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# Rapid detection of Hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers

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**Abstract**

A rapid reverse transcription-polymerase chain reaction (RT-PCR) procedure for the detection of Hepatitis E virus (HEV) RNA in serum is described. Total nucleic acids are extracted from a small volume of human serum and reverse transcribed using random hexamers. An aliquot of cDNA is then utilized in nested PCR employing degenerate HEV consensus primers. These primers are designed to sequences conserved between the Burma, Mexico, and US HEV strains, generating amplicons within each of the three open reading frames. Reactions are analyzed by agarose gel electrophoresis and samples showing an ethidium bromide stained band of the appropriate size in the first and second amplification, or in the second amplification only, are designated as positive. This protocol allows for the rapid and sensitive detection of HEV infection in human serum. 

*Keywords:* Hepatitis E virus; RT-PCR detection; Consensus primers

## 1. Type of research

1. Detection of HEV in human plasma (Erker et al., 1999; Wang et al., 1999);
2. HEV prevalence studies (Mateos et al., 1999);
3. Amplification of HEV sequences for genotypic analysis (Schlauder et al., 1999; Wang et al., 1999; Tsarev et al., 1999);
4. Studies to ascertain the association of HEV variants to non-A-GBV-C hepatitis (Schlauder et al., 1999; Wang et al., 1999);
5. Existence of non-human animal reservoirs (Clayson et al., 1996; Meng et al., 1998); and
6. Route of transmission of HEV infections (Pina et al., 1998; Singh et al., 1998)

## 2. Time required

For the entire protocol, approximately 7–9 h is required. Additional time may be needed depend-

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ing on the number of samples to be screened. The time for each step is estimated below (Section 4).

## 3. Materials

### 3.1. Special equipment

A 65°C water bath or incubator.
Microcentrifuge capable of 14 000 rpm.
DNA Thermocycler (Perkin–Elmer, Norwalk, CT 06859).
Apparatus for agarose gel electrophoresis.
UV Transilluminator/protective eye-wear.

### 3.2. Reagents

Total Nucleic acid extraction kit (United States Biochemical, Cleveland, OH)
Isopropanol.
Ethanol ( − 20°C).
Pellet Paint (Novagen, Madison, WI).
GeneAmp RNA PCR Kit (Perkin–Elmer, Norwalk).
HEV consensus PCR primers at a concentration of 10 μM each:
HEVORF1con-s1—5′ CTGGCATYACTACTGCYATTGAGC 3′
HEVORF1con-a1—5′ CCATCRARRCAGTAAGTGCGGTC 3′
HEVORF1con-s2—5′ CTGCCYTKGCGAATGCTGTGG 3′
HEVORF1con-a2—5′ GGCAGWRTACCARCGCTGAACATC 3′
HEVORF2/3con-s1—5′ GTATCGGKYKGAATGAATAACATGT 3′
HEVORF2/3con-a1—5′ AGGGGTTGGTTGGATGAATATAGGG 3′
HEVORF2/3con-s2—5′ CATGTCKTTTGCWKCGCCCATGG 3′
HEVORF2/3con-a2—5′ AKKGCGAAGGGCTGAGAATCAACC 3′
HEVORF2con-s1—5′ GACAGAATTRATTTCGTCGGCTGG 3′
HEVORF2con-a1—5′ CTTGTTCRTGYTGGTTRTCATAATC 3′
HEVORF2con-s2—5′ GTYGTCTCRGCCAATGGCGAGC 3′
HEVORF2con-a2—5′ GTTCRTGYTGGTTRTCATAATCCTG 3′
Taq DNA Polymerase Kit (Qiagen, Valencia, CA).
1 × TAE plus ethidium bromide (200 μg/ml final concentration).
Molecular Biology Grade Agarose.
6 × AGE loading dye (0.125% Bromophenol Blue, 0.125% Xylene Cyanole FF, 15% Ficoll).
Nucleic acid marker (ladders with markers between 100–400 bp aid in analysis).

## 4. Detailed procedure

### 4.1. Nucleic acid extraction (time: 1–1.5 h)

On ice, thaw a 25-μl aliquot of plasma or serum from the patient(s) to be tested. Transfer plasma to a labeled 1.5-ml microcentrifuge tube. To each sample, add 74.5 μl RNase-free/DNase-free water supplied in the Total Nucleic acid extraction kit, 0.5 μl pAW109 RNA from the GeneAmp RNA PCR kit, and 100 μl of Lysis solution. Also, similarly process a 25-μl aliquot of water to be used as a negative control. Vortex samples thoroughly. Add 500 μl of Extraction matrix (prepared as described by the manufacturer). Add 400 μl Extraction Buffer and vortex for 10 s.

