Virology 259, 428–436 (1999)

Marked Genomic Heterogeneity and Frequent Mixed Infection of TT Virus Demonstrated by PCR with Primers from Coding and Noncoding Regions[1]

Hiroaki Okamoto,* Masaharu Takahashi,† Tsutomu Nishizawa,* Masato Ukita,* Masako Fukuda,† Fumio Tsuda,‡ Yuzo Miyakawa,§ and Makoto Mayumi*,[2]

*Immunology Division and Division of Molecular Virology, Jichi Medical School, Tochigi-Ken 329-0498, Japan; †Institute of Immunology, Tokyo 112-0004, Japan; ‡Department of Medical Sciences, Toshiba General Hospital, Tokyo 140-8522, Japan; and §Miyakawa Memorial Research Foundation, Tokyo 107-0062, Japan

Received January 8, 1999; returned to author for revision February 25, 1999; accepted April 20, 1999

A nonenveloped, single-stranded, and circular DNA virus designated TT virus (TTV) has been reported in association with hepatitis of unknown etiology. TTV has a wide sequence divergence (~52%), by which it is classified into at least 16 genotypes separated by an evolutionary distance of >0.30. Therefore, the detection of TTV DNA by polymerase chain reaction would be influenced by primers deduced from conserved or divergent regions of the genome. Of the 30 sera from healthy individuals, up to 17% tested positive with primers deduced from coding region, much less frequently than up to 93% testing positive with primers from noncoding region. These differences were not attributable to the sensitivity of detection, because a cloned TTV DNA of genotype 1a was detected sensitively (up to 1 copy per test) with primers deduced from either the coding or the noncoding region of the same genotype. Sera testing positive only with noncoding region primers, or those showing higher titers with noncoding than coding region primers, contained TTV DNA strains with sequence divergence of 47–53% from the TA278 isolate of genotype 1a within the N22 region spanning 222–231 nucleotides. Some of the sera contained two or three TTV DNA strains of distinct genotypes. These results indicate TTV strains with extremely high sequence divergence prevailing in healthy individuals and frequent mixed infection with TTV strains of distinct genotypes.

INTRODUCTION

A novel DNA virus has been recovered from a patient with posttransfusion hepatitis of unknown etiology (Nishizawa *et al.,* 1998), by representational difference analysis (Lisitsyn *et al.,* 1993), and named TT virus (TTV) after the initials of the index case. Polymerase chain reaction (PCR) has been developed with primers deduced from a genomic area corresponding to the original N22 clone of 500 nucleotides (nt) that is conserved among TTV isolates of distinct genotypes (Okamoto *et al.,* 1998a,b). By PCR with heminested primers, TTV DNA was detected in patients with acute and chronic hepatitis of unknown etiology more frequently than in blood donors in various districts of the world (Okamoto *et al.,* 1998a; Charlton *et al.,* 1998). TTV is transmitted parenterally, by transfusions, intravenous drugs, and medical practices for the patients on hemodialysis and those with hemophilia (Okamoto *et al.,* 1998a). In addition, TTV may spread by a fecal–oral route, since it is secreted into feces with a buoyant density comparable to that in serum (Okamoto *et al.,* 1998b). Because of a high infectious capacity, by both parenteral and nonparenteral transmission, taken along with possible association with liver diseases of unknown etiology (Okamoto *et al.,* 1998a; Charlton *et al.,* 1998; Fujiwara *et al.,* 1998), TTV DNA should be determined in unexamined countries, especially in patients with hepatitis of unknown etiology. Such efforts may be justified, despite some concerns for lack of association of TTV with chronic liver disease (Naoumov *et al.,* 1998).

TTV is an unenveloped and single-stranded DNA virus for which a sequence of approximately 3.7 kb has been determined (Okamoto *et al.,* 1998a). Recently, Mushahwar *et al.* (1999) determined an additional GC-rich region of 113 nt to complete the genomic sequence of TTV, which was determined to be circular. Hence, TTV may be qualified as a member of the *Circoviridae* family (Lukert *et al.,* 1995). As sequence data accumulate on TTV, it has become increasingly evident that TTV has an extremely wide range of sequence divergence despite being a DNA virus. To date, at least 11 genotypes have been identified that are separated by a sequence divergence of >30% (Okamoto *et al.,* 1998a,b; Simmonds *et al.,* 1998; Tanaka *et al.,* 1998a,b; Takayama *et al.,* 1999; Viazov *et al.,* 1998).

A wide sequence divergence of TTV, which would not be distributed evenly over conserved and nonconserved regions in the genome, needs to be taken into account in selecting primers for detecting TTV DNA by

[1] The nucleotide sequence data in this paper, for the nine TT virus isolates of genotypes 12–16, will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under Accession Nos. AB021081–AB021089.

[2] To whom reprint requests should be addressed. Fax: +81-285-44-1557. E-mail: immundiv@jichi.ac.jp.

