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RESEARCH ARTICLE

High-throughput sequencing exclusively identified a novel Torque teno virus genotype in serum of a patient with fatal fever

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Torque teno virus (TTV) has been found to be prevalent world-wide in healthy populations and in patients with various diseases, but its etiological role has not yet been determined. Using high-throughput unbiased sequencing to screen for viruses in the serum of a patient with persistent high fever who died of suspected viral infection and prolonged weakness, we identified the complete genome sequence of a TTV (isolate Hebei-1). The genome of TTV-Hebei-1 is 3649 bp in length, encoding four putative open reading frames, and it has a G+C content of 49%. Genomic comparison and a BLASTN search revealed that the assembled genome of TTV-Hebei-1 represented a novel isolate, with a genome sequence that was highly heterologous to the sequences of other reported TTV strains. A phylogenetic tree constructed using the complete genome sequence showed that TTV-Hebei-1 and an uncharacterized Taiwanese strain, TW53A37, constitute a new TTV genotype. The patient was strongly suspected of carrying a viral infection and died eventually without any other possible causes being apparent. No virus other than the novel TTV was identified in his serum sample. Although a direct causal link between the novel TTV genotype infection and the patient's disease could not be confirmed, the findings suggest that surveillance of this novel TTV genotype is necessary and that its role in disease deserves to be explored.

KEYWORDS **Torque teno virus; genome; persistent high fever; high-throughput sequencing**

INTRODUCTION

Torque teno virus (TTV) is a non-enveloped virus with a negative, circular, single-stranded genome, which was first identified in 1997 by Nishizawa when searching for an as-yet-unidentified hepatitis virus in patients with post-transfusion hepatitis (Nishizawa T, et al., 1997). In serum, TTV particles form immune complexes by binding to IgG, but in feces they exist as free viral particles (Itoh Y, et al., 2000). TTV is currently classified into a new, 'floating' genus, *Anellovirus*, of the *Circoviridae* family (Bendinelli M, et al., 2001; Biagini P, et al., 2006). TTVs have been found to display a high degree of genomic variation, which can be as great as 40% (Okamoto H, 2009). The genome sizes of the original isolates were 3,600–3,900 bp; however, Torque teno mini virus (TTMV), identified in 2000, has a genome of 2,800–2,900 bp (Takahashi K, et al., 2000), and Torque teno midi virus (TTMDV) has a genome of 3,175–3,230 bp (Ninomiya M, et al., 2007). In addition to possessing genomes of widely variable length, TTVs have also been found to be prevalent in a range of body fluids and tissues, both in healthy individuals and in patients with a wide variety of pathologies, such as liver diseases,

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respiratory disorders, hematological disorders, cancers and multiple sclerosis (Biagini P, et al., 2001; Mancuso R, et al., 2013; Niel C, et al., 2001; Niel C, et al., 1999; Vasilyev E V, et al., 2009). So far, however, no direct causal relationship between TTV infection and the incidence of disease has been identified, and the pathogenic role of TTV, TTMV and TTMDV infections remains unknown (Okamoto H, 2009).

Here, we report the discovery of a novel TTV-genotype genome revealed through the analysis of unbiased, next-generation sequencing data obtained from a serum sample of a patient with a persistent high fever, who was suspected of having a viral infection and died eventually without any known etiological agents having been identified. Although the pathological role of this newly identified TTV has not yet been confirmed, we contend that the potential role of novel TTV genotypes in pathogenicity deserves to be explored further.

MATERIALS AND METHODS

Sample collection

A 59-year old male patient from Hebei Province, China, was admitted to the Department of Respiratory Disorders, PLA Hospital 307, Beijing, China, with severe pneumonia. The patient's body temperature reached a maximum of 40 °C and this elevated temperature persisted for 15 d, until death. During hospitalization, the patient was treated sequentially with anti-viral drugs, such as ribavirin, and with antibiotics, such as penicillin, piperacillin tazobactam, moxifloxacin hydrochloride, impenem cilastatin sodium, vancomycin hydrochloride and voriconazole; however, none of these treatments either alleviated the pneumonia symptoms or reduced the patient's temperature. Administration of a dexamethasone injection or an indomethacin suppository caused the patient's temperature to normalize transiently, but thereafter the fever returned and the patient's temperature climbed again to 39 °C. The neutrophil ratio and the level of hypersensitive C-reactive protein in the patient's serum were each found to increase. The patient had suffered from infarction for thirteen years, hypertension for ten years and osteonecrosis. In addition to the above underlying diseases, the patient had received blood transfusions on many occasions.

