

# Detection of Hepatitis E Virus Genotype 1 Among Blood Donors From Southwest of Iran

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## Abstract

**Background:** Infection with hepatitis E virus (HEV) is endemic in developing countries and reveals significant regional differences. Several studies have reported virus transmission via blood transfusion. To date, however, no cases of HEV RNA detection in blood donors have been reported from Iran.

**Objectives:** The aim of this study was to determine the presence of HEV RNA in plasma samples of blood donors referred to a blood transfusion center in Shiraz in the southwest of Iran. The HEV genotypes were also investigated using nucleotide sequencing.

**Patients and Methods:** Blood samples were collected from 700 blood donors who were referred to Fars blood transfusion organization from January to March 2014. Plasma samples were screened for the presence of HEV IgG and IgM antibodies by standard enzyme immunoassay. Samples seroreactive to anti-HEV were further tested for the presence of HEV RNA using nested polymerase chain reaction (PCR) with universal primers for detection of all four HEV genotypes. Positive PCR samples were then subjected to DNA sequencing for further analysis.

**Results:** Fifty (50, 7.1%) out of 700 plasma samples tested positive for anti-HEV antibodies. HEV RNA was detected in 7/50 (12%) of the antibody-positive samples, the majority of which were IgM positive. Sequence analysis of seven isolates of the HEV RNA ORF 2 gene region revealed > 80% similarity with genotype 1.

**Conclusions:** The analysis indicates that the HEV isolated from blood donors in the southwest of Iran belongs to genotype 1. However, more samples from other geographic regions of Iran are needed to confirm these findings. Because transmission of HEV by administration of blood or blood components is likely to occur, it may be sensible to screen donor blood for HEV to eliminate transfusion-transmitted HEV infection when the recipient is immunocompromised.

**Keywords:** Blood Donors, Genotype, Hepatitis E Virus, Iran

## 1. Background

Hepatitis E virus (HEV) infection is an acute self-limiting hepatitis in humans and is a major public health concern worldwide. A single-stranded positive-sense RNA genome of HEV encodes three open reading frames (ORFs). Based on variation in nucleotide sequence by > 20% in the ORF2 region, HEV has been classified into different genotypes. So far, four distinctive HEV genotypes have been recognized in human clinical samples (1). Genotypes 1 and 2 are restricted to humans and are prevalent in developing countries, where the virus is transmitted via water contaminated with feces (2). Genotypes 3 and 4 are transmitted zoonotically and are prevalent in many industrialized countries (3).

As an acute disease, the clinical features of HEV infection are similar to other hepatotropic viruses. Although the severity of HEV-associated acute hepatitis is believed to rely on the status of the host's immune system, viral factors may also play an important role in the pathogenesis of the disease. Indeed, the genotype of HEV contributes to the pathogenesis of HEV-associated hepatitis (4). Genotype 4 HEV infected patients show a more severe form of viral hepatitis than genotype 3 HEV infected patients. Thus, the genetic changes in HEV genotypes may affect the effectiveness of virus transmission and, in turn, the severity of HEV-associated hepatitis. To further determine the transmission and pathogenesis of HEV, molecular epidemiological study of HEV genotypes is needed (1).

Recently, some reports have suggested that HEV could be responsible for chronic hepatitis in organ transplant patients (5-8).

While the greatest risk for solid organ transplant recipients is chronic infection, direct blood transfusion from an infected blood donor is another route of HEV infection in this group of patients. Blood donors may be infected with HEV, as indicated by plasma pools testing positive for HEV RNA and by prevalence of antibodies to HEV among blood donors. Cases of transfusion-transmitted hepatitis E have been reported (9-13).

A systematic review conducted by the world health organization (WHO) indicated that the global seroprevalence of anti-HEV IgG among blood donors depends on geographical region; it ranges from 0.05% in Germany (14) to 52.5% in France (15). In Iran, depending on the geographical area, the seroprevalence of anti-HEV IgG among blood donors is between 7.8% and 14.3% (16-18).

## 2. Objectives

To date, no cases of HEV RNA detection in blood donors have been reported from Iran. In this study, we determined the presence of HEV RNA in plasma samples of recent blood donations with positive anti-HEV antibodies. The HEV genotypes were also investigated using nucleotide sequencing.

## 3. Patients and Methods

### 3.1. Sample Collection

Blood samples were collected from 700 blood donor volunteers who were referred to Fars blood transfusion centers in the southwest of Iran from January to March 2014. All donors tested negative for syphilis, hepatitis B and C, and HIV infections. They had no signs of acute virus hepatitis at the time of donation. Blood samples were centrifuged at  $900 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  and the plasma was recovered. Plasma samples were then prepared as aliquots on the day of collection and kept at  $-80^{\circ}\text{C}$  until used.