0042-6822/99 $30.00

TABLE 1

Positions and Nucleotide Sequences of Oligonucleotide Primers

Primer No.	Polarity	Nucleotide position[a]	Nucleotide sequence
NG054	Sense	3–22	5′-TTT GCT ACG TCA CTA ACC AC-3′
NG085	Sense	12–31	5′-TCA CTA ACC ACG TGA CAC CC-3′
NG049	Antisense	91–110	5′-CCA TTC GGA AGT GCA CAT AC-3′
NG133	Sense	91–115	5′-GTA AGT GCA CTT CCG AAT GGC TGA G-3′
NG134	Sense	114–136	5′-AGT TTT CCA CGC CCG TCC GCA GC-3′
NG132	Antisense	204–223	5′-AGC CCG AAT TGC CCC TTG AC-3′
NG147	Antisense	211–233	5′-GCC AGT CCC GAG CCC GAA TTG CC-3′
NG057	Sense	621–644	5′-ACG GTG GCG CAG GTG GAG ACG CAG-3′
NG058	Antisense	706–729	5′-TCT GCG GCG TCT CCT TAC GTT TCT-3′
NG015	Sense	1495–1514	5′-ACC ACA CAA ACT ATA GCC CA-3′
NG012	Antisense	1619–1638	5′-GGT GCC TGG ATA TGC ATA AG-3′
NG059	Sense	1900–1923	5′-ACA GAC AGA GGA GAA GGC AAC ATG-3′
NG061	Sense	1915–1938	5′-GGC AAC ATG YTR TGG ATA GAC TGG-3′
RD037	Sense	2008–2027	5′-GCA GCA GCA TAT GGA TAT GT-3′
NG063	Antisense	2161–2185	5′-CTG GCA TTT TAC CAT TTC CAA AGT T-3′
RD038	Antisense	2258–2277	5′-TGA CTG TGC TAA AGC CTC TA-3′
NG005	Sense	2958–2977	5′-TAC GAG GCC TGT AGA ATA TG-3′
NG009	Antisense	3246–3265	5′-CCC GAA GTG GTC TGA CAC GC-3′
NG148	Sense	3337–3360	5′-CGA AAG TGA GTG GGG CCA GAC TTC-3′
NG149	Sense	3362–3385	5′-CCA TAA GGC CTT TAT CTT CTT GCC-3′
NG136	Antisense	3444–3467	5′-ACG TCA CTG GAA CGG CCA TTT TGG-3′
NG135	Antisense	3454–3477	5′-GCG GCT GTG ACG TCA CTG GAA CGG-3′
NG022	Sense	3487–3502	5′-TAA GTA GCT GAC GTC AAG GA-3′
NG016	Sense	3522–3541	5′-TCA TCC TCG GCG GAA GCT AC-3′
NG021	Antisense	3690–3709	5′-AAA GAG GAA GGA AGT CAG CC-3′
NG065	Antisense	3713–3739	5′-GCC GAC GGT TTT TTG GCG CCT TTT TTC-3′

[a] Nucleotide positions were in accord with the TA278 isolate of 3739 nt (Okamoto *et al.,* 1998a).

PCR. In the case of hepatitis C virus (HCV), for instance, primers deduced from the 5′ untranslated region (UTR) are used for sensitively detecting HCV RNA (Bukh *et al.,* 1992; Okamoto *et al.,* 1990a), while those designed from coding regions enable the identification of various HCV genotypes (Chayama *et al.,* 1993; Okamoto *et al.,* 1992).

Various sets of primers were designed on distinct regions of the TTV genome, to evaluate their capacity for detecting TTV DNA by PCR. Furthermore, the nucleotide sequences of amplification products were determined and compared among TTV isolates of different genotypes. The results obtained highlighted a deep influence of primers used for PCR on the detection of TTV DNA, due to an extremely wide range of sequence divergence among TTV of distinct genotypes. They are hoped to help in epidemiological surveys of TTV and in correlating certain TTV genotypes with hepatitis of unknown etiology.

RESULTS

Detection of TTV DNA in sera from 30 healthy Japanese individuals by 15 different PCRs with primers deduced from various regions of the genome

Using primers deduced from noncoding and coding regions of the prototype TTV isolate (TA278) of genotype 1a (Table 1), 15 one-stage or two-stage PCRs were designed. They were evaluated for the ability to detect TTV DNA in sera from 30 healthy Japanese individuals; results are indicated in Table 2. The detection of TTV DNA varied widely from 10 to 93% in the PCRs, depending on the primers used.

The highest positive rate (93%) was achieved by three of the four primer sets deduced from UTR (A), which is positioned 3′ to the GC-rich region, followed by one of the six sets from UTR (B) located 5′ to the GC region (up to 87%) and the nested primers used in the 3.4-kb-long PCR (83%). The two (7%) sera testing negative for TTV DNA by UTR (A) primers were negative by PCR with any other primers.

By contrast, the primers deduced from the coding region and some of those from UTR (B) could detect TTV DNA in merely 10–17% of the 30 sera tested. Since the detection of TTV DNA was much influenced by regions of the genome from which primers were designed, PCR with heminested primers (NG059 or NG061/NG063) deduced from the N22 region will be referred to as N22 PCR. Likewise, PCR with nested primers (NG133/NG147; NG134/NG132) deduced from UTR (A) and that with those (NG148/NG135; NG149/NG136) from UTR (B) will be called UTR PCR (A) and UTR PCR (B), respectively, for differentiation (Table 2 and Fig. 1).

There were 2 one-stage PCRs with primer pairs

TABLE 2

Detection of TTV DNA in 30 Japanese Individuals by PCR with Various Primer Pairs

Primer pair (1st PCR; 2nd PCR)	Annealing	Extension	Length of PCR products	Positive (%)
UTR (A)				
NG054/NG049	58°C	40 s	108 bp	16 (53%)
NG054/NG132	58°C	45 s	211 bp	28 (93%)
NG133/NG147	60°C	40 s	143 bp	28 (93%)
NG133/NG147; NG134/NG132	60°C; 60°C	40 s; 40 s	143 bp; 110 bp	28 (93%)
UTR (B)				
NG005/NG009	58°C	50 s	308 bp	4 (13%)
NG148/NG135	58°C	40 s	141 bp	21 (70%)
NG148/NG135; NG149/NG136	60°C; 60°C	40 s; 40 s	141 bp; 106 bp	26 (87%)
NG148/NG065; NG149/NG021	60°C; 60°C	60 s; 60 s	403 bp; 348 bp	21 (70%)
NG022/NG021	58°C	45 s	223 bp	3 (10%)
NG016/NG065	58°C	45 s	218 bp	9 (30%)
Coding region				
NG057/NG058	58°C	40 s	109 bp	3 (10%)
NG015/NG012	58°C	40 s	144 bp	3 (10%)
NG059/NG063; NG061/NG063	60°C; 60°C	45 s; 45 s	286 bp; 271 bp	5 (17%)
NG061/NG063	60°C	45 s	271 bp	5 (17%)
RD037/RD038	58°C	45 s	270 bp	3 (10%)
3.4-kb-long PCR				
NG133/NG135; NG134/NG136	60°C; 60°C	150 s; 150 s	3387 bp; 3354 bp	25 (83%)

Note. PCR was performed on nucleic acids extracted from 25 μl of serum with AmpliTaq Gold for 55 cycles in the one-stage PCR and for 35 and 25 cycles in the two-stage PCR. The conditions for PCR were the same as described under Materials and Methods except for annealing temperature and extension times specified for each primer pair.

