IJBC 2014;6(3): 119-126

Frequency of Torque Teno Mini Virus in Hepatitis B and C Patients and Healthy Blood Donors in Isfahan, Iran

Ghazimorad A[1], Bouzari M *[1], Kardi MT [2]

1. Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran.
1. Mahdieh Medical Diagnostic Laboratory, Isfahan, Iran.

***Corresponding Author:** Bouzari M, Email: bouzari@sci.ui.ac.ir*

Submitted: 23-07-2013 , Accepted: 12-03-2014

Abstract

Background: Recently, some new viruses have been identified for their association with hepatitis which Torque Teno Mini Virus being among them. The aim of this study was to determine the frequency of Torque Teno Mini Virus in healthy individuals and hepatitis B and C patients in Isfahan, Iran.

Materials and Methods: One hundred serum samples of healthy individuals from Isfahan Blood Transfusion Organization were collected. A total of 25 human serum samples from hepatitis B and 25 samples from hepatitis C infected patients were also collected from Mahdieh diagnostic laboratory in Isfahan, Iran. Viral DNA was extracted and Torque Teno Mini Virus DNA was detected using a nested PCR with primer sets designed for a conserved region of the Torque Teno Mini Virus genome. PCR and Reverse transcriptase PCR were used for detection of HBV and HCV respectively.

Results: Torque Teno Mini Virus -DNA was detected in 17% of healthy individuals. It also was detected in 20% and 48% of serum samples from hepatitis B and C infected individuals, respectively. The frequency of Torque Teno Mini Virus was significantly higher in hepatitis C patients versus healthy individuals ($P < 0.05$). Also, the frequency of TTMV in hepatitis C patients was significantly higher than hepatitis B patients ($P < 0.05$).

Conclusions: The difference in Torque Teno Mini Virus frequency between the hepatitis C and healthy group was significant ($P< 0.05$). The etiology of the higher infection rate in hepatitis C individuals needs to be determined.

Keywords: Torque Teno Mini Virus, Hepatitis B, Hepatitis C, PCR.

Introduction

The term viral hepatitis is usually reserved for those infections caused by at least one of the six different and distinct agents collectively referred to as hepatitis viruses. These viruses are Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis E virus (HEV), Delta virus (HDV) and Hepatitis G Virus (GBV-C/HGV) [1]. Hepatitis B virus (HBV) is a sexually and vertically transmitted DNA virus. It is the cause of one million deaths due to liver failure, cirrhosis and hepatocellular carcinoma globally, each year [2]. Two hundred million people are infected with Hepatitis C virus (HCV) worldwide, which among them up to 85% may develop liver failure, chronic hepatitis and hepatocellular carcinoma. It is a major emerging health problem in developing countries [3].

Many cases of viral hepatitis occur but are not related to the well characterized hepatitis viruses A to E (non-A-E) [4]. Recently, some new viruses rather than hepatitis A to E have been identified for their association with hepatitis. These include hepatitis G, hepatitis SEN, hepatitis TT and hepatitis TT-like viruses [5]. One of TT-like viruses; Torque Teno Mini Virus (TTMV) is a small, non-enveloped spherical virus with circular single-stranded DNA genome of 2.8-2.9kb and diameter of less than 30 nm [6,7]. This virus was discovered using PCR with TTV-specific primers that partially matched homologous sequences and was designated as TTV-like mini virus (TLMV) [8]. Then the International Committee on Taxonomy of Viruses (ICTV) renamed it as Torque Teno Mini Virus (TTMV) [9, 10] ; and now it is classified in nine species in the Betatorquevirus Genus of Anelloviridae family [11]. A high conserved

region (~ 300nt) located upstream of ORF2 in the non-coding part of the genome is used for primer designing in studies of prevalence. Full-length genomic sequences of 17 isolates have been determined and four highly divergent groups have been proposed [12].

TTMV frequently and ubiquitously infects humans, and its infection is characterized by lifelong viremia and great genetic variability, but its pathogenic role in infections remains unknown[13]. This virus is reported in many countries [14, 15] including Iran where the virus was detected in 62% of chronic cervicitis and cervical tumors [16]. TTMV is transmissible by transfusion [15], and co-infection of a single individual by various strains of TTMV is frequent [15, 17].

The studies on the Anelloviruses are recent and their relation to particular pathologies is still unclear. More studies permit better understanding about TTMV and its effects on host. The aim of our study was to determine the frequency of this newly described virus in healthy individuals and hepatitis B and C patients in Isfahan, Iran.

