

Detection of TT virus (TTV) by three frequently-used PCR methods targeting different regions of viral genome in children with cryptogenic hepatitis, chronic B hepatitis and HBs carriers

Koray Ergünay¹, Figen Gürakan², Yusuf Usta², Aysel Yüce², Hasan Özen²
Erdem Karabulut³, Şemsettin Ustaçelebi¹

Departments of ¹Clinical Microbiology, ²Pediatrics, and ³Biostatistics, Hacettepe University Faculty of Medicine, Ankara, Turkey

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This study was designed so that three sensitive and widely-used polymerase chain reaction (PCR) methods for the detection of TT virus or Torque Teno virus (TTV) would be simultaneously applied to a large number of subjects to evaluate performances of the various PCR protocols with different genotype sensitivities. Sera were collected from 92 children admitted to Hacettepe University İhsan Doğramacı Children's Hospital Pediatric Gastroenterology Unit (17 cryptogenic chronic hepatitis, 17 asymptomatic HBs carriers, 18 chronic HBV patients and 40 healthy children). TTV DNA was detected via nested N22, nested 3'-UTR and 5'-UTR PCRs for all samples. Differences in TTV DNA detection prevalences were not statistically significant between the study groups with all methods. No significant correlation was detected between presence of TTV DNA and liver enzyme levels. A significant agreement between PCR methods that target UTR was observed. TTV detection rate increased with age, suggesting a non-parenteral, environmental exposure to the virus for the study population.

Key words: TT virus, TTV, cryptogenic hepatitis, chronic HBV, HBs carriers.

TT virus or Torque Teno virus (TTV) is a DNA virus of 30-50 nm, the first non-enveloped human virus with a circular single-stranded DNA genome¹. TTV strains identified to date comprise 5 genogroups with at least 23 genotypes. TTV is distributed widely in human populations throughout the world, with more than 80% of the population being infected in some areas². Although initial association of TTV with post-transfusion hepatitis and fulminant hepatitis of unknown etiology could not be verified for adults and children, high viral loads were detected in hospitalized children with severe acute respiratory disease³. Furthermore, TTV was suggested to be a contributing factor affecting the clinical parameters and outcome of pediatric asthma patients⁴. There are also reports that suggest TTV to be the causative agent for at least

some cases of non A-E hepatitis⁵. For TTV, no cell culture systems exist for isolation or animal model for experimental studies. Polymerase chain reaction (PCR) remains the only means of TTV detection, although certain serologic/molecular methods have been developed but not yet used in large-scale studies¹.

After the extraordinarily divergent genetic heterogeneity displayed by TTV was identified, PCR methods targeting more conserved regions of the viral genome were developed. The use of these methods that can amplify a broader spectrum of viral genotypic variants showed that TTV infection is more frequent in both hepatitis cases and healthy populations than previously suggested, and raised questions about the pathogenic potential of the agent^{1,6}. PCR protocols that have been developed for

the detection of TTV target either the N22 region (in ORF1), which is reported to be sensitive to certain viral genotypes, or the 5'/3'-UTR (untranslated) regions, in order to have increased specificity for more viral genotypes/subtypes compared to the N22 region⁶. Although there have been numerable studies that focus on the detection of the virus, few used more than one primer set on both healthy children and cases of hepatic injury⁷. This study was designed so that three sensitive and widely-used PCR methods would be simultaneously applied to a large number of subjects, including healthy children, cryptogenic chronic hepatitis cases, chronic hepatitis B cases and hepatitis B carriers.

