

# Molecular characterization of human Torque Teno virus

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**Abstract.** The present study analyzed the presence of human Torque Teno virus (TTV) in hospitalized patients from different departments. In total, 378 serum specimens were collected from the patients (171 with cardiovascular disease, 192 with tumor and 15 with gastroenteritis) and analyzed by ELISA and nest-polymerase chain reaction (PCR) to detect the presence of TTV. The results showed that 64 specimens (17%) were TTV positive from detection with the human ELISA kit, and the patients aged <30 years have a higher prevalence. TTV in males was more common than in female patients. In addition, nest-PCR was used to detect TTV within different phylogenetic groups among the 64 specimens, and the results showed that groups 1 (TA278 strain), 4 (KC009) and 5 (CT39) were much more prevalent than groups 2 (PMV isolate) and 3 (11 genotypes) in the different departmental patients.

## Introduction

Torque Teno virus (TTV) is a small, non-enveloped, single-stranded circular DNA virus that was first classified as a member of the family circoviridae, genus *Anellovirus*. TTV was first discovered in 1997 in Japan from the serum of a patient with acute post-transfusion hepatitis of unknown etiology (1). In addition to humans, TTV can also be detected from non-human primates (2-4), domestic animals (porcine, avian, bovine and ovine) (5,6), companion animals (feline and canine) (7), wild animals (wild boar and camels) (8,9) and marine animals (sea lions and sea turtle) (10,11). Thus, in 2009, TTV was classified by the International Committee on Taxonomy of Viruses into the family Anelloviridae, which contains 9 genera (from *Alphatorquevirus* to *Zetatorquevirus*).

TTV is widespread worldwide and ≥80% of healthy adults have persistent viraemia (12). A number of previous studies have reported that the prevalence of TTV in children was

10-40% (13,14). The infection and replication mechanisms and the pathogenicity of TTV remain unknown.

The genome of TTV has a range of 2.1-3.9 kb in length and contains 3 or 4 overlapping open reading frames (ORFs), as well as a short stretch of untranslated region with high GC content. Although TTV is a DNA virus, the TTV sequence exhibits a wide range of sequence divergence, particularly in human isolates. Based on the genetic diversity, TTV strains have been classified into 5 distinct phylogenetic groups (groups 1-5) (15). Additionally, certain novel TTV variants, which are widely distributed in China (16), have not yet been classified into genomic groups. Group 1 is represented by the TA278 strain of genotype 1 and group 2 is represented by the PMV isolate (17). Group 3 is composed of 11 genotypes and includes SANBAN, TUS01 and TYM9 isolates (18,19). Genogroups 1 and 3 are the most widespread, followed by 4 (KC009) and 5 (CT39), while genogroup 2 viruses are less common (20).

In the present study, in order to characterize the infection status of human TTV in China, serum samples were investigated and collected from hospitalized patients with cardiovascular disease, tumor or gastroenteritis, using ELISA and polymerase chain reaction (PCR) assays.

## Materials and methods

**Samples.** A total of 378 blood specimens were collected from the hospitalized patients, aged from 19 to 89 years, during the period between August and December in 2012, in Jiangxi, China. All 378 patients suffered from cardiovascular disease (171/378), tumor (192/378) or gastroenteritis (15/378) at the sampling time. No patients had a history of transfusions. Samples were centrifuged at 1,000 × g for 20 min, and supernatant serum aliquots were collected and stored at -80°C until the testing was performed.

**ELISA detection.** TTV antigen (Ag) was determined using a commercial ELISA kit (Huitebi Technology Development Co., Ltd., Beijing, China) according to the manufacturer's instructions. The ELISA plate was coated with purified TTV antibody to capture the TTV virus from the detected serum and screened by the horseradish peroxidase-tagged TTV antibody (catalogue number, 2R109). The kit contained positive and negative controls, and the cut-off values for the results assay were determined based on 0.15 plus the mean optical density 450 values of the negative control samples.

