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# Performance of three PCR methods targeting different regions of viral genome for the detection of TTV in Non A-E hepatitis, chronic B and C hepatitis and healthy blood donors

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**Abstract:** *TT virus* (TTV) was suggested to be the etiologic agent for non A-E hepatitis but this could not yet be proven due to high detection rates not only in hepatitis but also in healthy persons and sensitivity differences of PCR methods employed. The aim of this study was to evaluate TTV DNA positivity in non A-E hepatitis cases, chronic HBV and HCV hepatitis cases and healthy blood donors via PCR systems that target all regions of the viral genome used for viral detection. 23 non A-E hepatitis, 28 chronic HCV, 21 chronic HBV cases and 56 healthy blood donors were included in the study and evaluated by PCR protocols that target 5'-UTR, 3'-UTR and N22 (ORF1) regions. As a result, 3'-UTR and 5'-UTR PCR had comparable detection rates that were higher than N22 PCR. Differences in detection rates among study groups were not statistically significant for any PCR method. Hepatic enzyme levels of the patients were not correlated with the presence of TTV DNA. Detection rate was significantly higher for Non A-E hepatitis group when positivity rates from all methods were combined. These results suggest an alteration of viral genotypes in Non A-E hepatitis which might be associated with pathogenesis.


*Keywords: TT virus, TTV, hepatitis B, hepatitis C, non A-E hepatitis*

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## 1 Introduction

*TT virus* or *TorqueTeno Virus* (TTV) is the first human circular single-stranded DNA virus, isolated from an idiopathic post transfusion hepatitis patient from Japan in 1997 by Representational Difference Analysis (RDA) (1). Polymerase Chain Reaction (PCR) remains to be the only means of TTV detection although certain serologic/molecular methods were developed but not yet used in large-scale studies [2]. TTV's close association with post transfusion hepatitis and fulminant hepatitis of unknown etiology suggested that the agent might be a candidate virus responsible for some cases of cryptogenic hepatitis. Many reports suggest TTV to be the causative agent for at least some cases of non A-E hepatitis [3–6]. After the extraordinarily divergent genetic heterogeneity displayed by the virus is identified, PCR methods targeting more conserved regions of the viral genome are 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, even raised questions about the pathogenic potential of the agent [2, 7, 8]. TT virus strains identified to date comprise 5 genogroups with at least 23 genotypes [9]. The region of the viral genome that should be targeted for genotyping is also a topic of debate [10].

PCR protocols for the detection of TTV that have been developed target either N22 region (in ORF1) which is reported to be sensitive to certain viral genotypes or UTR (untranslated) region in order to have increased specificity for more viral genotypes/subtypes compared to N22 region [11, 12]. Although there have been numerable studies that focus on the detection of the virus, few used more than 1 primer set on both healthy subjects and cases of hepatic injury [4, 7, 13]. This study is designed so that three most widely-used and sensitive detection methods will be applied to a large number of subjects including healthy blood donors, non A-E hepatitis cases, chronic hepatitis C and hepatitis B cases.

## 2 Statistical methods and experimental procedures

### 2.1 Study population

23 patients admitted to Hacettepe University Hospital Department of Internal Medicine Gastroenterology Division with a diagnosis of Non A-E hepatitis were enrolled in the study. Other possible infectious, metabolic or toxic causes of hepatitis were ruled out by appropriate biochemical, serologic and molecular tests by gastroenterology specialists. 21 chronic hepatitis B and 28 chronic hepatitis C cases were evaluated along with Non A-E patients. 56 healthy persons qualified as blood donors by Hacettepe Hospital Blood Bank are also included in the study. All patients were included in the study with informed consent. The study was approved by Hacettepe University Medical Ethics Committee. Sera collected from all cases were aliquoted and stored at – 80°C until studied.

## 2.2 Detection of TTV DNA

DNA was extracted from sera by using Viral DNA Extraction Kit ™(Metis Biotechnology, Turkey) and High Pure Viral Nucleic Acid Kit ™(Roche Diagnostics, Germany) according to manufacturers' instructions.

For PCR amplification of N22 region, degenerate primers were employed [15]. Primers for the first round were used for the 50 $\mu$l mix containing 10 $\mu$l of template, 2 mM magnesium chloride, dNTPs and Taq polymerase. For the second PCR, inner set of primers was employed and magnesium chloride concentration was raised to 2.5 mM. 35 cycles of 30 seconds at 94°C were applied as the thermocycling conditions. 45 seconds at 60°C, 45 seconds at 72°C after a denaturation step of 2 minutes at 94°C as the thermocycling conditions. A last polymerization step of 10 minutes at 72°C was also performed. Amplicons of 277 basepairs 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 3'-UTR [7]. Primers for the first round were used for a 50 $\mu$l reaction mix containing 10$\mu$l of template, 2 mM magnesium chloride, dNTPs and Taq polymerase. For the second PCR, inner set of primers was employed and magnesium chloride concentration was decreased to 1.75 mM. Thermocycling program consisted of 35 cycles of 30 seconds at 94°C, 45 seconds at 55°C, 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 last. Amplicons of 243 basepairs were separated by electrophoresis on 2% agarose gel, and visualized under ultraviolet light after staining with ethidium bromide.