Place at 65°C for 5 min. Vortex for 10 s. Place at 65°C for an additional 5 min.

Centrifuge in a microcentrifuge at 14 000 rpm for 5 min.

Transfer aqueous phase (top) to a new 1.5-ml microcentrifuge tube with care not to disturb the interface. Add 500 μl Extraction matrix to the aqueous phase. Vortex for 10 s.

Place in a microcentrifuge set at 14 000 rpm for 5 min.

Transfer aqueous phase (top) to a new 1.5-ml microcentrifuge tube with care not to disturb the interface. Add 0.1 volumes of NaOAc (approximately 20–40 μl, supplied in the extraction kit), 1 μl pellet paint and 1.0 volume of Isopropanol (220–440 μl). Vortex briefly.

Centrifuge at 14 000 rpm for 10 min. Remove isopropanol with care not to disturb the nucleic acid pellet. Wash with 400 μl cold 70% ethanol.

Centrifuge at 14 000 rpm for 5 min. Remove ethanol and air dry nucleic acid pellet at room temperature until there are no traces of ethanol (approximately 5 min).

Dissolve nucleic acid pellet in 3.75 μl nuclease-free water.

### 4.2. RT procedure (time: approximately 1 h)

Transfer 3.75 μl of nucleic acid to an appropriately labeled 9600 GeneAmp tube.

Make a RT master mix containing the following reagents from the GeneAmp RNA PCR kit: (volumes are given per reaction) 2.5 μl 10 × PCR II buffer, 5 μl 25 mM $MgCl_2$, 2.5 μl each 10 mM dGTP, dATP, dTTP, dCTP, 1.25 μl random hexamers, 1.25 μl RNase Inhibitor, and 1.25 μl reverse transcriptase (MuLV). Gently vortex to mix.

Add 21.25 μl of master mix to each sample. Pipet up and down several times to mix reagents.

Place sample in 9600 DNA Thermal Cycler set to cycle: 20°C for 10 min, 42°C for 30 min, 99°C for 5 min, followed by a 4°C soak.

### 4.3. PCR procedure (time: approximately 4 h)

Transfer 5 μl of cDNA into four fresh appropriately labeled 9600 GeneAmp tubes (one for each of the following HEV PCR primer sets: ORF1, ORF2/3, and ORF2 as well as one for pAW109 PCR to test the extraction and RT efficiencies).

Set-up PCR1 ORF1 master mix containing the following reagents: (Perkin–Elmer; volumes are given per reaction) 2 μl 10 × PCR II buffer, 1 μl 25 mM $MgCl_2$, 2.5 μl 10 μM HEVORF1con-s1, 2.5 μl 10 μM HEVORF1con-a1, 0.125 μl AmpliTaq Polymerase, and 11.875 μl $H_2O$. Gently vortex. Similarly, set up ORF2 master mix substituting HEVORF2con-s1 and HEVORF2con-a1 for the ORF1 consensus primers. Also, set-up a pAW-PCR master mix as above substituting 0.4 μl DM151 and 0.4 μl DM152 for the HEV primers, add an additional 4.2 μl $H_2O$ per reaction.

Set-up HEV PCR1 master mix for ORF2/3 containing the following reagents: (Qiagen; volumes are given per reaction) 2 μl 10 × PCR buffer, 5 μl Q solution, 2.5 μl 10 μM HEVORF2/3con-s1, 2.5 μl 10 μM HEVORF2/3con-a1, 0.125 μl AmpliTaq Polymerase, and 7.875 μl $H_2O$.

On ice, add 20 μl of the appropriate PCR1 or pAW-PCR master mix to the cDNA samples. Pipet up and down several times to mix reagents.

Place sample in a 9600 DNA Thermal Cycler preheated to 94°C and set to cycle as follows: 94°C for 1 min, 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 30 s. Follow with a 10-min 72°C extension and a 4°C soak.

Transfer 1 μl of PCR1 to a fresh appropriately labeled 9600 GeneAmp tube.

Set-up PCR2 ORF1 master mix as follows: (Perkin–Elmer; volumes are given per reaction) 2.5 μl 10 × PCR II buffer, 2 μl 25 mM $MgCl_2$, 0.5 μl each 10 mM dGTP, dATP, dTTP, dCTP, 2.5 μl 10 μM HEVORF1con-s2, 2.5 μl 10 μM HEVORF1con-a2, 0.125 μl AmpliTaq Polymerase, and 12.375 μl $H_2O$. Gently vortex. Additionally, set up a similar ORF2 master mix using HEVORF2con-s2 and HEVORF2con-a2 primers in place of the ORF1 primers.