Material and Methods

Healthy blood donors

A total of one hundred human serum samples were randomly obtained from healthy blood

Table 1: Nucleotide sequence of primers for TTMV nested-PCR.

Primer	Sequence	PCR Product	Orientation
M 1359	GTTTATSMCGCYAGACGGAG	433 bp	Sense
M 1365	TYTGCGAAWAGGGCSTCTAA	433 bp	Antisense
M 1360	GAAGGTGAGTGAAACCACCG	340 bp	Sense
M 1366	AGGGCSTCTAAWTCTCCKTC	340 bp	Antisense

K: G or T, M: A or C, S: G or C, W: A or T, Y: C or T. (Source: 8)

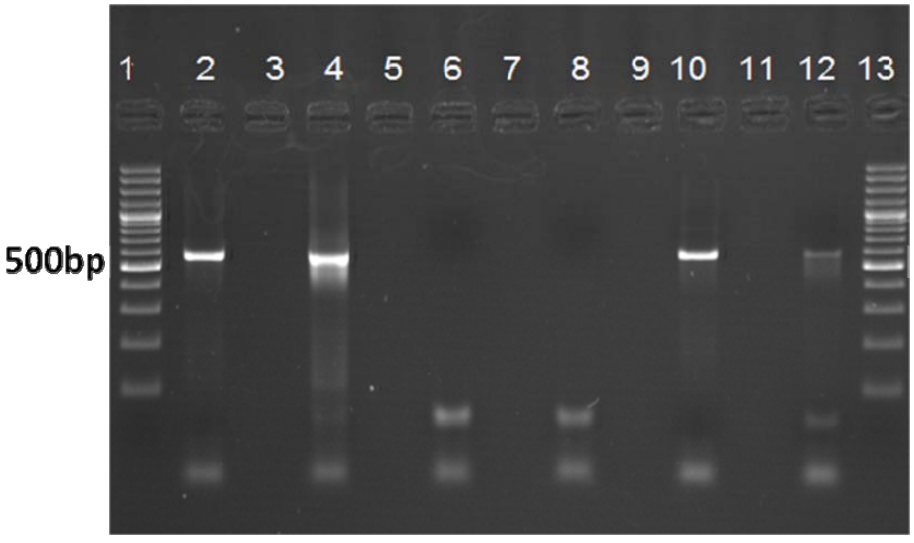

Figure 1: Detection of HBV by PCR. Columns 2, 4, 10 and 12: positive HBV cases, Columns 1 and 13: 100 bp Markers (Gene Ruler Fermentas), Columns 3, 5, 6-9 and 11 negative HBV cases

donors from blood bank of the blood transfusion organization in Isfahan, Iran. The sera were negative for HBs-Ag and anti HCV and anti HIV antibodies. The cases consisted of 33% (33) females and 67% (67) males with a mean age of 32.2 years (18-55).

Hepatitis B or C infected patients

A total of 25 human serum samples from hepatitis B and 25 from hepatitis C infected patients were collected from the Mahdieh Diagnostic Laboratory in Isfahan, Iran. The hepatitis B infected cases consisted of 4 (16%) females and 21 (84%) males. The hepatitis C infected cases included 5 (20%) females and 20 (80%) males.

DNA preparation for detection of TTMV

Viral DNA was extracted using phenol/ chloroform after treatment of 200 µL of serum with 0.5 mg mL^{-1} of proteinase K in the presence of 0.2 M NaCl, 0.25% Sodium Dodecyl Sulfate (SDS), for 2 h at 65oC. The pellet was dried and resuspended in distilled water or TE (Tris-HCl buffer (10mM, pH 8.0)

Table 2: Frequency of infection with TTMV DNA in different groups studied.