Material and Methods

Study Population

Sera collected previously from 92 children (male/female: 55/37; mean age \pm SD: 9.8 \pm 4.4) admitted to Hacettepe University İhsan Doğramacı Children's Hospital Pediatric Gastroenterology Unit with informed consent were reevaluated for the study. The study population consisted of 17 children with cryptogenic chronic hepatitis, 17 asymptomatic HBs carriers, 18 chronic hepatitis B virus (HBV) patients and 40 healthy children without any clinical and biochemical signs of liver disease. For all study groups, serologic parameters for hepatitis viruses and hepatic transaminase levels were determined. Asymptomatic HBs carriers had normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT), positive HBs and anti-HBe antibodies, negative anti-delta antibody and HBV DNA. Normal-high AST and ALT, positive HBV DNA, HBs and HBe antigens, and negative anti-delta and anti-HBe antibodies were noted for the chronic hepatitis B group. For the cryptogenic hepatitis group, all possible infectious, metabolic or toxic causes of hepatitis were ruled out by appropriate biochemical, serologic and molecular tests by pediatric gastroenterology specialists. The control group consisted of an age-matched group of children under evaluation in the Gastroenterology Clinic for gastroesophageal reflux, obesity, functional abdominal pain, and/or chronic constipation. Sera collected from all cases were aliquoted and kept at -80°C until studied. Results of the PCR study targeting the N22 region were published previously⁸.

Detection of TTV DNA

DNA purification was performed using Viogene DNA-RNA extraction kit (Viogene, USA) and High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) according to the manufacturers' instructions.

Three PCR protocols were employed simultaneously for each sample to detect TTV DNA. For PCR amplification of the N22 region, degenerate primers were employed⁹. Primers for the first round were used for the 50 μl mix containing 10 μl of template, 2 mM magnesium chloride, dNTPs and Taq polymerase. For the second PCR, inner set of primers were employed and magnesium chloride concentration was raised to 2.5 mM. Thermocycling consisted of 35 cycles of 30 seconds at 94°C , 45 seconds at 60°C , and 45 seconds at 72°C after a denaturation step of 2 minutes at 94°C . A last polymerization step of 10 minutes at 72°C was also performed. Amplicons of 277 base pairs were separated by electrophoresis on 2% agarose gel, and visualized under ultraviolet light after staining with ethidium bromide.

A nested PCR protocol was used for targeting the 3'-UTR¹⁰. Primers for the first round were used for a 50 μl reaction mix containing 10 μl of template, 2 mM magnesium chloride, dNTPs and Taq polymerase. For the second PCR, inner set of primers were employed and magnesium chloride concentration was decreased to 1.75 mM. Thermocycling consisted of 35 cycles of 30 seconds at 94°C , 45 seconds at 55°C , and 45 seconds at 72°C after a denaturation step of 2 minutes at 94°C and a polymerization step of 10 minutes at 72°C at the last. Amplicons of 243 base pairs were separated by electrophoresis on 2% agarose gel, and visualized under ultraviolet light after staining with ethidium bromide.

A single round PCR that targets the 5'-UTR was used for detection of viral DNA via this region¹¹. A 50 μl PCR mixture containing degenerate primers, 10 μl of template, 2.5 mM magnesium chloride, dNTPs and Taq polymerase was amplified using a thermocycling program that consisted of an initial denaturation for 9 minutes at 95°C ; and then 55 cycles of 20 seconds at 95°C , 20 seconds at 55°C , and 30 seconds at 72°C . Amplicons were also kept at 72°C for 5 minutes for further polymerization.

PCR products were subjected to electrophoresis in 2% agarose gel and the expected amplicons of 199 base pairs were investigated after staining with ethidium bromide under ultraviolet light.

Nucleic acid extraction, PCR amplification and electrophoresis were performed in separate laboratories in order to prevent contamination. Positive and negative controls were employed for each reaction. If the initial reaction was negative, PCR was repeated.

Sequences and genomic positions of the primers are provided in Table I.

In the cryptogenic hepatitis group, 11.7% (2/17) were positive and 23.5% (4/17) were negative by all three PCR systems. For the chronic B hepatitis group, 16.6% (3/18) and 27.7% (5/18) were positive and negative by all PCR systems, respectively. For HBs carriers, 5.8% (1/17) and 47% (8/17) were positive and negative by all PCR systems, respectively. In the control group, 2.5% (1/40) were positive and 40% (16/40) were negative by all three PCR systems. N22 PCR positivity was correlated to 3'-UTR PCR positivity in all and to 5'-UTR PCR positivity in 7 of 8 cases.