A single round PCR that targets 5'-UTR was used for detection viral DNA via this region [16]. A 50 $\mu$l PCR mixture containing degenerate primers, 10 $\mu$l 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; then 55 cycles of 20 seconds at 95°C, 20 seconds at 55°C, 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 seperate laboratories in order to avoid 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 1.

## 2.3 Statistics

Differences of descriptives and prevalences of TTV DNA positivity as determined by each PCR method were interpreted by statistical tests. P values $<0.05$ were assumed as statistically significant. Reliability of PCR tests was determined by Cronbach's alpha (Kuder-Richardson 20, KR20) coefficient and agreement between two PCR protocols were assessed by Kappa coefficient for each group. Data analyses were performed by SPSS® Version 12.0.

**Table 1** Sequences, genomic positions and references of the primers used for the detection of TTV DNA.

| | Target region | Type | Sequence[a] and genomic position [b] | Product Length | Basic Reference |
|---|---|---|---|---|---|
| **Protocol 1** | N22 | Nested | 1st round:<br>*CAGACAGAGGAGAAGGCAACATG*<br>(sense, nt:1901-1923)<br>*TACCAYTTAGCTCTCTATTCTWA*<br>(antisense, nt:2228-2206)<br>2nd round:<br>*GGMAAYATGYTRTGGATAGACTGG*<br>(sense, nt:1915-1938)<br>*CTACCTCCTGGCATTTTACCA*<br>(antisense, nt:2192-2171) | 277 bp | *15* |
| **Protocol 2** | 3' - UTR | Nested | 1st round:<br>*GTGGGACTTTCACTTGTCGGTGTC*<br>(sense, nt:3087-3110)<br>*GACAAATGGCAAGAAGATAAAGGCC*<br>(antisense, nt:3392-3368)<br>2nd round:<br>*AGGTCACTAAGCACTCCGAGCG*<br>(sense, nt:3120-3141)<br>*GCGAAGTCTGGCCCCACTCAC*<br>(antisense, nt:3362-3342) | 243 bp | *7* |
| **Protocol 3** | 5' - UTR | Single Round | *GCTACGTCACTAACCACGTG*<br>(sense, nt: 6–25)<br>*CTBCGGTGTGTAAACTCACC*<br>(antisense, nt: 185–204) | 199 bp | *16* |

[a] W: A or T; M: A or C; Y: C or T; R: A or G; B: G, C, or T);
[b] Genomic positions refer to TTV isolate TWH (GenBank accession NO: AB008394).

## 3 Results

Distribution of age and gender of the study groups and hepatic enzyme levels were summarized in Table 2. For patients belonging to hepatitis of unknown or known etiology groups, no statistical significant difference was detected between hepatic enzyme levels and TTV DNA positivity by any of the PCR methods (p values not shown).

10 of 23 Non A-E hepatitis cases (43.5%),10 of 28 chronic hepatitis C cases (35.7%), 4 of 21 chronic hepatitis B cases (19.1%) and 17 of 56 blood donors (30.4%) were found to be positive for TTV DNA by N22 PCR (Table 3). No statistically significant difference for TTV detection rates between study groups could be demonstrated.

15 of 23 Non A-E hepatitis cases (65.2%), 14 of 28 chronic hepatitis C cases (50%), 9 of 21 chronic hepatitis B cases (42.9%) and 35 of 56 blood donors (62.5%) were found to

**Table 2** Distribution of age, gender, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels of the study groups.

| Group | Number | Gender (Female /Male) | Age (year) (Mean ± SD[a]) | AST level (IU $L^{-1}$) (Mean ± SD[1]) | ALT level (IU $L^{-1}$) (Mean ± SD[a]) |
|---|---|---|---|---|---|
| Non A-E Hepatitis | 23 | 15 / 8 | 46.8 ± 15.91 | 52.0 ± 46.16 | 49.20 ± 38.51 |
| Chronic HCV Hepatitis | 28 | 16 / 12 | 48.2 ± 16.10 | 49.46 ± 34.50 | 56.84 ± 48.97 |
| Chronic HBV Hepatitis | 21 | 11 / 10 | 41.0 ± 13.27 | 46.77 ± 28.48 | 60.83 ± 51.05 |
| Healthy Blood Donors | 56 | 19 / 37 | 32.4 ± 8.1 | n.d.[b] | n.d.[b] |

[a] Standard Deviation;
[b] not determined.