Group	Number of cases	Sex (M/F)		Cases with infection	
		Male	Female	Male (%)	Female (%)
Healthy	100	67	33	13/67(19.4%)	4/33(12.1%)
HBV-Infected	25	21	4	4/21(19%)	1/4(25%)
HCV-Infected	25	20	5	11/20(55%)	1(25%)

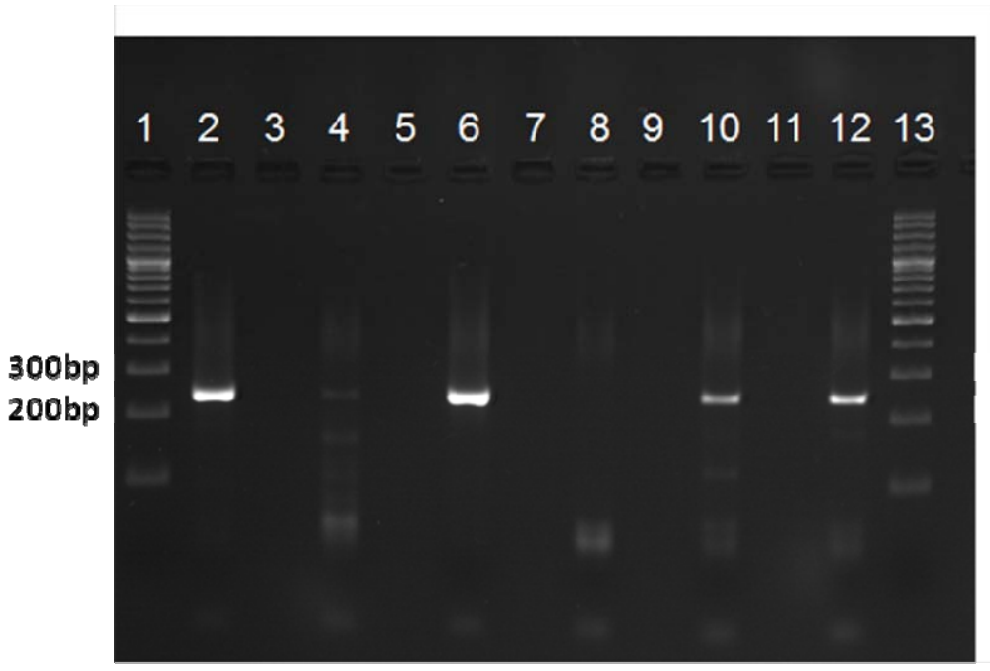

Figure 2: Detection Of HCV by RT-PCR. Columns 2, 6, 10 and 12: positive HCV cases, Columns 1 and 13:100 bp Markers (Gene Ruler, Fermentas).

containing 1mM EDTA) solution after precipitation with ethanol. The study was approved by the local ethics committee.

DNA and RNA preparation for detection of HBV and HCV

DNA and RNA were extracted using QIAamp DNA Mini Kit and QIAamp Viral (QIAGEN, Germany), according to their instructions, respectively.

Nested PCR amplification for detection of TTMV

The M1359, M1365, M1360 and M1366 primers[8] were used for amplification of UTR region of the viral genome (table 1). DNA was amplified by two rounds of PCR performed with 3 µL and 1 µL of DNA (in the first and the second round of PCR respectively) in a 25 µL reaction mixture containing 1U of Smar Taq DNA Polymerase (Cinnagen, Iran), 0.5 µM of each primers, 240 µM of each dNTPs, 20mM of Tris-HCl 3 mM MgCl2, 50 mM KCl and 29 mM Ammonium Sulfate. Thermal cycling conditions were as follows: denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 20 sec, annealing at 65°C for 25 sec and extension at 72°C for 30 sec. The amplification was followed by a final extension step at 72°C for 5 min. PCR products (10 µL) were loaded on a 1.5% agarose gel (Sigma, Germany) containing ethidium bromide and electrophoresed.

PCR for detection of HBV

Forward (5′-CAACCTCCAATCACTCACCAAC-3′) and reverse (5′-ATATGATAAAACGCCGCAGACAC-3′) primers [18] were used for amplification of the genome. DNA was amplified in one round of PCR performed with 20 µL of DNA in a 50 µL reaction mixture containing 25 µL of Hotstar Taq Plus (Qiagen, Germany), 1.5 µL of each primer (900nM) and 2 µL of DNAase free H_2O. Thermal cycling conditions were as follows: denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 30 Sec, annealing at 55°C for 30 Sec and extension at 72°C for 30 Sec. The amplification was followed by a final extension step at 72°C for 5 min.

RT-PCR for detection of HCV

The Reverse primer (5′-CTCGCAAGCACCCTATCAGGCAGT-3′) 19 was

Table 3: Nucleotide sequences of amplified amplicons.