(NG054/NG132 or NG133/NG147) deduced from UTR (A) that could detect TTV DNA with the same sensitivity as two-stage PCR with nested primers (93%). A one-stage PCR with primer pair (NG148/NG135) from UTR (B) also achieved a sensitivity comparable to PCR with nested primers (70%).

Detection of cloned TTV DNA of genotype 1a by N22 PCR, UTR PCR (A), and UTR PCR (B)

Six two-stage or one-stage PCRs were selected, which achieved high performance in detecting TTV DNA in 30 sera (Table 2). The PCRs were tested for their sensitivity in detecting 100 copies, 10 copies, or 1 copy each of cloned TTV DNAs (TRM1 and TA278) of genotype 1a (Table 3). All six PCRs could detect 100 and 10 copies per tube in duplicate and quadruplicate tests, respectively; they could detect a single copy in 2–5 of 10 tests. Hence, primers deduced from different genomic regions were no different in sensitivity when they were used for detecting cloned TTV DNAs.

Titration of TTV DNA in 10 sera testing positive and 5 testing negative by N22 PCR

There were 10 sera that tested positive by N22 PCR and contained TTV DNA of genotype 1a, having sequence similarity of >95% to the TA278 isolate within a fragment of 222 bp. Along with 5 sera negative by N22 PCR, they were tested by UTR PCR (A) and UTR PCR (B) with the results shown in Table 4. N22 PCR detected TTV DNA in titers from 10^2/ml to 10^4/ml (samples 1–10). UTR PCR (A) and UTR PCR (B) could detect TTV DNA in titers from 10 to 100 times higher than N22 PCR in eight and seven sera, respectively. Since cloned TTV DNA was detected with equal sensitivity by the three different PCR methods, two sera (samples 1 and 9) testing positive for TTV DNA with equal titers by them would contain at least one strain of TTV of genotype 1a. Higher titers detected by UTR PCR (A) and UTR PCR (B) in eight and seven sera, respectively, would be ascribable to TTV strains in these sera that would not be detected by N22 PCR. In support of this view, TTV DNA was detected by UTR PCR (A) and UTR PCR (B) in five sera (samples 11–15) that tested negative by N22 PCR.

Sequence analysis of TTV DNA isolates in three sera with higher titers by UTR PCR than N22 PCR and three sera testing positive by UTR PCR only

A long-distance PCR, capable of amplifying 3.4 kb (Fig. 1), was performed on three sera with titers higher by UTR PCR (A) and UTR PCR (B) than N22 PCR (samples 3–5 in Table 4). Amplification products from them were cloned, and the nucleotide sequence was determined within the region amplified by N22 PCR (Fig. 2). They contained TTV strains of genotype 1a, which were detectable by N22 PCR, and in addition, those with sequence divergence of 47–53% from genotype 1a that would hardly be detected by N22 PCR. There was one such variant (TS3) in sample 3 and two each in samples 4 and 5 (TS4-I and TS4-II as well as TS5-I and TS5-II).

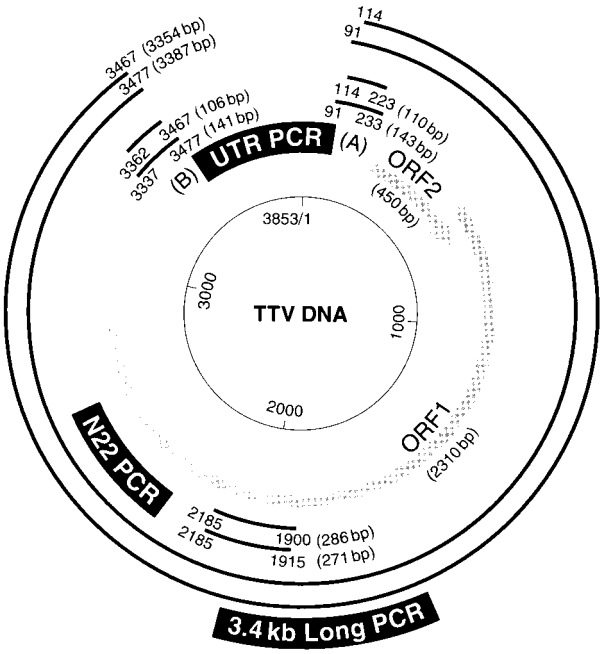

FIG. 1. Amplification products of PCR with primers deduced from distinct regions of the TTV genome and directed to different targets. The position and length of the amplification product for each PCR are indicated. Shaded arrows represent two open reading frames. Nucleotide positions are in accord with those in the TA278 isolate (Okamoto *et al.,* 1998a). UTR(A) was positioned 3′ to the GC-rich region (Mushahwar *et al.,* 1999), and UTR(B) was located 5′ to it.

The 3.4-kb-long PCR was also performed on three sera that tested positive by UTR PCR (A) and UTR PCR (B) but negative by N22 PCR (samples 13–15 in Table 4). They contained one TTV strain (samples 14 and 15) or two strains (sample 13), with sequence divergence of 50–53% from the TA278 isolate of genotype 1a.

In total, nine TTV variants with a marked sequence divergence from TA278 of genotype 1a were recovered from the six sera (Fig. 2). They showed a sequence divergence of 3–48% from one another and were classified into five groups by a difference of >30% within a sequence of 225–231 bp. The deduced amino acid sequences were much more divergent among the five groups and ranged from 29 to 59%.