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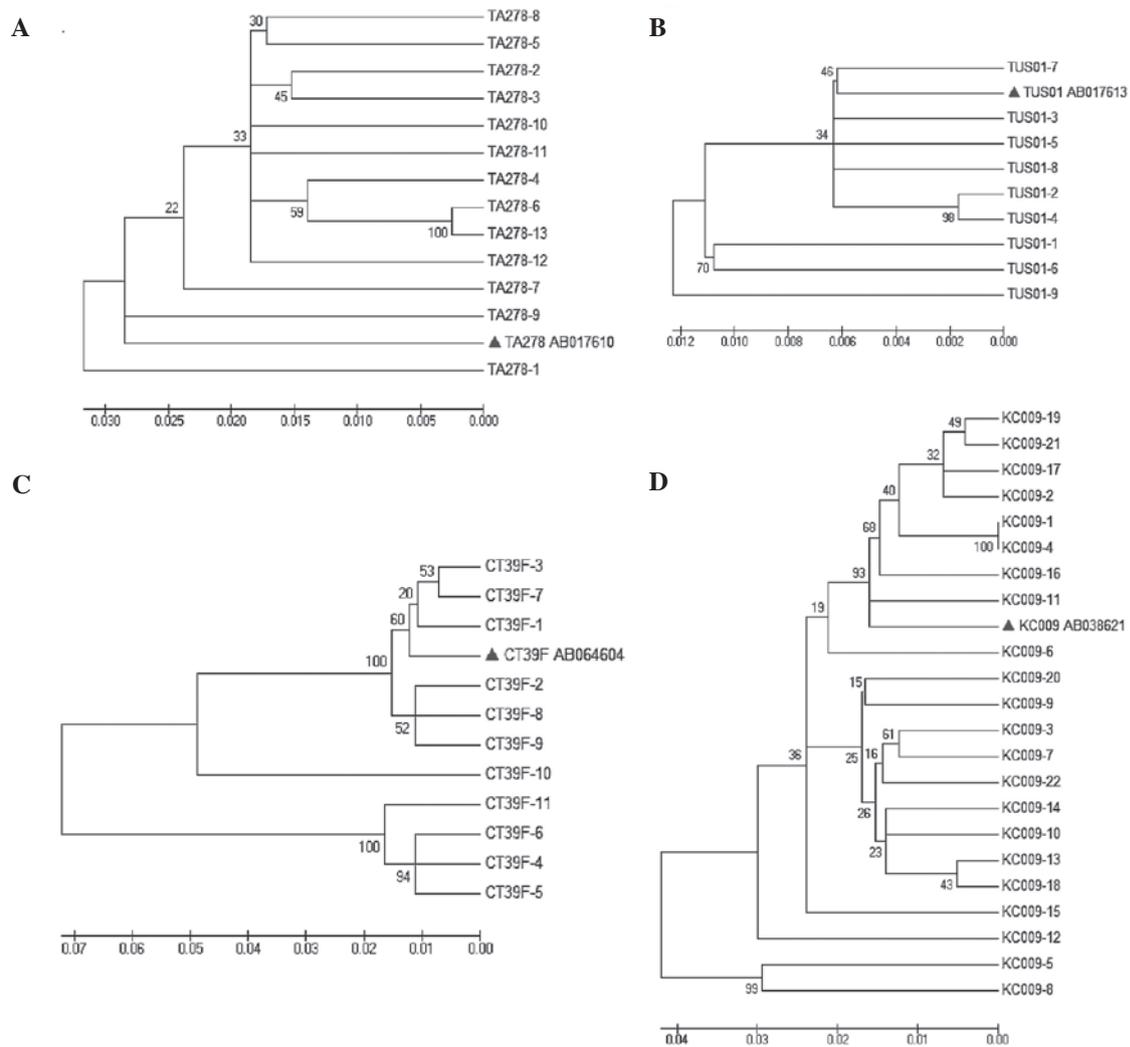


Figure 1. Phylogenetic tree constructed using the neighbor-joining method and evaluated using the interior branch test method with MEGA 4 software. Percent bootstrap support is indicated at each node. GenBank accession number, source are also indicated. The solid triangles indicate reference strains. Trees A, B, C and D were constructed based on part of the nucleotide sequence of TTV genotypes TA278, TUS01, CT39F and KC009, which stands for genogroups 1, 3, 5 and 4 of human TTV, respectively.