Nucleotide Sequence	Accession Number	Group studied
NTNNACCTAGCTCNAGCTNCTCGGCTGGCCGACGGCCACTAA ACTACCATATTACTTCTTAATATCTCTAGCTGCTCCCATTTTTAA ATTTCTTGAAAATTACTTGTCAGTAAGTTCTTGTTCGTTTATTTG TATAATAATGCACTTTAGTGGATGTTCACACTTGCAAGAGAGG TTTAGTCTACCCACAAAGAAATTCAAACTTACAAGAATTTTTTG TTTTTAGACCTTTTAAAATGGCTTNGCAGCTTGCACATCTGGAA GAATTGGTGGAAAAGACGAAGAAGGTTTTGCCGGACTGGACT TGCCCGCATTTTTCGCAGACGAC	GQ337060	Healthy
AAATCCATAAGNCTCTAGGCACTCGTCTGGACGACTTCCACTA AACTTAAATTCTTCTTCCCCTTAATATGACATTTTAAATTTTTAA TGCCTTCTTCATGTTTTTTAACTTTTCCTTGTCTGTTTAACACTGT TAGTAAGTGTAAAATACTGTCGTTGCGGCCGCAAAGTAGGTC GNGGGACCTAAATATGTTGTTAATCCATAGAGTTTCAGCTTGA TTTCCAGAAGAAGACGTTTCATGAAGTAGTTGTCTCATCTGGG AGAAGAAGAAATTTTAAGTTAAGAAGACGTCTCCGCCAGAGG GAACTAGCCGGAATTTTTCGCAGACTTC	GQ337059	Hepatitis B
NAATGCTGNATCGGNCTGGTAGTCTGGCGGACGGGCAAGAC ACTTAAAATAATTTTTTTTTNCATTGATAAACTGTGTTAAATTAC TATCTTTTCNAAATTTGACTCCNCTTAGTCNCCTCTAAANCCCT TTGTGTCNATATACACAAATCCCCACAANGACAACCATCCTAA NGAAATTACTGCATGTCTTATAGTTAGCTGATTCTTTTTTTATTA NCCAGTCNATNCAAACATTTTTTTTCATATATCTTNANNT	GQ337062	Hepatitis C

used to perform RT PCR with 10 µL of RNA in a 20 µL reaction mixture containing 1 µL of Transcriptor Reverse Transcriptase (Fermentas, Germany), 0.4 µL of the reverse primer, 2 µL of 10 mM dNTPs Mix (2.5 mM each), 2 µL of 10X PCR Buffer (10mM MgCl2), 0.5 µL of RNase inhibitor and 4.10 µL of nuclease free H_2O. The mixture was incubated at 42°C for 60 min. Forward (5′-GCAGAAAGCGTCTAGCCATGGCGT-3′) and Reverse (5′-CTCGCAAGCACCCTATCAGGCAGT-3′) primers 19 were used for amplification of cDNA with 5 µL of template cDNA in a 50 µL reaction mixture containing 2 µL of Taq DNA polymerase (Qiagen, Germany), 2 µL of each primer (200 nM), 1 µL of 10 mM dNTPs Mix (2.5 mM each) and 5 µL of 10X PCR Buffer (10 mM $MgCl_2$). Thermal cycling conditions were as follows: denaturation at 95°C for 5 min (1 cycle), followed by 45 cycles of denaturation at 95°C for 30 Sec, annealing at 57°C for 15 Sec and extension at 72°C for 30 Sec. The amplification was followed by a final extension step at 72°C for 5 min.

DNA sequencing and computer analysis of TTMV sequences

To confirm the presence of TTMV DNA detected in specimens, one amplicon from each group was sequenced (Gene Service Company, UK). A WU-BLAST-2 (NCBI-BLAST-2 Nucleotid) search of the determined sequences against a nucleotide sequence database (EMBL, European Bioinformatics Institute) was performed.

Molecular evolutionary analysis

Neighbor-joining method was used to construct phylogenic tree using MEGA4 (Molecular Evolutionary Genetics Analysis software version 4.1) [20] based on our sequences (GQ337059, GQ337060 and GQ337962) against sequences obtained from GenBank, including TTMV virus Isfahan isolates from tumor (JQ734980, JQ734981, JQ734982, JQ734984 and JQ734986), cervicitis (JQ734979 and JQ734985) and multiple sclerosis (GQ337061) cases and 13 other TTMV isolates from GenBank database.

Statistical analyses

Fisher's exact test was used for statistical analyses using Graph Pad Instat software (Graph Pad Software Inc. USA).