Phylogenetic analysis of TTV isolates within a partial sequence of 222–231 bp

Figure 3 depicts a phylogenetic tree constructed on 9 TTV variants in five groups, along with the 18 reported, within a fragment of 222–231 bp (nt 1939–2160 in the coding region); it corresponded to the target sequence of N22 PCR with NG061/NG063. The tree justified 5 new genotypes for the 9 TTV variants that were distinct from the previously reported genotypes. They were tentatively designated genotypes 12–16 and clustered in one of the two major branches, which included the TUS01 isolate from the United States that was not amplifiable by N22 PCR. The 16 distinct genotypes were confirmed by another phylogenetic tree constructed by the neighbor-joining method (Saitou and Nei, 1987).

Table 5 compares representative TTV isolates of genotypes 1–16 with 482 isolates for which the sequence of 222–231 nt is available in the DDBJ/GenBank/EMBL DNA databases through DDBJ (National Institute of Genetics, Mishima, Japan) as of April 5, 1999. Most of them (470 or 98%) are partial sequences of only 194–396 bp within the N22 region, and all of them are classified into genotypes 1–4, 6, and 8. Most of them (447 or 93%) are of genotype 1 or 2, and none possess genotypes 5, 7, or 9–16.

DISCUSSION

Although patients with various forms of liver disease have TTV DNA in serum significantly more frequently than appropriate controls (Okamoto *et al.,* 1998a; Charl-

TABLE 3

Detection of Cloned TTV DNA of Genotype 1a by Two-Stage or One-Stage PCR with Primer Sets Deduced from Various Regions[a]

Target region and primer set	Number of cycles	Length of PCR products (bp)	Number of cloned TTV DNA per tube			
			100 copies	10 copies	1 copy	None
UTR (A) in two-stage PCR						
NG133/NG147; NG134/NG132	35; 25	143; 110	2/2 (100%)	4/4 (100%)	4/10 (40%)	0/10
UTR (A) in one-stage PCR						
NG133/NG147	55	143	2/2 (100%)	4/4 (100%)	4/10 (40%)	0/10
UTR (B) in two-stage PCR						
NG148/NG135; NG149/NG136	35; 25	141; 106	2/2 (100%)	4/4 (100%)	4/10 (40%)	0/10
UTR (B) in one-stage PCR						
NG148/NG135	55	141	2/2 (100%)	4/4 (100%)	2/10 (20%)	0/10
N22 region in two-stage PCR						
NG059/NG063; NG061/NG063	35; 25	286; 271	2/2 (100%)	4/4 (100%)	5/10 (50%)	0/10
N22 region in one-stage PCR						
NG061/NG063	55	271	2/2 (100%)	4/4 (100%)	4/10 (40%)	0/10

[a] pTV-TRM1-1 clone (3254 bp: nt 12–3265) from the TRM1 isolate and T7 clone (614 bp: nt 3126–3739) from the TA278 isolate served as targets for PCR.

TABLE 4

Detection of TTV DNA by PCR with Primers Deduced from UTR in 10 Sera Positive and 5 Negative by PCR with N22 Primers[a]

Sample No.	TTV DNA titer by PCR with primers (comparison with N22)		
	N22	UTR (A)	UTR (B)
1	10^2/ml	10^2/ml	10^2/ml
2	10^2/ml	10^3/ml (10×)	10^2/ml
3	10^2/ml	10^3/ml (10×)	10^3/ml (10×)
4	10^2/ml	10^4/ml (100×)	10^4/ml (100×)
5	10^3/ml	10^4/ml (10×)	10^4/ml (100×)
6	10^3/ml	10^4/ml (10×)	10^4/ml (10×)
7	10^3/ml	10^4/ml (10×)	10^4/ml (10×)
8	10^3/ml	10^5/ml (100×)	10^5/ml (100×)
9	10^4/ml	10^4/ml	10^4/ml
10	10^4/ml	10^5/ml (10×)	10^5/ml (10×)
11	—	10^2/ml	10^2/ml
12	—	10^3/ml	10^2/ml
13	—	10^3/ml	10^3/ml
14	—	10^4/ml	10^4/ml
15	—	10^5/ml	10^5/ml

[a] Nucleic acids extracted from serum were serially diluted 10-fold and the end titer was determined. Nucleotide sequence of the N22 region was determined in samples with numbers 3, 4, 5, 13, 14, and 15, and indicated in Fig. 2 as TS3, TS4, TS5, TS13, TS14, and TS15, respectively.

ton *et al.,* 1998; Fujiwara *et al.,* 1998), it is common in healthy individuals represented by symptom-free blood donors. TTV DNA, detectable by PCR with heminested primers deduced from conserved areas of the N22 region, is reported in 34 of the 290 (12%) blood donors in Japan (Okamoto *et al.,* 1998a), 19 of the 1000 (2%) blood donors in England (Simmonds *et al.,* 1998), and 1 of the 100 (1%) blood donors in the United States (Charlton *et al.,* 1998). Taking into account a stringent selection of blood donors, excluding the donors with markers of hepatitis viruses and elevated transaminase levels in previous donations, the prevalence of TTV in the general population would be even higher.

Recently, Takahashi *et al.* (1998) reported detecting TTV DNA in 92% of the 100 healthy individuals in Japan by PCR with the primers deduced from the UTR of the TA278 isolate (Okamoto *et al.,* 1998a). They detected TTV DNA, however, in only 23% of the same cohort by PCR with the primers deduced from the N22 region (NG061/NG063). They found titers of TTV DNA by PCR with UTR primers, 10 to 100 times higher than those with N22 primers, in three samples containing TTV of genotype 1a or 1b. Based on these observations, they concluded that TTV would be highly prevalent among the Japanese population and that PCR with primers deduced from the N22 region would be much less sensitive than the PCR with primers from the UTR.

Their proposal has twisted the research on TTV into an unexpected phase and cast doubt on a hepatitis-inducing capacity of TTV, if any, which infects the great majority (92%) of healthy individuals. Given a significant association of TTV DNA with various forms of hepatitis of unknown etiology (Okamoto *et al.,* 1998a; Charlton *et al.,* 1998; Fujiwara *et al.,* 1998), one wonders how to reconcile ubiquitous TTV infection in the general population with the detection of TTV DNA preferentially in patients with hepatitis of unknown etiology.

