195 aa) and ORF1b (1,497 bp, 499 aa), and fragment N58.46 with ORF1a (414 bp, 138 aa) and ORF1b (1,815 bp, 605 aa). By comparison to all other isolates, we assumed the 3' region of the amplicons to result in the putative larger proteins as indicated (Fig. 2). In addition, 79 clones harbored scattered single nucleotide differences which did not influence the ORF structure. As the bacterial strains used in amplifying the plasmids may have induced such mutations into the plasmids, we transformed one clone with two different bacteria strains (TOP10F' and XL1-Blue) and sequenced the resulting clones from each strain (107 in total). All obtained sequences were identical to the input clone.

Databank searches with ProtSweep against Swissprot, TrEMBL, and EMBL without expressed sequence tags and sequence tagged sites revealed no significant hits for any of the resulting putative proteins, except for the ORF1a of N58.46 which shared some homology to the UL31-like protein of herpes simplex virus (accession number AAG14224).

a) *ta278*



b) *tth7*

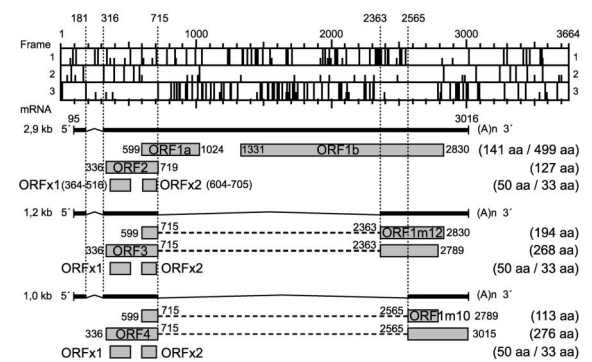


FIG. 3. Putative ORFs for TTV *ta278* (a) and *tth7* (b).

Replicative forms of TTV have been reported in bone marrow cells (42). Three mRNA species (2.9, 1.2, and 1.0 kb) have, in addition, been reported either in bone marrow cells (40) or in COS1 cells transfected with a TTV construct (19). We have

TABLE 7. Nucleotide and amino acids of additional smaller ORFs within ORF1 of various TTV isolates

Isolate	ORF1a				ORF1b			
	Position (nt) of:		Length in:		Position (nt) of:		Length in:	
	Start codon	Stop codon	bp	aa	Start codon	Stop codon	bp	aa
Full-length TTV isolates								
<i>tth7</i>	599	1022	423	141	1331	2828	1,497	499
<i>tth22g4^a</i>	599	824	225	75	935	1286	351	117
103	490	1015	525	175	1068	2730	1,662	554
<i>senv-b</i>	592	772	180	60	832	2869	2,037	679
<i>tchn-a</i>	502	685	183	61	767	2834	2,067	689
<i>tchn-c1</i>	478	1060	582	194	1050	2766	1,716	572
<i>tchnd2</i>	479	1757	1,278	426	1869	2709	840	280
<i>tt32b8</i>	416	863	447	149	1139	2645	1,506	502
Partial ORF1 isolates								
L2T1.57	599	956	357	119	986	2828 ^b	1,860	620
N57.72	599	677	78	26	935	2828 ^b	1,893	631
N58.11	599	845	246	82	935	2828 ^b	1,893	631
N58.15	599	1184	585	195	1331	2828 ^b	1,497	499
N58.46	599	1014	414	138	1013	2828 ^b	1,815	605
N58.5	599	1082	483	161	1331	2828 ^b	1,497	499

^a ORF1 is divided into 3 ORFs in the *tth22g4* sequence. The start codon of ORF1c is at nt 1331, and the stop codon is at nt 2828 (total length, 1,497 bp or 499 aa).

^b Assumed stop codon of ORF1b by comparison to all other isolates.