Results

Detection of HBV and HCV by PCR and RT-PCR

As shown in figure 1 expected 550bp products were detected in HBV positive cases. A 220bp product was detected in HCV positive cases in agarose gel electrophoresis (Figure 2).

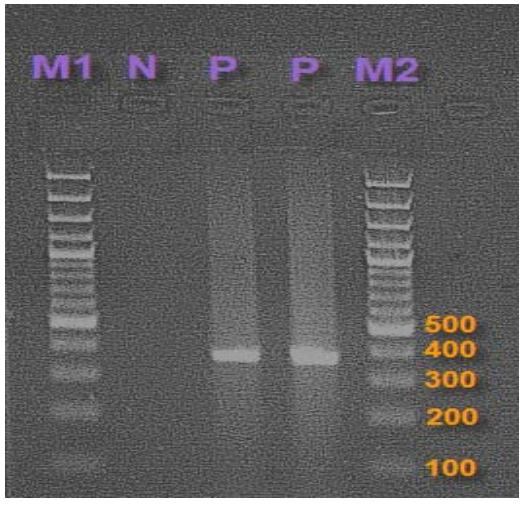

Figure 3: TTMV gene amplification by nested PCR. M1 and M2:100bp markers (Gene Ruler Fermentas Germany), N: negative control, P: positive control (left) and positive case (right).

Detection of TTMV-DNA by nested PCR

As shown in figure 3, in nested PCR a 340 bp product was detected in the second round of PCR. Among healthy individuals, the detection rate was 17% (17/100). The frequency of TTMV DNA was found to be 20% for hepatitis B and 48% (12/25) for hepatitis C infected patients. The frequency of TTMV in different sex groups are shown in table 2.

Sequences and construction of phylogenetic tree

The sequences of amplicons are shown in Table 3. The sequences were submitted in the Gene bank with accession numbers GQ337060, GQ337059, GQ337062. In WU-blast-2 alignment sequence homology of up to 93, 68 and 58 percent were observed for sequences obtained from hepatitis C, hepatitis B and healthy individuals respectively.

In the phylogenetic construct the HBV isolate (GQ337059) and the HCV isolate (GQ337062) were placed in the same cluster with already reported multiple sclerosis isolates from the same region in Iran (GQ337061). Also, healthy individual isolated sequence (GQ337060) was placed in another cluster. This cluster also included already reported sequences (JQ734980 and JQ734981) from cervical tumors of the same region. They were far from isolates (JQ734983, JQ734984, JQ734985 and JQ734986) from the same region which were placed in another cluster. These sequences which were placed in two distinct clusters were distant from the other sequences reported form other countries (Figure 4).

Statistical results

The results of the statistical analyses are shown in table 4. The frequency of TTMV was significantly higher in hepatitis C patients versus healthy individuals ($P < 0.05$). Also, the frequency of TTMV in hepatitis C patients was significantly higher than hepatitis B patients ($P < 0.05$).

Discussion

TTMV was identified as a transfusion transmissible agent present in a group of healthy blood donors and hepatitis B and C patients in Isfahan, Iran. In this study the frequency of TTMV was significantly higher in hepatitis C infected patients compared to the healthy blood donors and hepatitis B patients ($P< 0.05$). This may be as a result of synergistic interaction between TTMV and hepatitis C virus but the confirmation of this interaction needs more sophisticated studies. On the other hand this may indicate that specific transmission routs common in the two viruses may be involved. The lack of relationship between Hepatiteis B and TTMV in the present study might be due to the limited sample size in our study and studies with a bigger sample size are encouraged to further evaluate this relationship.

Using the same primers and methodology in healthy blood donors in Korea [21] the prevalence of the virus has been reported to be 41.3% (62/150) among healthy individuals which is higher than 17% detected in the present study. This could be correlated to different geographical locations. Changani et al. detected the virus in 26 out of 41 cervical tumors (65%) and 34 out of 67 cervicitis (50.7%) cases in Isfahan, Iran[16] which also is higher than the healthy individuals. This was correlated to the special conditions of the tissues examined.

In this study great diversity was observed among the samples sequenced, with homology rate from

Table 4: Statistical analyses of the frequency of TTMV infection in different groups studied.