The present study addresses this query and gives answers. TTV has an extraordinarily wide range of sequence divergence, giving rise to at least 16 genotypes separated by sequence difference of >30% and evolutionary distance (number of nucleotide substitutions per site) of >0.30. The 16 genotypes include 5 new genotypes identified in the course of this study (Fig. 3). Hence, the areas of the genome on which primers are designed can critically influence the detection of TTV DNA by PCR. Therefore, a positive rate found by Takahashi *et al.* (1998) that was much higher with UTR primers (T801/T935) than N22 primers (NG061/NG063) is not attributable to different sensitivities between these two sets of primers as they claim. Rather, the difference is ascribed to distinct specificities between the two sets of primers they used.

The fact that the issue is specificity, and not sensitivity, is most clearly demonstrated in the titration of two cloned TTV DNAs of genotype 1a. They were detected with the same sensitivity by PCR with N22 primers and by those with various sets of primers deduced from UTR, in either one-stage or two-stage PCR (Table 3); all PCRs could detect nearly a single copy of cloned TTV DNA per test. Hence, the conclusion drawn by Takahashi *et al.* (1998) for a higher sensitivity of PCR with UTR primers than that with N22 primers is unjustified. On the contrary, the differences they observed would be reasonably attributed to TTV strains of distinct genotypes in the sera they tested. PCR with N22 primers would be able to detect the TTV strains of some genotypes, but not those of the others.

Some sera from healthy Japanese individuals have TTV DNA in titers higher by PCR with UTR primers than N22 primers. Furthermore, there were at least 23 (77%) sera from normal individuals among the 30 sera tested in which TTV DNA was negative by PCR with N22 primers, but nevertheless was positive by PCR with UTR primers. Overall, TTV DNA was detected in 28 of the 30 (93%) healthy individuals in Japan, thereby indicating a deep and wide penetration of TTV having various genotypes into the community. Such a high prevalence rate would be due to dual-transmission modes of TTV, parenterally and by a fecal–oral route (Okamoto *et al.,* 1998a,b).

It should be pointed out, also, that mixed infection with TTV of distinct genotypes is common. Of the 10 healthy Japanese individuals infected with TTV of genotype 1a, at least 3 (30%) had mixed infection with TTV of two or three genotypes. Mushahwar *et al.* (1999) reported a similarly high rate of mixed TTV infection. It is not certain whether the carriers of TTV strains of distinct genotypes were coinfected with the strains at the outset or whether they

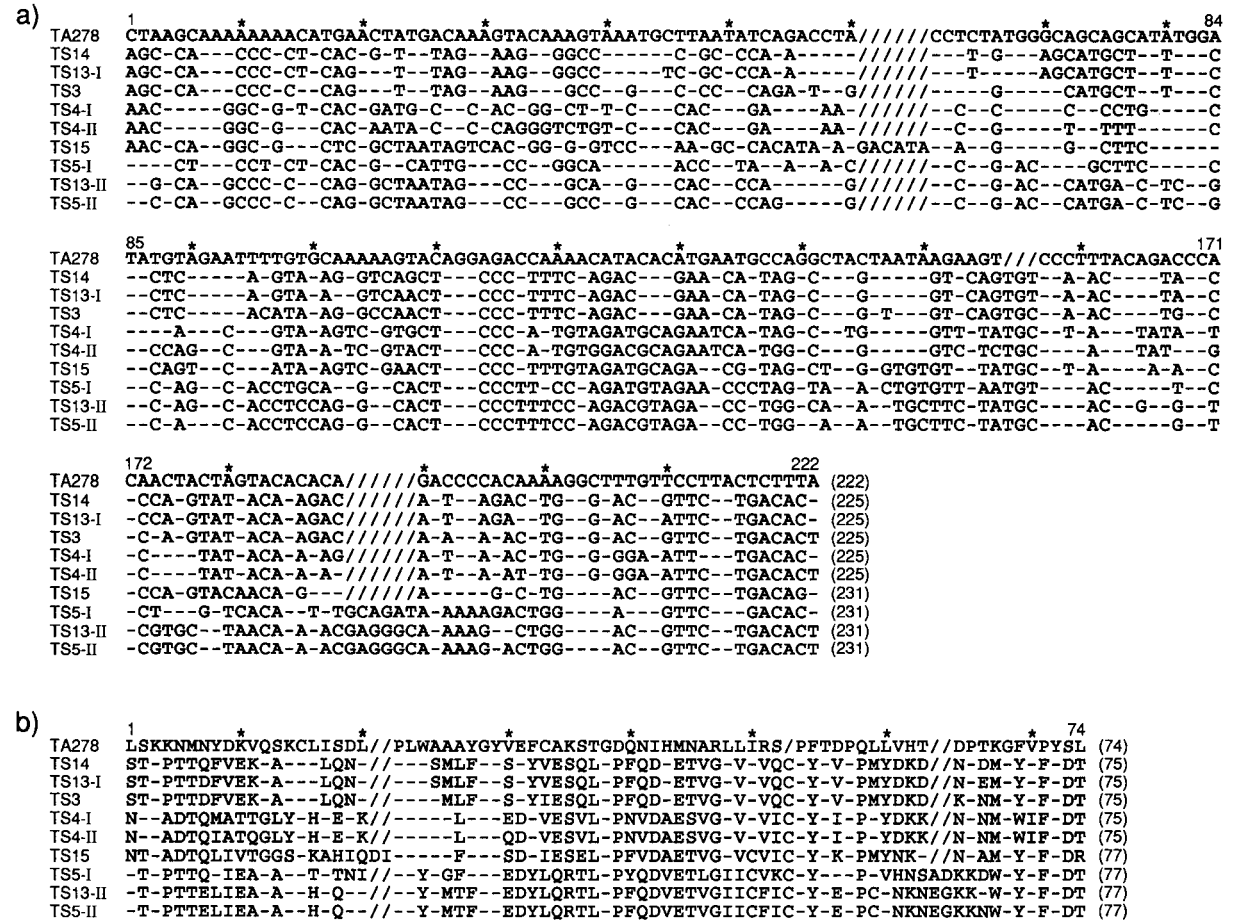

FIG. 2. Nucleotide (a) and amino acid (b) sequences of nine TTV variants classifiable into five distinct groups. Nine TTV isolates obtained from six sera (Table 4) were sequenced for 225 or 231 bp corresponding to the product of PCR with NG061/NG063 (primer sequences at both ends excluded). They were classified into five groups with divergence of >30% in the nucleotide sequence. Two different TTV isolates recovered from the same sample are distinguished by Roman letters (I and II). The sequence of TA278 of genotype 1a (Okamoto *et al.,* 1998a) is indicated at the top. Dashes represent the same nucleotides or amino acids as in the TA278 isolate, and slashes represent deletion of nucleotides or amino acids. The total number of nucleotides or amino acids is indicated in parentheses after each representative isolate.