**DNA extraction.** Viral DNA was extracted from 100  $\mu$ l serum samples using the MiniBEST Viral RNA/DNA Extraction kit version 4.0 (Takara, Dalian, China), according to the manufacturer's instructions.

**PCR detection of TTV DNA.** All the positive samples detected by ELISA were tested via nested PCR using specific primers corresponding to the 5 TTV genogroups (groups 1-5) (Table I), and the reference sequences were as follows: Group 1: TA278 (accession no. AB017610) (21), group 2: PMV (AF261761) (22), group 3: TUS01 (AB017613) (23), group 4: KC009 (24) (AB038621) and group 5: CT39F (AB064604) (25). All PCR reactions were carried out as follows: 32 cycles of denaturation at 94°C for 40 sec with an additional 7 min in the first cycle, annealing at 55°C for 40 sec, extension at 72°C for 70 sec and with an additional 7 min in the last cycle. The amplification products were excised from 1% agarose gels containing ethidium bromide (0.5  $\mu$ g/ml), purified with the AxyPrep DNA Gel Extraction kit (Axygen Biotechnology Co., Ltd., Silicon Valley, CA, USA), cloned into the pMD-18T vector (Takara) and sequenced (Takara).

**Phylogenetic analysis.** The sequences of the TTV isolates in the present study were analyzed using the MegAlign software (DNASTar Inc., Madison, WI, USA). Phylogenetic trees were constructed by the alignment of the TTV isolates in the study and the referenced strains (GenBank number and source of regions are shown in Fig. 1). They were evaluated using the neighbor-joining method with 1,000 bootstrap replicates in a heuristic search with the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.0; Oxford University Press, New York, NY, USA).

## Results

**Detection of TTV by ELISA.** A total of 378 patient serum specimens were collected from different departments (171 with cardiovascular disease, 192 with tumor and 15 with gastroenteritis) and were divided into 3 groups according to age. TTV was detected by the human ELISA detection kit following the manufacturer's instructions and 64 specimens were positive for TTV. The prevalence of TTV was 14.0% (24/171), 18.8% (36/192) and 26.7% (4/15) in cardiovascular, tumor

Table I. Primers employed in the analysis of Torque Teno virus.

Group	Polarity	Nucleotide position	Nucleotide sequence
1 (TA278)	Sense-1	867-886	5'-ATG TGG CGA AAA TAC TGT CA-3'
	Antisense-1	1903-1923	5'-CAT GTT GCC TTC TCC TCT GTC-3'
	Sense-2	1102-1125	5'-ATT AAT ACC ATG CCT CCT TTT CTA-3'
	Antisense-2	1903-1923	5'-CAT GTT GCC TTC TCC TCT GTC-3'
2 (PMV)	Sense-1	107-126	5'-TGA GTT TTC CAC GCC CGT CC-3'
	Antisense-1	1424-1433	5'-ATC CGG CGG TTA TAC CAG TA-3'
	Sense-2	473-492	5'-CAG TGT GGC GGC TCG TGT TG-3'
	Antisense-2	1364-1384	5'-TTG TGG TGA GCA GAA CGG AAA-3'
3 (TUS01)	Sense-1	322-341	5'-GAA GGC ACC TGC CAT GAG CT-3'
	Antisense-1	1936-1956	5'-GCC TTT GCC CTT GTC CAT TAG-3'
	Sense-2	477-496	5'-TGC CTG CTA CCT CTT CGC CT-3'
	Antisense-2	1358-1378	5'-AGC AGA ACG GAT ACC GCA AGT-3'
4 (KC009)	Sense-1	137-158	5'-AGG CCA ATG AGG ATC TTC TAC G-3'
	Antisense-1	982-1001	5'-GGG AGG GAA GTC GTC CAT GT-3'
	Sense-2	360-381	5'-TTA AAA AAT TCC CCC GCT CTG T-3'
	Antisense-2	918-937	5'-AGG GGC ATC CAT CCT GTA AT-3'
5 (CT39)	Sense-1	106-125	5'-GAG TTT ATG CTG CCC GTC CG-3'
	Antisense-1	722-742	5'-TCC ACC TCC TCC GCC TCC TTA-3'
	Sense-2	191-210	5'-CGC AGT CAA GGG GCA ATT CG-3'
	Antisense-2	671-690	5'-TCT AGG CCA TCG TCT GCG AA-3'

Table II. ELISA detection result of Torque Teno virus in cardiovascular, tumor and gastroenteritis patients.