**Table 3** Detection rates of TTV DNA in study groups by different PCR methods.

| Group | TTV PCR | | | | | | |
|---|---|---|---|---|---|---|---|
| | Protocol 1 | | Protocol 2 | | Protocol 3 | | |
| | Positive | Negative | Positive | Negative | Positive | Negative | Total |
| Non A-E Hepatitis | 10 (43.5%) | 13 (56.5%) | 15 (65.2%) | 8 (34.8%) | 13 (56.5%) | 10 (43.5%) | 23 |
| Chronic HCV Hepatitis | 10 (35.7%) | 18 (64.3%) | 14 (50%) | 14 (50%) | 13 (46.4%) | 15 (53.6%) | 28 |
| Chronic HBV Hepatitis | 4 (19.1%) | 17 (80.9%) | 9 (42.9%) | 12 (57.1%) | 9 (42.9%) | 12 (57.1%) | 21 |
| Healthy Blood Donors | 17 (30.4%) | 39 (69.6%) | 35 (62.5%) | 21 (37.5%) | 28 (50%) | 28 (50%) | 56 |

be positive for TTV DNA by 3'-UTR PCR (Table 3). The difference of TTV detection rates between study groups were not statistically significant.

13 of 23 Non A-E hepatitis cases (56.5%), 13 of 28 chronic hepatitis C cases (46.4%), 9 of 21 chronic hepatitis B cases (42.9%) and 28 of 56 blood donors (50%) were found to be positive for TTV DNA by 5'-UTR PCR (Table 3). No statistically significant difference for TTV detection rates between study groups were demontrated.

In non A-E hepatitis group; 13.04% (3 / 23) were positive and 4.34% (1 / 23) were negative by all three PCR systems and 47.8% (11/ 23) gave positive results by two of PCR systems employed. For chronic C hepatitis group, 14.2% (4 / 28) and 28.5% (8 / 28) were positive and negative by all PCR systems respectively where 32.1% (9 / 28) were positive by two systems. 9.5% (2 / 21) and 42.8% (9 / 21) were positive and negative by all PCRs for chronic B hepatitis group respectively and simultaneous detection rate percentage by two of the systems used in the study was 28.5% (6 / 21). In healthy blood donors; 25% (14 / 56) were positive and 26.7% (15 / 56) were negative by all three PCR systems. 17.8% (15 / 56) of this group gave positive results by two of PCR systems. Reliability analyses by Cronbach's alpha coefficient revealed low level of consistency of PCR tests for only blood donors. Agreement between PCR systems was below average for all groups as determined using Kappa coefficient.

If the positive detection rates from each group, regardless of the primer sets are interpreted, TTV DNA was detected in 22 of 23 Non A-E hepatitis cases (95.7%), 20 of 28 chronic hepatitis C cases (71.4%), 12 of 21 chronic hepatitis B cases (57.1%) and 41

of 56 blood donors (73.2%) (Table 4). This time, the detection rate for TTV DNA was found to be significantly higher for Non A-E hepatitis cases when compared to chronic C / B hepatitis groups and blood donors (p=0.031).

**Table 4** Combined detection rates of TTV DNA in study groups.

| Group | TTV DNA | | |
|---|---|---|---|
| | Positive | Negative | Total |
| Non A-E Hepatitis | 22 (95.7%) | 1 (4.3%) | 23 |
| Chronic HCV Hepatitis | 20 (71.4%) | 8 (28.6%) | 28 |
| Chronic HBV Hepatitis | 12 (57.1%) | 9 (42.9%) | 21 |
| Healthy Blood Donors | 41 (73.2%) | 15 (26.8%) | 56 |

## 4 Discussion

Although TTV was initially shown to be associated with hepatitis of unknown etiology; identification of unusual genetic heterogeneity of the virus and very high detection rates in healthy people led to questioning of the pathogenic potential of the virus [2]. This work combines data obtained from three sensitive and widely-used PCR protocols for the detection of TTV from non A-E hepatitis cases, chronic B and C hepatitis cases and healthy blood donors.