Set-up PCR2 master mix for ORF2/3 as follows: (Qiagen; volumes are given per reaction) 2.5 μl 10 × PCR buffer, 5 μl Q solution, 0.5 μl each 10 mM dGTP, dATP, dTTP, dCTP, 2.5 μl 10 μM HEVORF2/3con-s2, 2.5 μl 10 μM HEVORF2/3con-a2, 0.125 μl AmpliTaq Polymerase, and 9.375 μl $H_2O$.

On ice, add 24 μl of PCR2 master mix to the appropriate sample. Pipet up and down several times to mix reagents.

Place samples in a 9600 DNA Thermal Cycler preheated to 94°C and set to cycle as for PCR1.

### 4.4. Agarose gel electrophoresis (time: 1.5 h)

Pour a 2% agarose gel by melting 2.0 g agarose in 100 ml 1 × TAE containing ethidium bromide. After the gel solidifies, submerge in 1 × TAE containing ethidium bromide.

Add 4.0 μl 6 × AGE loading dye to each PCR reaction (pAW-PCR, PCR1 and PCR2). Load 10 μl of each reaction along with the appropriate volume of molecular weight markers.

Electrophorese at a constant 100 V until bromophenol blue travels 75% of the gel length.

Visualize on an UV Transilluminator.

## 5. Results

If the nucleic acid extraction and RT reactions were successful, an ethidium bromide stained band of 308 bp should be present in each of the pAW-PCR reactions. Any sample that does not produce this product, or is weakly positive, should be reextracted regardless of the HEV RT-PCR results.

A sample is considered HEV positive if either of the following is true: (a) reaction produces the respective ethidium bromide stained bands for both PCR1 and PCR2 for any one set of reactions: approximately 418 and 287 bp (ORF1), 237 and 191 bp (ORF2/3), or 197 and 145 bp (ORF2); or (b) the desired product is not observed in PCR1, however, an appropriate ethidium bromide band is present in at least one of the PCR2 reactions: approximately 287 bp (ORF1), 191 bp (ORF2/3), or 145 bp (ORF2).

A sample is designated HEV RNA negative when ethidium bromide bands of the expected sizes are not observed for any of the PCR1 or PCR2 reactions.

Results are inconclusive when any of the following occurs: (a) the pAW-PCR fails as described above; (b) if the water control becomes positive in PCR1 or PCR2, any data generated is invalid due to possible contamination; or (c) samples which are positive in PCR1 but negative by PCR2 should be designated indeterminate until further testing can be performed.

These primer sets where shown to be sensitive to several HEV strains. When testing a panel of known HEV positive sera, four of four known Burmese-like sequences were identified with the use of all three primer sets. ORF1 and ORF2 were most sensitive to these samples, each detecting 75%. HEV-US1 and HEV-US2 sequences were each detected with all three primer sets. While screening sera collected from acute non-A-GBV-C hepatitis patients, not only were Burmese-like sequences amplified, but significantly divergent isolates from China, Italy, and Greece were identified (Schlauder et al., 1999; Wang et al., 1999). Sensitivity to Mexican-like sequences could not be determined as serum known to be infected with the Mexican isolate was unavailable for testing.

## 6. Discussion

### *6.1. Trouble shooting*

The base pair lengths given here are derived from a Burma isolate (GenBank Accession Number M73218). It is possible to identify products which are several bases longer or shorter, as HEV isolates have been identified with nucleotide insertions and deletions within the regions being analyzed (i.e. HEV-US1 and HEV-US2 possess a codon deletion within the ORF2/3 amplicon). These differences are typically not clearly resolved on a 2% agarose gel.

If samples continue to produce negative results for the pAW109 control RNA, it is possible that the RT and/or PCR reactions are being inhibited by material within the sample. Serial dilutions of the serum or RT reactions may reduce this inhibition significantly.

To avoid contamination, segregate the nucleic acid extraction and RT-PCR set-up from PCR product analysis. Ideally, use different laboratories for each procedure. If contamination does occur, it is often best to discard any reagents used for extraction, RT, and PCR.

While non-specific amplification was not generally observed, all PCR products were cloned and sequenced for verification. This is particularly important when screening samples which are anti-HEV IgG/IgM negative.

### *6.2. Alternative procedures*

These degenerate oligonucleotide primers have been shown to be specific and sensitive to significantly divergent strains of HEV. However, additional sensitivity can be obtained using touchdown PCR (Roux, 1995). While this procedure may allow the PCR primers to anneal more

readily, an increase in non-specific products was generally observed. Thus all products of the expected size need to be sequence confirmed.

## 7. Essential literature references

Original papers: Erker et al. (1999), Wang et al. (1999), Schlauder et al. (1999).

Book chapter: Mushahwar and Dawson (1997).

Review: Mast and Krawczynski (1996).

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