were superinfected later in their lives. Taken along with a high prevalence in the general population, the infection with TTV may not confer protection against reinfection, especially with TTV strains of different genotypes.

A high prevalence (>90%) of ongoing infection with TTV in the general population compares with that of cytomegalovirus (Larsson *et al.,* 1998) and a high rate of exposure to Epstein–Barr virus (Evans, 1974), both of which induce hepatitis in a fraction of infected individuals (Edwards *et al.,* 1978; Hanshaw *et al.,* 1965).

Animal viruses have a wide range of sequence divergence by which they are classified into various genotypes. For instances, human papillomaviruses, enteroviruses, and adenoviruses have many genotypes or serotypes, which induce various forms of clinical diseases. In particular, human papillomaviruses have >70 types, of which types 16, 18, 31, and 45 are found in close association with cervical carcinoma of the uterus (Howley, 1996). Likewise, of the 47 human adenovirus serotypes so far identified, types 1, 2, and 5 are known to be related to hepatitis (Horowitz, 1996).

An association of TTV genotypes, detectable by PCR with N22 primers, with hepatitis of unknown etiology (Okamoto *et al.,* 1998a; Charlton *et al.,* 1998; Fujiwara *et al.,* 1998) suggests that TTV of only certain genotypes would be important clinically. Now that a wide range of sequence divergence of TTV is known, dispersing into at least 16 genotypes, future attempts should be directed to the development of PCR methods with genotype-specific primers deduced from the coding region, as has been accomplished for HCV (Chayama *et al.,* 1993; Okamoto *et al.,* 1992). Such efforts are hoped to disclose a capacity of TTV dependent on genotypes, in inducing hepatic as well as extrahepatic diseases, which would need to be put into a medical perspective.

MATERIALS AND METHODS

Extraction of nucleic acids

Plasma or serum (50–200 μl) was heated at 70°C for 2 h in Tris–HCl buffer (10 mM, pH 8.0) containing 0.5 mg/ml proteinase K, 0.5% (wt/vol) SDS, and 5 mM EDTA,

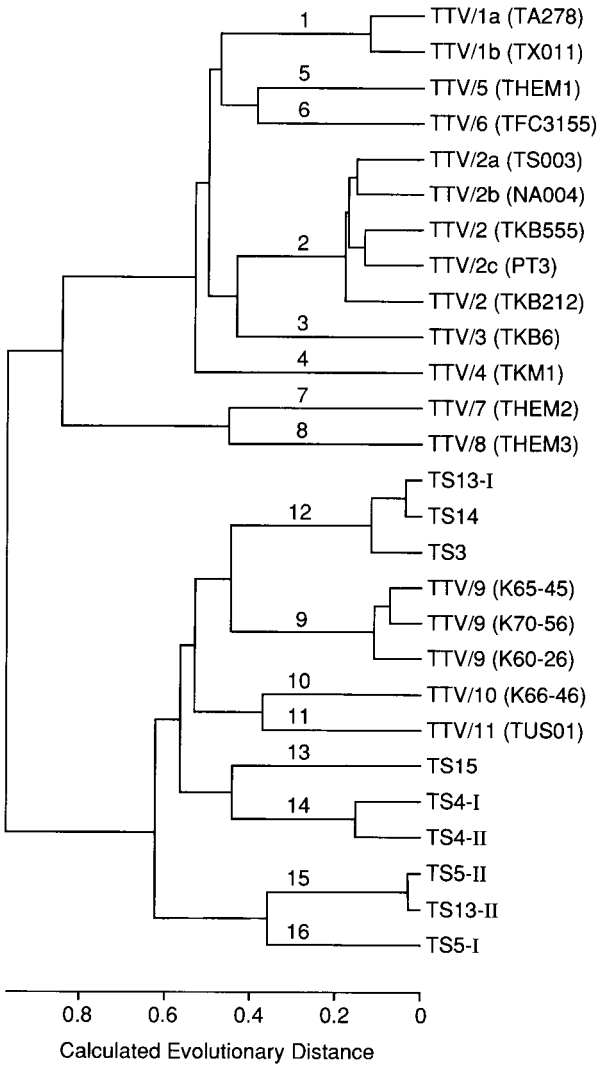

FIG. 3. Phylogenetic analysis of TTV isolates. A phylogenetic tree was constructed for 225- or 231-bp fragments from the 9 TTV variants in Fig. 2 and the 18 TTV isolates of genotypes 1–11 reported.

and nucleic acids were extracted with phenol/chloroform by the method reported previously (Okamoto *et al.,* 1990b). The extracted nucleic acids were dissolved in 20–100 μl of Tris–HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA.

Preparation of a TTV DNA clone (TRM1)

TTV DNA extracted from feces (Okamoto *et al.,* 1998b) was subjected to PCR with primer pair NG085/NG009 (Table 1), and products of 3254 bp were obtained. The products were inserted into plasmid vector and a TTV clone named TRM1 of genotype 1a was obtained. Closed circular DNA of the recombinant plasmid (pTV-TRM1-1) was recovered by equilibrium ultracentrifugation in a gradient of CsCl and ethidium bromide (Sambrook *et al.,* 1989) and dissolved in Tris–HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA.