Groups		Frequency	P_{value}
Healthy	V. Hepatitis B	17/100 V. 5/25	0.7
Healthy	V. Hepatitis C	17/100 V. 12/25	0.002
Hepatitis B	V. Hepatitis C	5/25 V. 12/25	0.007

V: Versus

57% to 93%, which confirms the former reports by Neil et al. [15] , who reported high variability in one of the most conserved regions of the TTMV genomes detected in the sera of blood donors in Brazil, and Takahashi et al. [8] , who reported high degree of genomic divergence for this virus and Changani et al. [16], who reported the same results in cervical tumors and cervicitis cases. Phylogenetic analyses showed that the isolate from the healthy individual (GQ337060) was placed in a cluster containing most of the sequences already reported from cervical tumors and cervicitis cases from the same region (JQ734980, JQ734981, JQ734983, JQ734984, JQ734985 and JQ734986). On the other hand the HBV and HCV isolates were placed in another distant cluster with high homology with the sequence obtained form a multiple sclerosis case (GQ337061) from the same region. This not only confirms the variability of the virus but also shows that probably two distinct genotypes are circulating in the studied area and that they differ from the isolates reported from other countries.

These data demonstrate that TTMV is present in the sera of healthy and hepatitis B and C infected individuals in Isfahan, Iran. Future studies for determination of TTMV pathogenesis, especially in hepatitis C infected individuals, is suggested. In the case of hepatitis B infected individuals studies with higher sample size are recommended.

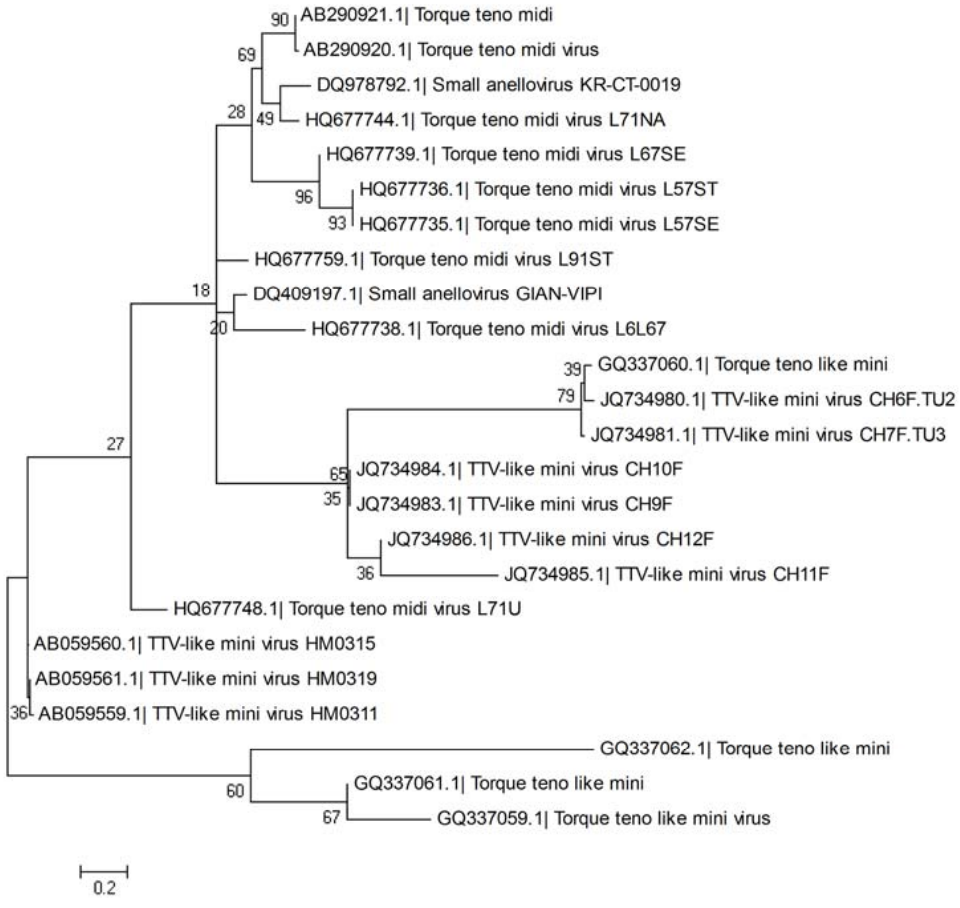

Figure 4: The tree was constructed using the neighbor-joining method with 1000 bootstrap replicates using MEGA 4.1 software. The maximum composite likelihood model based on the units of the number of base substitutions per site was used to compute the evolutionary distances. The tree is unrooted.