Peripheral blood samples were collected with the consent of the patient and in accordance with the guidelines of the Ethics Committee of the Beijing Institute of Microbiology and Epidemiology. The patient was orally informed that the specimens would be used to screen for viruses and that the laboratory tests listed in the schedule were optional. The anti-agglutination peripheral blood was collected, and serum was separated and stored at -80 °C. Sputum and throat swab samples were collected into Eagle's Minimum Essential Medium and swabs were agitated in the medium for several minutes. Both clinical manifestation and laboratory tests were indicative of infection, especially viral infection, but routine tests for pathogens (bacterial and viral cultures, nucleic acid tests and enzyme-linked immunosorbent assays [ELISAs]) had excluded the possibility of infection by common pathogens, such as hepatitis B and C viruses, human immunodeficiency virus 1 and 2, herpes viruses, influenza A and B viruses, adenovirus, respiratory syncytial virus, human parainfluenza virus, hantavirus, polyomavirus, *Mycobacterium tuberculosis*, and *Mycoplasma pneumoniae*.

Nucleic acid extraction

Serum (0.4 mL) was first mixed with 150 μL phosphate-buffered saline (PBS) solution and the mixture was centrifuged for 5 min at 10,000 rpm. The supernatant was filtrated through a 0.45 μm filter and nucleic acid extraction was undertaken using a MagMAX™ Viral RNA Isolation Kit (Life Technologies). Briefly, 400 μL supernatant was mixed with 802 μL lysis buffer (comprising 400 μL lysis/binding solution, 2 μL carrier RNA and 400 μL 100% *iso*-propanol) and 20 μL magnetic beads mixture (comprising 10 μL RNA-binding beads and 10 μL lysis/binding enhancer), and then agitated for 4 min using a vortex to achieve thorough lysis and nucleic acid binding. The mixture was then placed on the magnetic stack until it became clear. The supernatant was then removed and the pellet was first washed twice with 300 μL Wash Solution I and then twice with 450 μL Wash Solution II. The nucleic acid pellet was finally dissolved in 20 μL DNase- and RNase-free water.

Reverse transcription

Extracted nucleic acid (8 μL) was mixed with 1 μL 10 mmol/L dNTP mixture and 1 μL random hexamers (50 ng/μL, Life Technologies), followed by incubation at 65 °C for 5 min and on ice for 2 min successively. It was then added with 2 μL 10 × reverse transcription buffer, 4 μL 25 mmol/L $MgCl_2$, 2 μL 0.1 mol/L dithiothreitol, 1 μL RNaseOUT™ (40 U/μL, Life Technologies) and 1 μL SuperScript™ III reverse transcriptase (200 U/μL, Life Technologies). The reverse transcription reaction was carried out in a thermocycler (reaction conditions: 25 °C for 10 min, 50 °C for 50 min, 85 °C for 5 min, and then chilled on ice for 2 min). Finally, 1 μL RNase H (2 U/μL, Life Technologies) was added and the reaction mixture was incubated at 37 °C for 20 min.

Multiple displacement amplification

Multiple displacement amplification (MDA) is a non-PCR-based DNA amplification technique that can rapidly amplify minute amounts of DNA samples to a level

sufficient to permit genomic analysis. The MDA reaction was performed as follows. 1 μL reverse transcription product was mixed with 2.5 μL random hexamers modified with thiophosphate (Sangon) at the 3'-ends, 1 μL 10 × phi29 DNA buffer (Thermo Scientific) and 4.3 μL deionized water. The mixture was incubated at 95 °C for 3 min and then chilled on ice for 15 min, followed by being added with 0.5 μL 10 mmol/L dNTP, 0.2 μL 100 × BSA and 0.5 μL phi29 DNA polymerase (Thermo Scientific). The mixture was incubated at 30 °C for 16 h, and then the phi29 DNA polymerase was inactivated by heating at 65 °C for 10 min. The reaction products were separated on agarose gel and the band corresponding to a product > 1000 bp was withdrawn.