Table I. Sequences, Genomic Positions and References of the Primers Used for the Detection of TTV DNA

Target region	Type	Sequence ¹ and genomic position ²	Product length
N22	Nested	1 st round: CAGACAGAGGAGAAGGCAACATG (sense, nt:1901-1923) TACCAYTTAGCTCTCTATTCTWA (antisense, nt:2228-2206) 2 nd round: GGMAAYATGYTRTGGATAGACTGG (sense, nt:1915-1938) CTACCTCCTGGCATTTTACCA (antisense, nt:2192-2171)	277 bp
3'-UTR	Nested	1 st round: GTGGGACTTTCACTTGTTCGGGTGTC (sense, nt:3087-3110) GACAAATGGCAAGAAGATAAAGGCC (antisense, nt:3392-3368) 2 nd round: AGGTCACTAAGCACTCCGAGCG (sense, nt:3120-3141) GCGAAGTCTGGCCCCACTCAC (antisense, nt:3362-3342)	243 bp
5'-UTR	Single Round	GCTACGTCACTAACCACGTG (sense, nt:6-25) CTBCGGTGTGTAACCTCACC (antisense, nt:185-204)	199 bp

Statistics

Prevalence of TTV DNA positivity determined by each PCR protocol was interpreted by statistical tests. Chi-square and Mann-Whitney tests were used for comparisons whenever appropriate. P values <0.05 were assumed as statistically significant. Reliability of PCR tests were determined by Cronbach alpha (Kuder-Richardson 20, KR20) coefficient and agreement between PCR protocols was assessed by Kappa coefficient for each group. Data analyses were performed by SPSS ® package Version 12.0.

Results

Detection rates of TTV DNA in study groups are given in Table II. The differences in TTV DNA positivity between the study groups for each PCR method were not statistically significant.

The means and standard deviations for AST and ALT levels in study groups were 33.6 ± 12.6 IU/L and 26.4 ± 18.4 IU/L for controls, 43.4 ± 20.2 IU/L and 45.6 ± 31.8 IU/L for chronic B hepatitis cases, 30.3 ± 9.4 IU/L and 32.3 ± 29.97 IU/L for HBs carriers, and 101.0 ± 117.3 IU/L and 60.2 ± 44.7 IU/L for cryptogenic hepatitis cases, respectively. No significant correlation was detected between presence of TTV DNA and liver enzyme levels in any of the study groups (p: 0.833 for AST, p: 0.460 for ALT).

For controls, cryptogenic hepatitis cases and HBs carriers, detection of viral DNA with 3'-UTR and 5'-UTR PCR revealed a statistically significant increase with mean age (p: 0.007, 0.006 and 0.003 for 3'-UTR and p: 0.001, 0.001 and 0.008 for 5'-UTR, respectively). The same distribution could not be demonstrated in children with chronic hepatitis B infection.

Table II. Detection Rates of TTV DNA in Study Groups by Different PCR Methods and Calculated p Values

Group	TTV PCR						Total
	N22		3'-UTR		5'-UTR		
	Positive	Negative	Positive	Negative	Positive	Negative	
Cryptogenic hepatitis	2 (11.8%)	15 (88.2%)	11 (64.7%)	6 (35.3%)	10 (58.8%)	7 (41.2%)	17
HBs carriers	2 (11.8%)	15 (88.2%)	8 (47.1%)	9 (52.9%)	8 (47.1%)	9 (52.9%)	17
Chronic HBV hepatitis	3 (16.7%)	15 (83.3%)	10 (55.6%)	8 (44.4%)	11 (61.1%)	7 (38.9%)	18
Controls	1 (2.5%)	39 (97.5%)	19 (47.5%)	21 (52.5%)	18 (45%)	22 (55 %)	40
	p: 0.286		p:0.641		p:0.657		

Reliability analyses by Cronbach alpha coefficient revealed high level of consistency between 3'-UTR and 5'-UTR PCR for all study groups. Agreement between 3'-UTR and 5'-UTR PCR systems was above average for all groups as determined using Kappa coefficient.