Amplification by PCR

Amplification was performed with the primers specified in Table 1 by the following three methods.

(1) With the use of primers deduced from the prototype TTV isolate (TA278), for which a sequence of 3739 nt has been determined (Okamoto *et al.,* 1998a), 11 versions of one-stage PCR and 4 versions of two-stage PCR were developed (Table 2). Nucleic acids were extracted from sera (400 μl) of 30 healthy Japanese individuals, and using its portion (corresponding to 25 μl of serum) as a template and along with Perkin–Elmer AmpliTaq Gold (Roche Molecular Systems, Inc., Branchburg, NJ), amplification was performed for 55 cycles in one-stage PCR or 35 cycles for the first round and 25 cycles for the second round in two-stage PCR [95°C for 30 s (plus 9 min in the first cycle); 58 or 60°C for 30 s; 72°C for 40–60 s (plus 7 min in the last cycle)]. Annealing temperature and extension time were adjusted for individual primers as indicated in Table 2. The PCR products were run on electrophoresis in 2–3% (wt/vol) NuSieve 3:1 agarose (FMC BioProducts, Rockland, ME), stained with ethidium bromide, and photographed under ultraviolet light.

(2) A long-distance PCR was performed for the amplification of 3.4-kb products with nested primers, with sense deduced from UTR (A) and antisense from UTR (B), in the presence of TaKaRa Ex *Taq* (TaKaRa Shuzo, Shiga, Japan) as indicated in Fig. 1. The first round of PCR was performed for 35 cycles and the second round for 25 cycles [95°C for 30 s (plus 2 min in the first cycle); 60°C for 30 s; 72°C for 150 s (plus 7 min in the last cycle)].

(3) A cloned TTV DNA (pTV-TRM1-1) of 3254 bp (nt 12–3265) and a reported clone (T7) from TA278 of 614 bp (nt 3126–3739) (Okamoto *et al.,* 1998a) were amplified by two-stage or one-stage PCR with primer pairs deduced from UTR or N22 region indicated in Table 3 and target regions depicted in Fig. 1. PCR was performed with Perkin–Elmer AmpliTaq Gold and under conditions specified under item 1 in this section and in the footnote to Table 2.

Cloned TTV DNAs were tested at 1, 10, and 100 copies per tube. For the titration of TTV DNA in serum, extracted nucleic acids were serially diluted 10-fold, and the highest dilution (10^N/ml) testing positive by PCR was determined (Table 4).

Determination of nucleotide sequence

Amplification products were separated by agarose gel electrophoresis and ligated to pT7BlueT-Vector (Novagen Inc., Madison, WI). Both strands were sequenced employing plasmid DNA extracted from transformed *Escherichia coli,* and a ThermoSequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Buckinghamshire, England). Sequence was determined for three clones each and the consensus sequence was adopted. For 3.4-kb

TABLE 5

Comparison between the Representative TTV Isolates of Genotypes 1–16 and Reported Isolates within a Sequence of 222–231 nt in the N22 Region[a]

Genotype	Reference isolate	Accession No.	Same genotype		Other genotypes	
			No. of isolates	Nucleotide similarity	No. of isolates	Nucleotide similarity
1	TA278	AB008394	303 (62.9%)	84.9–100%	179	<70%
2	TS003	AB017770	144 (29.9%)	75.8–100%	338	<71%
3	TKB6	AB017774	26 (5.4%)	80.1–99.6%	456	<71%
4	TKM1	AB017775	2 (0.4%)	95.9–97.3%	480	<67%
5	THEM1	AB017776	0		482	<69%
6	TFC3155	AB017777	4 (0.8%)	89.2–97.2%	478	<70%
7	THEM2	AB017778	0		482	<61%
8	THEM3	AB017779	3 (0.6%)	77.8–91.9%	479	<55%
9	K70-56	AB017781	0		482	<56%
10	K66-46	AB017783	0		482	<60%
11	TUS01	AB017613	0		482	<61%
12	TS3	AB021081	0		482	<61%
13	TS15	AB021089	0		482	<60%
14	TS4-I	AB021082	0		482	<61%
15	TS5-II	AB021085	0		482	<59%
16	TS5-I	AB021084	0		482	<61%

[a] The sequence of 222–231 nt is not available for TX011(A) isolate (Accession No. AB011492) for genotyping, and therefore, it was excluded from comparison.

products of long-distance PCR, the sequence amplifiable with primers NG061/NG063, spanning ~300 bp in the N22 region, was determined.

Computer analysis of TTV sequences

Sequence analysis of TTV strains was performed with Genetyx-Mac version 10.1 (Software Development Co., Tokyo, Japan) and ODEN program version 1.1.1 (Ina, 1994) of DDBJ (National Institute of Genetics, Mishima, Japan). An evolutionary distance was estimated by the 6-parameter method (Gojobori *et al.,* 1982), and phylogenetic trees were constructed by the unweighted pair-group method with arithmetic mean (Nei, 1987) and the neighbor-joining method (Saitou and Nei, 1987).

REFERENCES

Bukh, J., Purcell, R. H., and Miller, R. H. (1992). Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. *Proc. Natl. Acad. Sci. USA* **89,** 187–191.

Charlton, M., Adjei, P., Poterucha, J., Zien, N., Moore, B., Therneau, T., Krom, R., and Wiesner, R. (1998). TT-virus infection in North American blood donors, patients with fulminant hepatic failure, and cryptogenic cirrhosis. *Hepatology* **28,** 839–842.

Chayama, K., Tsubota, A., Arase, Y., Saitoh, S., Koida, I., Ikeda, K., Matsumoto, T., Kobayashi, M., Iwasaki, S., Koyama, S., Morinaga, T., and Kumada, H. (1993). Genotypic subtyping of hepatitis C virus. *J. Gastroenterol. Hepatol.* **8,** 150–156.

Edwards, J. M., Vandervelde, E. M., Cohen, B. J., and McSwiggan, D. A. (1978). Laboratory diagnosis of EB virus infection in some cases presenting as hepatitis. *J. Clin. Pathol.* **31,** 179–182.