Library preparation and genome sequencing

Sequencing of the purified amplification products was performed using an Ion Torrent™ PGM Sequencer (Life Technologies). Adapter-ligated libraries were made using the kit named NEBNext® Fast DNA Library Prep Set for Ion Torrent (New England Biolabs), following the manufacturer's instructions. Briefly, 100 ng purified MDA amplification products were dissolved in deionized water to a total volume of 50 μL and fragmented using a Bioruptor™ Sonication System (Diagenode) to produce a size distribution of 300–400 bp. The sonicated fragments were end-repaired and ligated with Ion Torrent™ adapters P1 and A (Life Technologies). Following electrophoretic separation on 2% agarose gel, the 350–370-bp adapter-ligated fragments were selected using E-Gel™ Size Select (Invitrogen). The selected products were then PCR-amplified using the following regime: 98 °C for 30 s (initial denaturation), followed by 9 cycles of amplification (denaturing at 98 °C for 10 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s) and, finally, extension at 72 °C for 5 min. The concentration of the amplified product was determined using a Qubit™ 2.0 fluorometer (Life Technologies). Prior to sequencing, quality control analyses of the constructed libraries were performed to examine the fragment-size distribution using a Bioanalyzer 2100 instrument (Agilent Technologies). Template preparation was carried out with an Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies) according to the manufacturer's instructions. Briefly, the libraries were diluted to 3 ng/mL and applied to the surface of Ion Sphere™ particles (ISPs) for clonal amplification by emulsion PCR. Emulsion breaking and enrichment were processed subsequently. The quality of the enriched ISPs was estimated using an Ion Sphere™ Quality Control Kit (Life Technologies). An Ion PGM™ Sequencing 300 Kit (Life Technologies) was then used in conjunction with a PGM™ sequencer, according to the kit protocol. Enriched ISPs were loaded onto an Ion 318™ chip and sequenced using the PGM™ instrument for 640 flows.

Bioinformatic analysis

The obtained sequence data were filtered, using the PGM™ software to trim adapter sequences and remove low quality sequences. The filtered data were exported as FastQ format reads (clean reads), which were then assembled into fasta format contigs, using Newbler version 2.5 (Roche). All the large contigs (greater than 500-nt in size) were searched against the NCBI nt database and nr database (ftp://ftp. ncbi.nlm.nih.gov/blast/db/) respectively, using the Basic Local Alignment Search Tool (BLAST) programs BLASTN and TBLASTX, to screen for putative virus sequences.

Phylogenetic analysis

The full genome of TTV-Hebei-1 was subject to NCBI BLASTN analysis against previously-reported TTV genomes. For phylogenetic analysis, we retrieved full-length genome sequences of 74 strains, representing different TTV groups, which had been used in a grouping analysis in 2008 (Ninomiya M, et al., 2008). A phylogenetic tree was constructed with MEGA5.05 (www.megasoftware.net), using the maximum likelihood method. The robustness of the tree was assessed by bootstrap analysis of 1000 replicates, and indicated as a percentage on each branch.

RESULTS

Unbiased deep sequencing revealed a novel Torque teno virus

The PGM™ sequencing generated a loading density of 30%, 1,184,204 raw reads and 251 Mbp in total, of which 226 Mbp had a Phred quality score higher than 20. Prior to quality filtering, the mean read length was 212 bp. After data cleaning and sequence assembly using the Roche Newbler program, 15 large contigs were obtained. BLASTN analysis demonstrated that, of all the large contigs, only one contig of 3649 bp displayed sequence homology with viruses. This contig, with an average coverage of 36 folds, was assembled from 524 reads, comprising 0.05% of the total clean reads. Both the BLASTN and BLASTX analyses showed that this contig shared some sequence identity with Torque teno viruses (Table 1 and Table 2). The BLASTN analysis, however, showed that of the 10 closest matches, 9 covered less than 25% of the length of the query sequence with 86%–92% homology. The best match was an uncharacterized TTV strain, TW53A37, which showed coverage of 83% of the query sequence, with 80% sequence identity. In the BLASTX analysis, 9 of the top 10 matched open reading frames (ORFs) only showed an identity no larger than 45%, and the best matched ORF was again of the

Table 1. The 10 closest matches revealed by the BLASTN analysis of the putative virus contig

Match	Max score	Total score	Query cover	E value	Identity	Accession	Reference
TT virus TW53A37	2226	2226	83%	0	80%	FJ392117	
TT virus P/1C1	704	1102	24%	0	88%	AF298585	
TT virus TJN02	697	969	20%	0	92%	AB028669	(Ukita M, et al., 2000)
TT virus HD13c	691	1040	24%	0	87%	FR848327	(de Villiers E M, et al., 2011)
TT virus HD19	682	969	21%	0	91%	FR751491	(de Villiers E M, et al., 2011)
TT virus HD13b	680	1028	23%	0	88%	FR848326	(de Villiers E M, et al., 2011)
TT virus HD13a	680	1028	24%	0	87%	FR848325	(de Villiers E M, et al., 2011)
TT virus sle1957	671	1028	24%	0	87%	AM711976	(Leppik L, et al., 2007)
TT virus uncultured	664	1078	25%	0	86%	JN231329	(Willner D, et al., 2012)
TT virus sle1932	656	945	23%	0	86%	AM712004	(Leppik L, et al., 2007)