Discussion

The prevalence of TTV detection is influenced dramatically according to the region of the viral genome targeted for PCR. This work combines data obtained from three sensitive and widely-used PCR protocols for the detection of TTV in children with cryptogenic hepatitis and chronic B hepatitis, HBs carriers and children without signs of hepatic injury.

None of our data from any of the PCR protocols indicates an increased detection of TTV in cryptogenic hepatitis, and thus a possible etiologic role for hepatitis development, or an enhancement of existing hepatic damage (Table II). It was generally observed in initial studies that by using methods that target the N22 region, prevalence of TTV infection in healthy subjects was below 15%, and N22 PCR generally had a lower detection rate than UTR PCR due to its limited genotype specificity, as also previously reported by our group^{5,8,10}. Previous studies that compared the performances of different PCR methods revealed that the methods that detect 3' or 5'-UTR regions usually have comparable degrees of sensitivity⁵. These observations were confirmed in our study in children, where prevalence rates observed for each group with 5' and 3'-UTR PCR were found to have statistically significant agreement and high level of consistency and a higher detection rate compared to N22 PCR.

In our study, mean age of TTV-infected children was significantly higher in most study groups by UTR PCRs, which strongly supports the existence of a non-parenteral transmission route and coincides with the starting of primary school and increased interaction with the environment. The relationship with age could not be demonstrated in children with chronic HBV hepatitis, which is probably due to earlier exposure to TTV.

Torque Teno virus prevalence in Turkey is known to vary according to study group and detection method employed. With PCR that

targets the N22 region, detection rates ranging from 4.5% to 51.6% were reported in healthy adults and 2.5% in children^{8,12-14}. UTR PCR increases prevalence rates dramatically (as high as 82.7%) as expected^{15,16}. These data, besides differences in PCR methodology, probably imply the effect of genotypic distribution from different parts of the country on detection rates. In eastern Anatolia, TTV genotypes 1-4 are frequently encountered, with genotype 2 being the most prevalent¹⁴. Although a preferential detection of genotypes 1 and 2 was also reported, the entire genotypic spectrum of Turkish TTV isolates and their distribution remains to be determined¹³.

It is reported that TTV can be found in infected persons as quasispecies, and some undefined factors may alter the predominant genotype/types^{17,18}. Certain genotypes of the virus may also be more pathogenic, and infection with only these genotypes might cause tissue damage or related consequences, as in the example of human papilloma virus^{19,20}. Although genotype 1 was thought to be the candidate pathogenic subtype, based on the fact that studies observing an increased detection rate of TTV in non A-E hepatitis commonly used N22 PCR protocols sensitive to this genotype, convincing data on this topic is still lacking²⁰⁻²³. Interaction among genotypes that results in enhancement of the pathogenicity of certain genotypes has also been considered². We have previously reported that for adult non A-E hepatitis patients, there exists a statistically significant difference in prevalence rates according to the primers used, possibly indicating a different genotypic distribution in non A-E hepatitis cases²⁴. Similar findings of variations in distribution of TTV genotypes could not be shown in children with cryptogenic chronic hepatitis in this study.

Finally, for identifying the exact role of TTV in cases of hepatocellular dysfunction, detection of all existing viral genotypes and their distribution in study populations along with predominant genotypes in that geographic region need to be defined. Viral load detection for different genotypes will be of help when determining virologic dynamics of TTV infection.

The presence of TTV can not be suggested as a cause of hepatitis of unknown etiology; infection with TTV is found to be frequent in Turkish children and prevalence increases with

age. PCR methods targeting the UTR region are similar in sensitivity and better suited for general prevalence studies than N22 PCR.

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