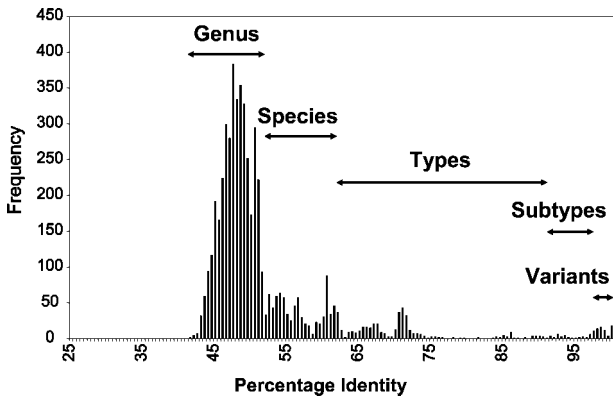


FIG. 4. Frequency distribution of pairwise identity percentages from nucleotide sequence comparisons (3,200 nt) of 103 TTV isolates.

analyzed the TTV genomes of our isolates and that of TA278 (accession no. AB017610) for additional initiation codons according to the Kozak rules (22). The use of the start codon at nt 372 in the third reading frame of the TA278 sequence

would result in an additional ORF_x (stop codon nt 689), coding for a putative protein of 105 aa in length (Fig. 3a). This ORF_x is not present in all TTV types. Donor and acceptor splice sites used for the transcription of the 1.2- and 1.0-kb mRNA species may also lead to additional ORFs. The splice sites for the 1.2-kb mRNA may result in an additional protein coded for on the second reading frame of TA278 (Fig. 3a). The splicing site at nt 712 linking with the acceptor site at nt 2373 will result in an ORF_{1m12} mRNA coding for a putative protein of 216 aa (ORF_{1m12}, nt 589 to 712 linked to nt 2373 to 2898). Similarly, the 1.0-kb mRNA splice sites located in reading frame 2 at nt 712 and linked to nt 2566 will create the ORF_{1m10} mRNA coding for a putative protein of 143 aa in length (ORF_{1m10}, nt 589 to 712 linked to nt 2566 to 2872). These two additional ORFs can be generated in all known TTV genomes. The corresponding putative proteins expressed by transcription of the *tth7* genome will be smaller in size, i.e., 195 and 98 aa, respectively. Additional studies have to be performed to determine whether these mRNA species are present in cells infected in vivo with TTV.