Table 2. The 10 closest matches revealed by the BLASTX analysis of the putative virus contig

Match	Max score	Total score	Query cover	E value	Identity	Accession	Reference
TT virus TW53A37	1028	1028	54%	0	77%	ACR20277	
TT virus tth20	547	547	54%	1.00E-175	45%	CAF05724	(Jelcic I, et al., 2004)
TT virus tth6	544	544	54%	3.00E-174	45%	CAF05718	(Jelcic I, et al., 2004)
TT virus tth18	533	533	54%	3.00E-170	44%	CAF05722	(Jelcic I, et al., 2004)
TT virus tth10	530	530	54%	4.00E-169	44%	CAF05720	(Jelcic I, et al., 2004)
TT virus Pt-TTV6	528	528	49%	4.00E-168	45%	YP_003587828	(Okamoto H, et al., 2000)
TT virus tth21	521	521	54%	1.00E-165	44%	CAF05726	(Jelcic I, et al., 2004)
TT virus tth8	516	516	54%	2.00E-163	42%	CAF05750	(Jelcic I, et al., 2004)
TT virus tth23	514	514	54%	8.00E-163	42%	CAF05760	(Jelcic I, et al., 2004)
TT virus tth19	512	512	54%	9.00E-162	42%	CAF05754	(Jelcic I, et al., 2004)

uncharacterized strain TW53A37, which exhibited 77% amino acid identity. The low percentage values for both amino-acid sequence identity and nucleotide sequence coverage indicated that the newly identified virus was a novel Torque teno virus. It was designated as TTV isolate Hebei-1, since it was identified in a patient from Hebei province, China.

Genomic organization of TTV isolate Hebei-1

The genome of TTV isolate Hebei-1 has a GC content of 50%, and the frequencies of adenine (A), cytosine (C), guanine (G) and thymine (T) are 29%, 26%, 23% and 21%, respectively (Figure 1). The genome of TTV-Hebei-1 contains four putative ORFs (Figure 2). Four potential promoter sites upstream of ORFs were predicted using the Neural Network Promoter Prediction method (http://www.fruitfly.org/seq_tools/promoter.html).

ORF1 was predicted to encode a peptide of 734 amino acids (aa), and a BLASTP search revealed that the amino acid sequence of Hebei-1 ORF1 was closest to that of TW53A37 (ACR20277), isolated from Taiwanese indigenes in 2008 (coverage, 90%; identity, 78%; maximum score, 1604) (Table 3). An aminotransferase class I and II domain (PF00155) was also predicted to be present in ORF1 with CLC Genomics Workbench version 3.6.1 (Qiagen) using the 100 most common domains from the Pfam database (Punta M, et al., 2012). ORF2 was predicted to encode a peptide of 154 aa, and shared the highest homology (coverage, 100%; identity, 48%; maximum score, 118) with the corresponding region of TTV strain TCHN-D1 (AAK11703), isolated in China in 2002, through BLASTP search. A zinc knuckle domain

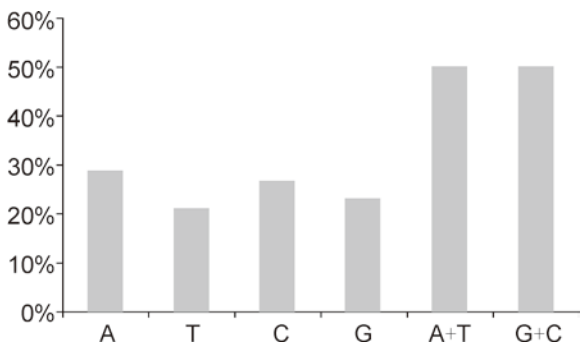

Figure 1. Base composition of the TTV-Hebei-1 genome.