Conclusions

The difference in Torque Teno Mini Virus frequency between the hepatitis C and healthy group was significant (P< 0.05). The etiology of the higher infection rate in hepatitis C individuals needs to be determined.

Acknowledgement

This study was supported by the grants from the postgraduate office of the University of Isfahan. Authors would like to thank the head and the staff of the Isfahan blood transfusion organization for providing serum samples and also Mahdieh Diagnostic Laboratory in Isfahan and also Sayedeh Azimeh Hosseini for their help in the construction of phylogenetic tree.

References

1. Khuroo MS. Discovery of hepatitis E virus- The untold story. JK Pract 2004; 11(3): 291-4.
2. Dienstag, JL. Hepatitis B Virus Infection. N Engl J Med. 2008; 359(14):1486-500.
3. Akbar H, Idrees M, Manzoor S, Ur Rehman I, Butt S, Yousaf MZ, et al. Hepatitis C virus infection: A review of the current and future aspects and concerns in Pakistan. 2009. J Gen Mol Virol. 2009;1(2): 12-18.
4. Mushahwar IK. Recently discovered blood borne viruses: Are they hepatitis viruses or merely endosymbionts? J Med Virol. 2000; 62(4): 399-404.
5. Wiwanitkit V. Hepatitis G, hepatitis SEN, hepatitis TT and hepatitis TT-like viruses: New emerging hepatitis viruses in pediatric patients. J P Infect Dis. 2006;1, 83-88.
6. Peng YH, Nishizawa T, Takahashi M, Ishikawa T, Yoshikawa A, Okamoto H. Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. Arch Virol. 2002;147(1): 21-41.
7. Takahashi K, Hoshino H, Ohta Y, Yoshida, N, 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. 1998;12(3): 233-9.
8. Takahashi K, Iwasa Y, Hijikata M, Mishiro S. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch Virol. 2000;145(5): 979-93.
9. Biagini P, de Micco P. Anellovirus. In: Mahy BWJ, Van Regenmortel, MHV (eds). Human and Medical Virology. San Diego, Academic Press Linacre House, 2010, pp: 17-22.
10. Hino S. TTV, a new human virus with single stranded circular DNA genome. Rev Med Virol. 2002;12(3):151-8.
11. Biagini P, Gallian P, Cantaloube J, Attoui H, de Micco P, de Lamballerie X. Distribution and genetic analysis of TTV and TTMV major phylogenetic groups in French blood donors. J Med Virol 2006;78(2):298-304.
12. Biagini P, Uch R, Belhouchet M, Attoui H, Cantaloube JF, Brisbarre N, et al. Circular genomes related to anelloviruses identified in human and animal samples by using a combined rolling-circle amplification/sequence-independent single primer amplification approach. J Gen Virol. 2007;88(Pt 10):2696-701..
13. Okamoto H. History of discoveries and pathogenicity of TT viruses. Curr Top Microbiol Immunol. 2009;331:1-20..
14. Moen E, Huang L, Grinde B. Molecular epidemiology of TTV-like mini viruses (TLMV) in Norway. Arch Virol. 2002;147(1):181-5.
15. Niel C, Lampe E. High detection rates of TTV-like mini Virus sequences in sera from Brazilian blood donors. J Med Virol. 2001;65(1):199-205.
16. Changani L, Bouzari M and Talebi A. Torque teno mini virus infection in chronic cervicitis and cervical tumors in Isfahan, Iran. Intervirology. 2013;56(4):265-70.
17. Biagini P, Gallian P, Touinssi M, Cantaloube JF, Zapitelli JP, de Lamballerie X, et al. High prevalence of TT virus infection in French blood donors revealed by the use of three PCR systems. Transfusion. 2000;40(5):590-5.
18. Fan YF, Lu CC, Chen WC, Yao WJ, Wang HC, Chang TT, et al. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology. 2001;33(1):277-86.
19. Blackard JT, Smeaton L, Hiasa Y, Horiike N, Onji M, Jamieson DJ, et al. Detection of hepatitis C virus (HCV) in serum and peripheral-blood mononuclear cells from HCV-monoinfected and HIV/HCV-coinfected persons. J Infect Dis. 2005;192(2):258-65.
20. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24(8):1596-9.
21. Chung JY, Han TH, Seong HK, Paik IK, Kim MJ. Transfusion-transmitted virus and TTV-like mini virus infection in blood donors. Korean J Lab Med. 2004; 24(4): 250-4.