Evans, A. S. (1974). New discoveries in infectious mononucleosis. *Mod. Med.* **135,** 18–24.

Fujiwara, T., Iwata, A., Iizuka, H., Tanaka, T., and Okamoto, H. (1998). Transfusion transmitted virus. *Lancet* **352,** 1310. [Correspondence]

Gojobori, T., Ishii, K., and Nei, M. (1982). Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J. Mol. Evol.* **18,** 414–423.

Hanshaw, J. B., Betts, R. E., Simon, G., and Boynton, R. C. (1965). Acquired cytomegalovirus infection association with hepatomegaly and abnormal liver function tests. *N. Engl. J. Med.* **272,** 602–609.

Horowitz, M. S. (1996). Adenoviruses. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), 3rd ed., pp. 2149–2171. Lippincott–Raven, Philadelphia.

Howley, P. M. (1996). *Papillomavirinae:* The viruses and their replication. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), 3rd ed., pp. 2045–2076. Lippincott–Raven, Philadelphia.

Ina, Y. (1994). ODEN: A program package for molecular evolutionary analysis and database search of DNA and amino acid sequences. *Comput. Appl. Biosci.* **10,** 11–12.

Larsson, S., Soderberg-Naucler, C., Wang, F. Z., and Moller, E. (1998). Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. *Transfusion* **38,** 271–278.

Lisitsyn, N., Lisitsyn, N., and Wigler, M. (1993). Cloning the differences between two complex genomes. *Science* **259,** 946–951.

Lukert, P. D., de Boer, G. F., Dale, J. L., Keese, P., McNulty, M. S., Randles, J. W., and Tisher, I. (1995). Family *Circoviridae*. *In* "Virus Taxonomy: Classification and Nomenclature of Viruses, Sixth Report of the Intentional Committee on Taxonomy of Viruses" (F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers, Eds.), pp. 166–168. Springer-Verlag, New York.

Mushahwar, I. K., Erker, J. C., Muerhoff, A. S., Leary, T. P., Simons, J. N., Birkenmeyer, L. G., Chalmers, M. L., Pilot-Matias, T. J., and Desai, S. M. (1999). Molecular and biophysical characterization of TT virus: Evidence for a new virus family infecting humans. *Proc. Natl. Acad. Sci. USA* **96,** 3177–3182.

Naoumov, N. V., Petrova, E. P., Thomas, M. G., and Williams, R. (1998). Presence of a newly described human DNA virus (TTV) in patients with liver disease. *Lancet* **352,** 195–197.

Nei, M. (1987). Phylogentic trees. *In* "Molecular Evolutionary Genetics" (M. Nei, Ed.), pp. 287–326. Columbia Univ. Press, New York.

Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., and Mayumi, M. (1997). A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem. Biophys. Res. Commun.* **241,** 92–97.

Okamoto, H., Okada, S., Sugiyama, Y., Tanaka, T., Sugai, Y., Akahane, Y., Machida, A., Mishiro, S., Yoshizawa, H., Miyakawa, Y., and Mayumi, M. (1990a). Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5′-noncoding region. *Jpn. J. Exp. Med.* **60,** 215–222.

Okamoto, H., Yotsumoto, S., Akahane, Y., Yamanaka, T., Miyazaki, Y., Sugai, Y., Tsuda, F., Tanaka, T., Miyakawa, Y., and Mayumi, M. (1990b). Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J. Virol.* **64,** 1298–1303.

Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1992). Typing hepatitis C virus by polymerase chain reaction with type-specific primers: Application to clinical surveys and tracing infectious sources. *J. Gen. Virol.* **73,** 673–679.

Okamoto, H., Nishizawa, T., Kato, N., Ukita, M., Ikeda, H., Iizuka, H., Miyakawa, Y., and Mayumi, M. (1998a). Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. *Hepatol. Res.* **10,** 1–16.

Okamoto, H., Akahane, Y., Ukita, M., Fukuda, M., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1998b). Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A–G hepatitis. *J. Med. Virol.* **56,** 128–132.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for constructing phylogenetic trees. *Mol. Biol. Evol.* **4,** 406–425.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Simmonds, P., Davidson, F., Lycett, C., Prescott, L. E., MacDonald, D. M., Ellender, J., Yap, P. L., Ludlam, C. A., Haydon, G. H., Gillon, J., and Jarvis, L. M. (1998). Detection of a novel DNA virus (TTV) in blood donors and blood products. *Lancet* **352,** 191–195.

Takahashi, K., Hoshino, H., Ohta, Y., Yoshida, N., and Mishiro, S. (1998). Very high prevalence of TT virus (TTV) infection in general population of Japan revealed by a new set of PCR primers. *Hepatol. Res.* **12,** 233–239.

Takayama, S., Yamazaki, S., Matsuo, S., and Sugii, S. (1999). Multiple infection of TT virus (TTV) with different genotypes in Japanese hemophiliacs. *Biochem. Biophys. Res. Commun.* **256,** 208–211.

Tanaka, H., Okamoto, H., Luengrojanakul, P., Chainuvati, T., Tsuda, F., Tanaka, T., Miyakawa, Y., and Mayumi, M. (1998a). Infection with an unenveloped DNA virus (TTV) associated with posttransfusion non-A to G hepatitis in hepatitis patients and healthy blood donors in Thailand. *J. Med. Virol.* **56,** 234–238.

Tanaka, Y., Mizokami, M., Orito, E., Ohno, T., Nakano, T., Kato, T., Kato, H., Mukaide, M., Park, Y. M., Kim, B. S., and Ueda, R. (1998b). New genotypes of TT virus (TTV) and a genotyping assay based on restriction fragment length polymorphism. *FEBS Lett.* **437,** 201–206.

Viazov, S., Ross, R. S., Niel, C. de Oliveira, J. M., Varenholz, C., Da Villa, G., and Roggendorf, M. (1998). Sequence variability in the putative coding region of TT virus: Evidence for two rather than several major types. *J. Gen. Virol.* **79,** 3085–3089.