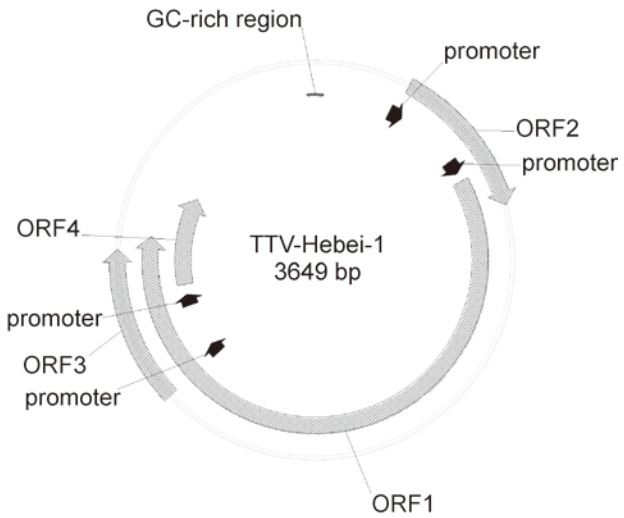

Figure 2. The genomic organization of the TTV-Hebei-1 genome. The circumference of the circle represents the relative size of the genome. The shaded closed arrows represent ORFs (ORF1-ORF4). The black arrow represents the position of the putative promoter and the black line indicates the GC-rich region.

(Accession number: PF00098) and a response regulator receiver domain (Accession number: PF00072) were also predicted to be present within ORF2. ORF3 was predicted to encode a peptide of 156 aa. The BLASTP search revealed that the putative peptide encoded by ORF3 contained a domain of unknown function (DUF755) that showed highest homology to ORF3 of TTV KAV (YP_003587907), a strain isolated in Germany (coverage, 69%; identity, 48%; maximum score, 112). Finally, ORF4 was predicted to encode a peptide of 127 aa and shared the highest homology with ORF2/3 of TTV HEL32 (YP_003587864), isolated in Finland (coverage, 55%; identity, 70%; maximum score, 112).

Phylogenetic analysis

Following the discovery of the original TTV isolate in 1997, many variants of TTV were reported, showing a high degree of diversity. On the basis of sequence differences that were greater than 30% or 50%, TTV could be classified into at least 39 genotypes, or 5 major genetic groups (Ninomiya M, et al., 2008; Okamoto H, 2009; Peng Y H, et al., 2002). A phylogenetic tree based on the complete genomes of TTVs was constructed (Figure 3) and the results indicated that neither the TTV isolate, Hebei-1, nor the recent Taiwan isolate, TW53A37, belonged to any of the five major genetic groups. We therefore suggest that these two recent TTV isolates should be classified as a new TTV genetic group, as well as a novel TTV genotype.

DISCUSSION

TTV has been proven to replicate in a range of tissues or organs, such as liver, lung tissue, pancreas, bone marrow, spleen and other lymphoid tissues. TTV also shows a broadly cellular tropism towards lymphoid tissues, including T and B lymphocytes, monocytes, natural killer cells, granulocytes and polymorphonuclear cells. Lifelong viremia is a characteristic of TTV infection and the viral titer may reach 10^6 copies/mL in serum from individuals of a healthy population (Hu Y W, et al., 2005; Pistello M, et al., 2001). Although a potential association of TTV infection with liver diseases, respiratory diseases, hematological disorders, cancer and multiple sclerosis has been reported, a direct causal link between TTV infection and these diseases remains undetermined, owing to the high-level prevalence of TTV in the healthy population. Genetic diversity has been recognized as another characteristic of TTV and the homology among TTV strains has been shown to be only 40%–60%. At least 39 genotypes or 5 major genetic groups of TTV have been reported up to now. Although no direct causal link has been proven, it has been suggested that some genotypes of TTV may be a contributory factor in some diseases. Mixed infections with different strains of TTV have already been reported in many cases (Okamoto H, et al., 2001), making identification of the etiological role of TTV more difficult. The latest development in sequencing techniques – unbiased, high-throughput sequencing – has been demonstrated to be a very powerful tool in screening for both known and unknown pathogens. In this report, we have identified a new genotype of

Table 3. Closest BLASTP matches to the open reading frames of TTV-Hebei-1

ORFs	Length	Isolate	Isolation year	Isolation region	Max score	Query cover	Identity	Accession	Reference
ORF1	734 aa	TW53A37	2008	Taiwan	1604	90%	78%	ACR20277	
ORF2	154 aa	TCHN-D1	2001	Guangzhou	118	100%	48%	AAK11703	(Luo K, et al., 2002)
ORF3	156 aa	KAV	2001	Germany	112	69%	48%	YP_003587907	(Heller F, et al., 2001)
ORF4	127 aa	HEL32	2001	Finland	112	55%	70%	YP_003587864	(Kakkola L, et al., 2002)

The abbreviation “aa” represents “amino acids”.