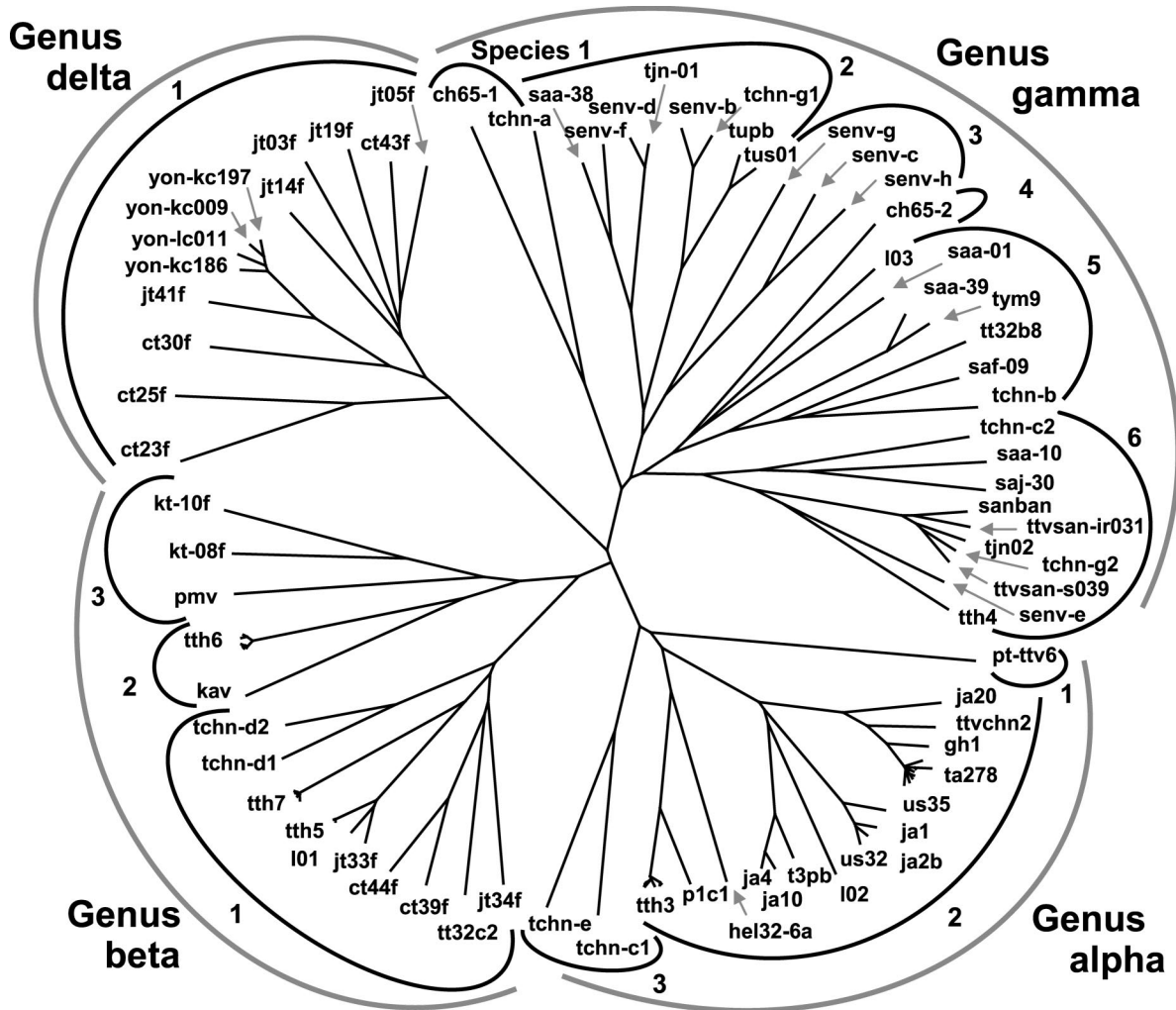


FIG. 5. Phylogenetic tree containing sequences of 103 TTV. The outermost semicircular symbols identify TTV genera, and the number at each inner semicircular symbol refers to the TTV species.

TABLE 8. Proposed classification of TTV

Genus	Species	Type (accession no.)
Alpha	1	pt-ttv6 (AB041957)
	2	ta278 (AB017610)
		ttvchn2 (AF129887)
		ja20 (AF122914)
		ja1 (AF122916)
		102 (AY026466)
		ja4 (AF122917)
		hel32-6a (AY034068)
		p1c1 (AF298585)
		tth3 (AJ620218)
	3	tchn-c1 (AF345523)
		tchn-c (AF345522)
	Beta	1
		tt32c2 (AX781379)
		ct39f (AB064604)
		ct44f (AB064605)
		101 (AY026465)
		tth7 (AJ620212)
		tchn-d1 (AF345524)
		tchn-d2 (AF345525)
2		kav (AF435014)
		tth6 (AJ620212)
3		pmv (AF261761)
		kt-08f (AB054647)
		kt-10f (AB054648)
Gamma	1	ch65-1 (AB037926)
		tchn-a (AF345526)
	2	saa-38 (AB060593)
		senv-f (AX025822)
		senv-d (AX025730)
		senv-b (AX025677)
		tus01 (AB017610)
	3	senv-g (AX025830)
		senv-c (AX025718)
		senv-h (AX025838)
	4	ch65-2 (AB049607)
	5	103 (AF371370)
		saa-01 (AB060597)
	saa39 (AB060592)	
	tt32b8 (AX781362)	
	saf-09 (AB060596)	
	tchn-b (AF348409)	
6	tchn-c2 (AF345527)	
	saa-10 (AB060594)	
	saj-30 (AB060595)	
	sanban (AB025946)	
	ttvsan-ir031 (AB038619)	
	tjn02 (AB028669)	
	ttvsan-s039 (AB038620)	
	senv-e (AX025761)	
	tth4 (AJ620226)	
Delta	1	ct23f (AB064595)
		ct25f (AB064596)
		ct30f (AB064597)
		jt41f (AB064603)
		ttvyon-kc009 (AB038621)
		jt14f (AB064601)
		jt03f (AB064599)
		jt19f (AB064602)
		ct43f (AB064598)
		jt05f (AB064600)