In concordance with previous reports, our data from any of the PCR protocol does not indicate an increased detection of TTV, thus a possible etiologic role for hepatitis development in any of the groups studied (Table 3) [17–19]. This conclusion is also supported by similar levels of hepatic enzyme levels detected in patients with hepatitis. The PCR method that targets N22 region used in the study is based on probably the most commonly used method for TTV detection: a semi-nested PCR that uses NG059, NG061 and NG063 primers [3]; but with increased sensitivity for different subtypes of TTV owing to the degenerate bases [15, 30]. TTV DNA positivity in 1.9% of blood donors and 19% in fulminant hepatic failure was reported with this primer set [15]. It is generally observed in initial studies that by using methods that target N22 region, prevalence of TTV infection in healthy subjects was below 15% and N22 PCR generally has a lower detection rate than UTR PCR due to its limited genotype specificity [11, 12]. Our data show that N22 PCR has indeed a lower detection rate, but we have a higher (30.4%, Table 3) prevalence in healthy blood donors than expected. Biagini et al. noted that the use of degenerate primers and/or longer annealing and extension times increases detection rates of N22 PCR, which supports our data and the use of degenerate primers for targeting N22 region [20].

PCR method that targets 3'-UTR in our study was reported to be one of the sensitive detection methods for TTV detection with a wide genotype range and used frequently in comparison studies [7, 12, 20]. Prevalence rates of non A-E hepatitis cases were reported

to increase from 2.1% to 59.6% and increase from 12.8% to 39.6% was also noted for commercial blood donors with the utilization of these primers [7]. In our study, this protocol recognized TTV DNA in 65.2% and 62.5% of non A-E hepatitis patients and healthy blood donors respectively (Table 3).

By using the single step PCR method that targets 5'-UTR , a surprising increase in prevalence, from 23% to 92% was reported for blood donors in Japan [16] and from 11% to 51% for HCV infected subjects in Taiwan [21]. 49.5% positivity among blood donors could be detected in France with this set [20]. Here, we also report a very close prevalence 50%, (Table 3) that would imply a similar distribution of the virus in our region in Turkey.

Previous studies that compare the performances of PCR methods targeting different regions of the viral genome reveal that the methods that detect 3'or 5'-UTR regions usually have comparable degrees of sensitivity [11, 12, 20]. In our study, prevalence rates observed for each group with 5' and 3'-UTR PCR were also similar and higher than N22 PCR (Table 3). It is also important to note that despite N22 PCR is considered to be less sensitive than UTR PCR; studies with cloned DNAs of specific TTV genotypes showed that different detection rates of these methods should be attributed to their specificities for different TTV genotypes, instead of sensitivities; N22 PCR having a narrow genotype range [11, 22]. Many researchers also report that TTV may cause infections where more than one genotype can be detected simultaneously with different levels of viral loads, that can possibly influence viral DNA detection with various primer sets [9, 23, 24]. Although we did not perform DNA sequencing and phylogenetic analyses for our isolates due to high number of samples; our results with 3 primer sets clearly imply the genetic heterogeneity of TTV infection in our study groups.

TTV prevalence in Turkey must also be addressed and is known to vary according to study group and detection method employed. With PCR that targets N22 region detection rates ranging from 4.5% to 51.6% was reported for healthy adults and 2.5% for children [25–28].UTR PCR increases prevalence rates dramatically (as high as 82.7%) as expected [29, 30]. These data, besides differences in PCR methodology, probably imply the effect of genotype 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 prevalent [28]. Although a preferential detection of genotypes 1 and 2 was also reported [26], entire genotypic spectrum of Turkish TTV isolates and their distribution still awaits to be determined.

Our study revealed that, the sole presence of TTV cannot be suggested as a cause of hepatitis of unknown etiology. But there seems to be a difference of genotypic distribution of the virus, either causing liver injury or occurring as a consequence, in non A-E hepatitis cases. There are reports suggesting that TTV may be found in infected persons as quasispecies, and some undefined factors may alter the predominant genotype(s) [31, 32]. We have previously reported that for the results of non A-E patients from N22 and 3'-UTR PCR, there exists a statistically significant difference in distribution according to the primers used when compared to healthy blood donors ang chronic

HBV/HCV patients that indicates a different genotypic distribution in non A-E cases [33]. This hypothesis is also supported by our current results; which reveal an significant increase of viral detection when 3 sets of primers with different genotype sensitivities are combined (Table 4). The factor affecting the genotypic distribution may be at the same time triggering hepatic injury or in vivo genotypic change may be the factor for liver dysfunction. 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 [22, 34]. Although genotype 1 was thought to be the candidate pathogenic subtype, based on the fact that studies which observe an increased detection rate of TTV in non A-E hepatitis commonly used N22 PCR protocols sensitive to this genotype; convincing data about this concept is still lacking [3, 4, 6, 34]. Interactions among genotypes that result in enhancement of the pathogenicity of certain genotypes must also be considered [9].

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

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