### 3.2. Enzyme Immunoassay (IEA) for the Detection of anti-HEV IgM and IgG Antibodies

Plasma samples were examined for the presence of anti-HEV immunoglobulins (IgM and IgG) using a commercially available enzyme-linked immunosorbent assay kit (Dia.Pro Diagnostic Bioprobes Srl, Milan, Italy), according to the manufacturer's instructions. The cut-off value was calculated according to the manufacturer's instruction. All the HEV IgM and IgG positive samples were retested and

then subjected to total RNA extraction and real-time polymerase chain reaction (RT-PCR) assay for further investigation.

### 3.3. Construction of Plasmid Containing HEV Sequence

A 700 bp synthetic fragment within HEV open reading frame 2 (ORF2) was cloned in pBHA vector (pBHA-ORF2) and used as a template for optimization of nested PCR and detection of HEV genome in clinical samples. By using the consensus complementary primers for the amplification of the synthetic fragment, each HEV genotype could be detected in plasma samples with the same PCR product size. The plasmid containing HEV ORF2 was also used for determining PCR sensitivity. A dilution series of  $10^{-1}$  to  $10^{-8}$  of this plasmid, equal to  $2 \times 10^8$  to  $2 \times 10^1$  copies of plasmid DNA/reaction, was used to determine the detection limit and sensitivity of the PCR assay.

### 3.4. Detection of HEV RNA and Genotyping of HEV

#### 3.4.1. RNA Extraction

Total RNA was extracted from plasma samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The extracted RNA was dissolved in 25  $\mu\text{L}$  RNase free tris-EDTA (TE) buffer and kept at  $-80^{\circ}\text{C}$  until used.

#### 3.4.2. First Strand cDNA Synthesis

Extracted RNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania) with random hexamer in a final volume of 20  $\mu\text{L}$  according to the manufacturer's protocol. The synthesized cDNA was directly used in PCR by addition of 2  $\mu\text{L}$  of the cDNA reaction mixture to a 25  $\mu\text{L}$  PCR reaction.

#### 3.4.3. Nested PCR Amplification

Nested PCR was performed to detect HEV from the prepared cDNA samples. Based on the multiple sequence alignment of ORF2 genes, two sets of degenerate HEV primers were used for the universal nested RT-PCR assay (19). The expected product of the universal nested RT-PCR was 348 bp. The PCR reaction mixture and thermal cycling conditions were identical to those described previously (19), except that the annealing temperature in both runs was  $58^{\circ}\text{C}$ .

The reaction products were then analyzed using 1.5% agarose gel electrophoresis and ethidium bromide staining. In addition, all products of expected size were gel-purified using a Bioneer Gel recovery DNA kit (Bioneer, Korea) and cloned using Fermentas InTA clone™ Cloning Kit (Fermentas, Lithuania) containing TA cloning vector pTZ57R/T.

All plasmids containing PCR products were sequenced to confirm that samples were positive for HEV. Sequences of the PCR products were determined for both DNA strands. For further investigation, sequences obtained from these PCR products of the ORF2 gene were analyzed and compared with the corresponding region of all strains available in the Genbank database (<http://www.ncbi.nlm.nih.gov>).

## 4. Results

### 4.1. Seroprevalence of Anti-Hepatitis E Virus IgG and IgM Among Blood Donors

Plasma samples from 700 blood donors were screened for the presence of HEV IgM and IgG antibodies. Of these 700 blood donors, there were 560 males (80%) with median age of  $44.5 \pm 15.3$  years and 140 females (20%) with median age of  $40.5 \pm 12$  years. Overall, 5/700 (0.71%) of the blood donors were seropositive for IgM and 42/700 (6.0%) were positive for HEV IgG antibodies. Anti-HEV IgM and IgG together were found in 3/700 (0.42%) of the samples. In this study the seroprevalence of total HEV IgM and IgG antibodies among blood donors was determined to be 7.1%. All the seropositive blood donors were male.

### 4.2. Sensitivity of Nested PCR

Sensitivity of the nested PCR assay was assessed by serial dilution of pBHA-ORF2. Sensitivity assessments demonstrated that the 348 bp HEV ORF2 fragment was detectable to a dilution of  $10^{-8}$ , which was equivalent to  $2 \times 10^1$  copies of plasmid DNA/reaction volume.

### 4.3. Detection and Genotyping of Hepatitis E Virus RNA

We did not verify the seronegative samples for the presence of HEV RNA. However, plasma samples of 50 seropositive blood donors were screened for the presence of HEV RNA by nested PCR. HEV RNA was detected in 4 out of 5 (80%) of the HEV IgM positive plasma samples and 2 out of 42 (4.8%) of the HEV IgG positive plasma samples. HEV RNA was also detected in one patient who was positive for both anti-HEV IgM and IgG antibodies. Overall, HEV RNA was detected in 7/50 (14%) seropositive patients, and the majority of IgM-positive individuals (Table 1).