DISCUSSION

The present study describes the isolation of 24 full-length TTV from a single spleen biopsy sample from a patient with Hodgkin's disease. They were grouped into 5 different TTV

genotypes, based on the sequence analyses of the complete genomes. A group of 6 different isolates constituted the tth6 group, 8 isolates constituted the tth3 group, 3 isolates constituted the tth5 group, and 6 isolates constituted the tth7 group. The isolate tth4 represented another TTV genotype.

It became very evident from sequence comparisons between our isolates, tth3, tth9, tth16, tth17, tth25, tth26, tth27, and tth31, that the sequence divergence restricted to the HVR, a small region located in the ORF1, does not influence the definition of a genotype. It is, however, of great interest that the sequence identity in this HVR varied between 80 and 99% between the isolates, leading to a modification in the amino acid composition. Only single and scattered insignificant nucleotide changes were present in the remaining part of the genomes. This demonstrates the circulation of TTV as quasi-species (36). The modification in the structure of the putative capsid protein points to an interesting possible mechanism by which these persisting virus infections may escape immune surveillance.

The modifications observed in the tth7 group of TTV isolates described here may be of significance for a pathogenic role of these viruses. It is interesting that this modification of ORF1 into separate smaller ORFs was restricted to this one group of isolates, although the genomes of all of the other TTV isolates described here were generated in exactly the same way. Additional experiments also verified that these modifications had originated within the patient and did not result from any experimental artifacts. It is also of interest that the ORF1 of certain species contains as many as 14 individual start sites, which when used, will all result in proteins larger than 100 aa. The possible existence of interrupted ORF1s has previously been reported (7, 21, 26). Other known TTV with an interrupted ORF1 are 103 (AF371370) (25), senv-b (AX025677) (55), tchn-a (AF345526), tchn-c1 (AF345523), tchn-d2 (AF345525) (26), us32 (AF122921) (7), and tt32b8 (AX781361) (6; unpublished data). The smaller ORFs resulting from the premature stop codons are summarized in Table 7. Our laboratory has previously described the identification of TTV in several malignant tumors (6). The complete TTV genome from a case of lung cancer, isolate tt32b8, also revealed an interrupted ORF1, resulting in smaller ORFs in this region. In vitro functional studies with these newly created proteins may assist in defining a possible role for TTV in the etiology of some malignancies.

Multiple reports have dealt with the isolation and characterization of a large number of TTV, and many authors have attempted to classify these viruses into groups and/or genotypes (1, 12). The justification for different genera and species has, in addition, been suggested (45, 55). The use of different regions of the genome for establishing phylogeny has frequently been questioned and debated (3, 39, 45, 55). Recombination between TT isolates has been discussed (28, 59), although according to our experience this may represent PCR artifacts (generating genomes or partial genomes by PCR amplification on DNA templates harboring several closely related TTV genomes) (unpublished data). The TT-like mini viruses have been lumped together with the TTV into the proposed genus *Anellovirus*, based on a similar genome organization and similar modes of transmission and infection (12). Not only are the genomes of the TT-like mini viruses about 30% smaller