Patients	Age, years	Infection rate			Total rate for patients
		Male	Female	Total	
Cardiovascular disease (n=171)	≤30	33.3 (1/3)	66.7 (2/3)	50.0 (3/6)	14.0 (24/171)
	31-60	18.5 (5/27)	12.0 (3/25)	15.4 (8/52)	
	≥60	20.0 (10/50)	4.7 (3/63)	11.5 (13/113)	
Tumor (n=192)	≤30	100.0 (1/1)	0.0 (0/2)	33.3 (1/3)	18.8 (36/192)
	31-60	4.7 (2/43)	15.7 (11/70)	11.5 (13/113)	
	≥60	30.9 (13/42)	26.5 (9/34)	28.9 (22/76)	
Gastroenteritis (n=15)	≤30	0.0 (0/0)	0.0 (0/0)	0.0 (0/0)	26.7 (4/15)
	31-60	33.3 (2/6)	0.0 (0/1)	28.6 (2/7)	
	≥60	33.3 (2/6)	0.0 (0/2)	25.0 (2/8)	
Total rate for gender	No.	20.2 (36/178)	14.0 (28/200)	No.	

Data are % (detected/total).

and gastroenteritis patients, respectively. The patients aged <30 years had a higher prevalence in cardiovascular (50.0%, 3/6) and tumor patients (33.3%, 1/3) and TTV in males, 20.2% (36/178), was more common compared with female patients, 14.0% (28/200) (Table II).

**PCR detection of TTV DNA.** According to the result of ELISA, 64 patients (24 with cardiovascular disease, 36 with tumors and 4 with gastroenteritis) were detected to be carrying TTV. In order to know the most prominent genogroup, 64 serum

specimens were analyzed by nest-PCR with group-specific primer sets. The results showed that the positive samples were 48 among the 64 samples (56.3%), and in the 24 cardiovascular disease patients, the infection rate of TTV groups 1-5 were 20.8% (5/24), 0.0% (0/24), 16.7% (4/24), 20.8% (5/24) and 8.3% (2/24), respectively (Table III). A total of 4 patients had co-infection of groups 3 and 4, or groups 3 and 5, or groups 1 and 5, or groups 1, 3 and 4 (Table IV). The prevalence in the 36 tumor patients were 22.2% (8/36), 0.0% (0/36), 13.9% (5/36), 41.2% (15/36) and 19.4% (7/36), respectively.

Table III. Frequency of Torque Teno virus detected by nest-polymerase chain reaction assay in cardiovascular, tumor and gastroenteritis patients.

Patients	Group 1	Group 2	Group 3	Group 4	Group 5
Cardiovascular disease (n=24)	20.8 (5/24)	0.0 (0/24)	16.7 (4/24)	20.8 (5/24)	8.3 (2/24)
Tumor (n=36)	22.2 (8/36)	0.0 (0/36)	13.9 (5/36)	41.7 (15/36)	19.4 (7/36)
Gastroenteritis (n=4)	0.0 (0/4)	0.0 (0/4)	0.0 (0/4)	50.0 (2/4)	50.0 (2/4)
Total (n=64)	21.7 (13/60)	0.0 (0/60)	15.0 (9/60)	34.4 (22/64)	17.2 (11/64)

Data are % (detected/total).

Table IV. Torque Teno virus infection from different groups in cardiovascular, tumor and gastroenteritis patients.