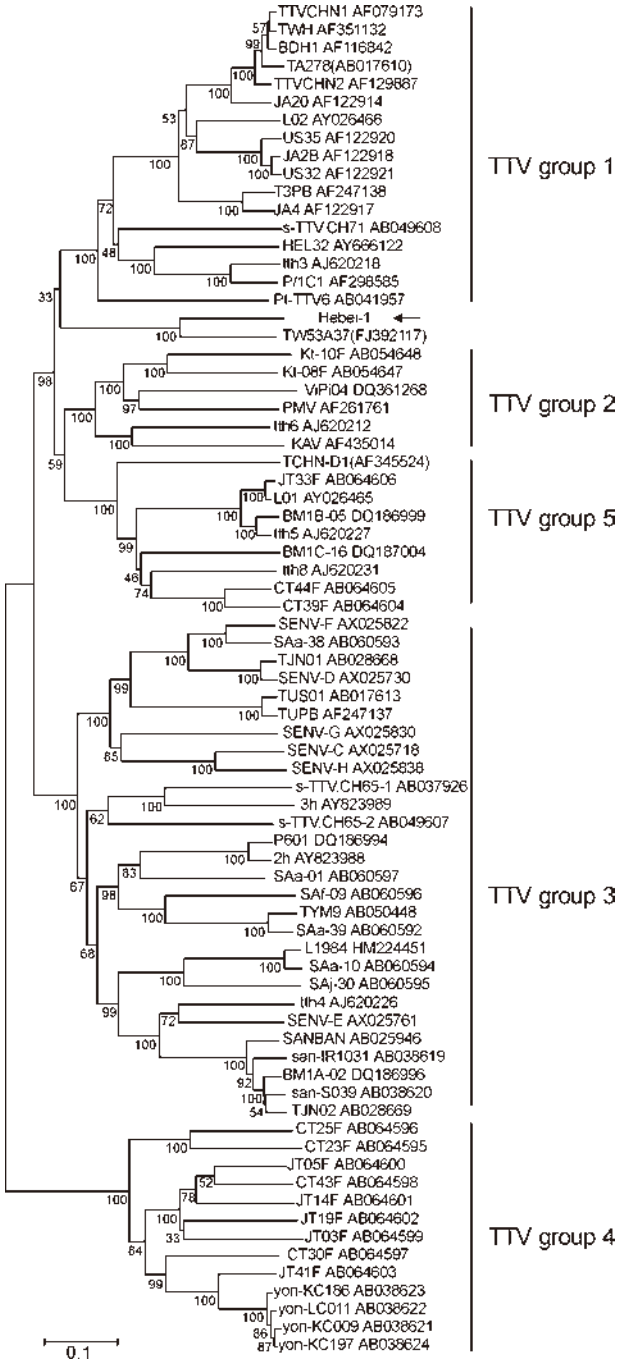

Figure 3. Phylogenetic tree based on the entire genomic sequences of 75 strains of TTV constructed using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were undertaken using MEGA5.05.

TTV which represents a novel TTV genetic group. Using unbiased deep sequencing, this TTV was exclusively detected in the blood sample of a patient with a fatal fever who was suspected of suffering from a viral infection, suggesting that this novel genotype of TTV might be different from other characterized TTVs that are widely prevalent in healthy populations. Since the patient died soon after the blood sample was collected, no further investigation of the TTV as the possible causative agent could be pursued. Nevertheless, even though no direct causal link of the persistent high fever and pneumonia with the novel TTV infection was determined, the patient had been strongly suspected to be pathogen-infected and no other pathogens were detected in the blood sample by unbiased high-throughput sequencing. Therefore, the possible role of infection with this novel genotype of TTV in cases of persistent high fever or pneumonia deserves to be explored further.

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COMPLIANCE WITH ETHICS GUIDELINES

All the authors declare that they have no competing interest. Additional informed consent was obtained from the patient for which identifying information is included in this article.

AUTHOR CONTRIBUTIONS

ZM carried out data analysis and drafted the manuscript. XY, GP, XA, YH and FP performed the experiments. WW, ZZ and SL participated in data analysis. FP collected samples. CB conceived and coordinated the study. YT conceived and supervised the study, and wrote the manuscript.

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