TABLE 9. Definition of prototypes with subtypes and variants

Genus	Species	Prototype (accession no.)	Subtype (accession no.)	Variant(s) (accession no.)
Alpha	2	ta278 (AB017610)	gh1 (AF122913)	ja9 (AF122915), bdh1 (AF116842), trm1 (AB026345), tk16 (AB026346), tp1-3 (AB026347), ttvchn1 (AF079173), twh (AF351132)
		ja1 (AF122916)	us35 (AF122920)	ja2b (AF122918), us32 (AF122921)
		ja4 (AF122917) tth3 (AJ620218)	t3pb (AF247138)	ja10 (AF122919) tth9 (AJ620219), tth16 (AJ620220), tth17 (AJ620221), tth25 (AJ620222), tth26 (AJ620223), tth27 (AJ620224), tth31 (AJ620225)
Beta	1	101 (AY026465)	jt33f (AB064606)	tth14 (AJ620228), tth29 (AJ620229)
		tth7 (AJ620230)	tth5 (AJ620227)	tth8 (AJ620231), tth13 (AJ620232) tth19 (AJ620233), tth22g4 (AJ620234), tth23 (AJ620235)
	2	tth6 (AJ620212)		tth10 (AJ620213), tth11g2 (AJ620214), tth18 (AJ620215), tth20 (AJ620216), tth21 (AJ620217)
Gamma	2	senv-d (AX025730)	tjn01 (AB028668)	
		senv-b (AX025677)	tchn-g1 (AF345521)	
		tus01 (AB017613)	tupb (AF247137)	
	5	tym9 (AB050448)	saa-39 (AB060592)	
	6	ttvsan-s039 (AB038620)	tchn-g2 (AF345529)	
Delta	1	ttvyon-kc009 (AB038621)		ttvyon-kc197 (AB038624)
			ttvyon-kc186 (AB038623) ttvyon-lc011 (AB038622)	

than those of TTV but the viral particles of these two groups differ accordingly in size (53). This development is very analogous to that previously experienced in the papillomavirus field (5). The family *Papillomaviridae* consists of more than 100 isolates with a large divergence in genome sequence, very similar to that seen for TTV. The rules currently applied to the classification of viruses (e.g., pathology and serology) do not apply for any of these groups because too little is known about the biological functions of each individual isolate. In addition, the absence of suitable in vitro systems for culturing the individual virus types indicates that the desired information about these characteristics may not be obtained. The ubiquitous nature of TTV and the rapid isolation of novel types may create substantial taxonomic confusion.

We have compared the overlapping 3,200 bp (nt 136 to 3264 of TTV type TA278) of 79 full-length TTV genomes and the 24 TTV described here and determined the percentage of nucleotide sequence identity between all isolates (data available upon request). The frequency distribution of the percent identities of the TTV is presented in Fig. 4. A phylogenetic tree has been compiled of these sequences by using neighbor-joining analysis (Fig. 5). Based on this information and the above-mentioned reasons, we propose the classification of TTV as a separate family, *Anelloviridae*, including TTV groups with an identity of less than 50% constituting individual genera and TTV genomes with sequence identity greater than 50% and less than 60% belonging to the same genus. A species will be defined as a group of TTV types sharing greater than 60% and less than 90% sequence homology to each other. A TTV type is defined by a complete viral genome sharing less than 90% homology with the closest related known TTV type. Subtypes

may be defined as sequences sharing more than 90% homology, and those sharing homologies of 96% and higher may be defined as variants of each other. Applying this, the 5 previously named groups (45) will be reduced to 4 genera (α , β , γ , and δ), each harboring a number of species (Table 8 and Fig. 5). The isolates in groups 2 and 5 (45) share more than 50% and less than 60% sequence identity and will therefore be combined into one genus, β . A number of hitherto known TTV types become variants of a prototype when applying the proposed criteria. We propose a priority according to the date of submission to a databank for selection of a specific type as the prototype. The TTV types which will be affected by this classification are summarized in Table 9.

The demonstration of the in vivo modification within the HVR of a single TTV type opens a possibility for further investigation into the role these changes may play in immune evasion and the persistence of these viruses in its host. A second mechanism by which the TTV genome is modified within its host, and which needs further investigation, is the induction of single nucleotide mutations in ORF1 leading to stop codons. The smaller proteins resulting from the usage of the additional start codons in this ORF may have different additional functions which may play an important role in the pathogenesis of these viruses.

ACKNOWLEDGMENTS

We thank Corinna Whitley, Elsbeth Schneider, Sabine Serick, Imke Grewe, Karin Gunst, Sonja Stephan, Helene Rahn, Christine Nitsch, Martin Dietrich, and Romana Cop for excellent technical assistance.

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