Group	Patient no.	Gender	Age, years	Groups
Tumor	2	F	65	1,4,5
	4	F	31	N
	5	F	72	1,4,5
	6	F	38	1,5
	8	F	43	N
	9	M	66	3,4
	10	M	63	1,5
	11	F	71	4
	12	F	37	N
	15	F	56	1
	16	M	85	3
	17	F	80	N
	18	M	63	3
	19	M	62	4
	20	F	71	4,5
	21	M	75	4,5
	23	M	67	N
	25	F	68	N
	29	M	65	1,4
	30	F	31	4,5
	31	M	62	N
	34	M	66	N
	36	F	66	1,4
	39	F	62	4
	43	M	28	5
	45	M	77	4
	47	F	37	4
	48	M	65	3,4
	49	F	55	N
	51	M	58	N
	58	F	59	N
	60	M	65	N
63	F	55	N	
64	M	50	N	
65	F	65	N	
66	F	56	1,3	
Cardiovascular disease	1	M	49	N
	3	F	54	4

Table IV. Continued.

Group	Patient no.	Gender	Age, years	Groups
Tumor	7	F	56	3,4
	13	M	53	3,5
	14	F	54	4
	22	M	69	N
	24	M	60	1
	26	M	47	N
	27	M	21	N
	33	F	64	N
	38	M	76	1,3,4
	40	M	77	1
	44	F	29	N
	46	F	79	4
	50	M	44	N
	52	M	61	1,5
	53	M	69	N
Gastroenteritis	8	M	64	4
	37	M	64	4
	41	M	43	5
	42	M	55	5

M, male; F, female; N, none.

Among the tumor patients, 2 were co-infected with 3 groups (groups 1, 4 and 5), and 10 were co-infected with 2 groups (groups 1 and 3, groups 1 and 4, groups 1 and 5, groups 3 and 4 or groups 4 and 5). However, in gastroenteritis patients, groups 4 and 5 were detected with the prevalence of 50.0% (2/4) (Table IV).

*Sequence analysis of TTVs.* The phylogenetic tree was constructed (Fig. 1) based on the sequences of the isolates and referenced representative strains (GenBank number and

source of regions are shown). In tree A, all the 13 isolates were found to share 92-95% nucleotide homology with the genogroup 1 strain TA278 (AB017610). In tree B, the genogroup 3 strain TUS01 (AB017613) shared 97-99% nucleotide homology with the isolates. The tree C was grouped into 2 clusters, and the 4 isolates (CT39F-4, 5, 6 and 11) were found to share 86-88% nucleotide homology with the referenced genogroup 5 strain CT39F (AB064604), the other isolates were clustered with the strain CT39F and shared 90-98% nucleotide homology with it. In tree D, all the isolates shared 92% nucleotide homology at least with the genogroup 4 strain KC009 (AB038621) besides one isolate, which shared 85-89% nucleotide homology with KC009 strain and other isolates.

## Discussion

Human TTV is globally distributed, and TTV strains have been divided into 5 distinct phylogenetic groups (groups 1-5). PCR is the usual detection method for TTV DNA, and the choice of primers used may significantly influence the level of detection. High rates of infection (60-100%) have been identified among healthy populations worldwide by primers T801 and T935. These were designed in the 3' end of the conserved untranslated region (UTR) (12) that is able to amplify the genomes of a number of TTV genotypes. However, the phylogenetic classifications of TTV isolates based on the 2 most prominently studied regions of the genome (N22 and UTR PCR regions) are unreliable (26). Therefore, genogroup-specific or genotype-specific primers were designed to detect the prevalence of TTV. Genogroups 1 and 3 are the most widespread, followed by 4 and 5, while genogroup 2 viruses are less common (20).