Cut-off = Negative control mean OD 450nm + 0.350

### 4.4. Sequence Analysis of HEV Isolates

The sequences of a 348-bp region within the ORF2 gene fragment of seven HEV isolates from plasma samples of blood donors were determined. A BLAST search was carried out to confirm the identity of the strains. The isolates were then compared with different

**Table 1.** Characteristics of Blood Donors Testing Positive for Hepatitis E Virus RNA

Donor	Age, y	Anti-HEV IgM/OD, 450 nm <sup>a</sup>	Anti-HEV IgG/OD, 450 nm <sup>a</sup>
1	33	3.21 (+)	0.06 (-)
2	30	0.06 (-)	2.14 (+)
3	43	0.25 (-)	1.18 (+)
4	55	3.11 (+)	0.32 (-)
5	26	2.94 (+)	0.07 (-)
6	46	2.31 (+)	0.12 (-)
7 <sup>b</sup>	38	3.16 (+)	1.51 (+)

<sup>a</sup>IgG and IgM anti-HEV signals were considered according to measured sample-to-cut-off (S/CO) ratios, as follows: S/CO < 0.9 = negative; 0.9 - 1.1 = Equivocal, >1.1 = positive.

<sup>b</sup>Sample positive for both IgM and IgG antibodies.

HEV complete genome sequences available in GenBank (<http://www.ncbi.nlm.nih.gov>) including M73218, D10330, AF459438, AF076239, AF051830, X99441, AF185822, M80581, AF444002, L25595, D11092, D11093, and L08816 genotype 1 related sequences, and the other three HEV genotypes. Sequence analysis of all the isolates revealed 88% to 99% nucleotide sequence identity with HEV genotype 1.

## 5. Discussion

The overall seroprevalence of HEV IgG and IgM antibodies among blood donors was 7.1%, which is almost similar to data reported from other regions in Iran using the same enzyme immunoassay method (16-18). The lower number of seropositive donors from rural areas (26%) as compared to urban areas (74%) could explain the lower seropositivity (26%) for HEV antibodies among residents of rural Iran.

Subclinical infection of HEV has been reported in voluntary blood donors in different studies by detection of HEV RNA in donor blood samples. Pooled sera or plasma samples have been investigated for the presence of anti-HEV antibodies or HEV RNA. In several studies, HEV RNA was detected in blood donors with anti-HEV IgM and IgG (11, 20, 21). In one study, HEV RNA was detected in 67% of donors with anti-HEV IgM (20). However, it is possible to detect HEV RNA in plasma samples of blood donors who are negative for HEV antibodies as well. The follow-up samples of this group of blood donors demonstrated seroconversion after a while and confirmed recent HEV infection and viremia (11).

In our study, detection of HEV RNA in 7 of 50 seropositive blood donors demonstrates a relatively high incidence of HEV infection in this part of Iran. Evidently, the donors were asymptomatic, and the serological screening

test indicated that 4 of 7 individuals were HEV IgM positive, which indicates recent infection. Moreover, detection of HEV-RNA in two plasma samples positive for anti-HEV IgG antibody indicates that the donors were still in the acute phase of infection. Measurement of viral load would help to determine the severity of infection. We did not perform RNA detection among seronegative blood donors because it is likely that HEV RNA could be detectable even among this group of blood donors.

Pooled sera have been used in several studies for the screening of large numbers of blood samples. However, the dilution factor may affect the sensitivity of the assay. Because we tested each plasma sample separately, possible detection of a high number of positive sample for HEV antibodies and subsequently HEV RNA increased.

There are four genotype recognized within the genus of hepatitis E virus as species. Genotypes 1 and 2, which are exclusively identified in humans, include strains from Asia, Africa, and Mexico. Genotypes 3 and 4 include human and swine strains of HEV that have been found in humans and several species of animals in industrialized countries and Asia (particularly in China) (22, 23). There is a suggestion that variants differing in nucleotide sequence by > 20% in the ORF2 region should be classified into different genotypes (24).

In this study, sequence analysis of seven isolates of HEV RNA ORF2 region were aligned by HEV strains with the complete or nearly complete genome sequence available in GenBank. When we compared the percentage of identity of the sequences, all the sequences matched more than 80% with genotype 1.

Although HEV genotype 1 has been exclusively identified in humans, as the majority of urban residents are immigrants from rural areas, other sources of transmission especially in rural areas should also be considered.

In conclusion, the data indicates that the isolated HEV from blood donors in the southwest of Iran belongs to genotype 1. However, more samples from other parts of the geographical region are needed to confirm these finding. In addition to this, seronegative samples should also be tested for the presence of HEV RNA for further epidemiological study. At the molecular level, further genomic characterization of the particular HEV strains in the region is recommended.

Because transmission of HEV by administration of blood or blood products is likely to occur, it may be sensible to screen donor blood for the presence of HEV RNA to eliminate transfusion-transmitted HEV infection, especially when the recipient is immunocompromised.

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