Thus far, there are few studies regarding TTV detection with different genogroup-specific primers. Devalle and Niel (27) used the oligonucleotide primers T1S (sense) and T1A (antisense) designed at the 3' and 5' ends of the conserved UTR for the first cycle to detect and differentiate TTV isolates belonging to each of the 5 genomic groups (groups 1-5). In the second cycle of PCR, T2S (sense), immediately downstream of T1S, was designed in the UTR. The 5 PCR assays differed by their internal antisense primers, T2G1A, T2G2A, T2G3A, T2G4A and T2G5A, which were designed to be specific for TTV genomic groups 1-5, respectively. The results showed that TTV DNA from  $\geq 1$  genomic group was detected in 11 (46%) blood donors, 13 (54%) hepatitis B virus (HBV) carriers and 24 (100%) human immunodeficiency virus-1 (HIV-1)-infected patients. The genomic group 5 TTV was the most prevalent (46%, 33/72), followed by group 3 (43%, 31/72), group 1 (35%, 25/72), group 2 (18%, 13/72) and group 4 (17%, 12/72), and the prevalence of TTV in HIV-1 patients was higher compared to HBV carriers and blood donors. Through aligned submitted full-length TTV nucleotide sequences from GenBank, Biagini *et al* (28) designed the specific primer sets (TTG1S1/R1, TTG1S2/R2- TTG5S1/R1, TTG5S1/R1) for each of the representative phylogenetic groups to detect TTV from plasma samples. The above results indicated that the overall prevalence value for TTV DNA totaled 48%, and TTV belonging to group 1 was the most frequently detected (34%), followed by group 3 (24%,

TUPB prototype) and group 5 (12%, JT33F prototype). By contrast, viruses belonging to group 2 (2%, KAV prototype) and group 4 (2%, JT41F prototype) were only detected occasionally.

In the present study, a total of 378 patient serum specimens were collected from different departments (171 cardiovascular disease patients, 192 tumor patients and 15 gastroenteritis patients) and 64 specimens (17%) were positive for TTV, as detected by the human ELISA detection kit. The prevalence of TTV was 14.0% (24/171), 18.8% (36/192) and 26.7% (4/15) in cardiovascular, tumor and gastroenteritis patients, respectively. The patients aged <30 years have a higher prevalence in cardiovascular (50.0%, 3/6) and tumor patients (33.3%, 1/3), and TTV in males, 20.2% (36/178), was more common compared to female patients, 14.0% (28/200).

Although the ELISA kit can detect TTV Ag, the genogroups are not clear, and in order to know the most prominent genogroup, 64 serum specimens were analyzed by nest-PCR with group-specific primers sets. The results showed that the positive samples were 48 among the 64 samples (56.3%), but not 100%, which confirmed that certain other genogroups may exist, or all the genotypes in one genogroup cannot be detected by the primers.

However, the genogroups were still analyzed in the positive samples. In the 24 patients with cardiovascular disease, groups 1 and 4 were most prevalent with the infection of 20.8% (5/24), followed by group 3 at 16.7% (4/24) and group 5 at 8.3% (2/24). In the 36 patients with tumors, group 4 was most prevalent with the infection of 41.2% (15/36), followed by group 1 at 22.2% (8/36), group 5 at 19.4% (7/36) and 13.9% (5/36). However, in the patients with gastroenteritis, only groups 4 and 5 were detected with the prevalence of 50.0% (2/4). The discrepancy between groups may be due to the study of different populations or by methodological differences in the protocols used. No samples belonging to group 2 were detected from all the 64 patients, indicating that group 2 had a low prevalence, which is consistent with the previous study.

The phylogenetic tree was constructed (Fig. 1) based on the sequences of the isolates and referenced representative strains. In tree A, all the 13 isolates were found to share 92-95% nucleotide homology with the genogroup 1 strain TA278 (AB017610). In tree B, the genogroup 3 strain TUS01 (AB017613) shared 97-99% nucleotide homology with the isolates. The tree C was grouped into 2 clusters, and the 4 isolates (CT39F-4, 5, 6 and 11) were found to share 86-88% nucleotide homology with the referenced genogroup 5 strain CT39F (AB064604). The other isolates were clustered with the strain CT39F and shared 90-98% nucleotide homology with it. In tree D, all the isolates shared 92% nucleotide homology at least with the genogroup 4 strain KC009 (AB038621) besides one isolate, which shared 85-89% nucleotide homology with KC009 strain and other isolates. These results confirm that genetic variability rather than geographical variance exists among TTVs in infected humans.

In the present study, different Chinese human TTV isolates were detected and investigated. These findings provide novel insights and foundations for further studies to characterize the territorial presence and prevalence of TTV within China.

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