Detection of negative sense RNA in packaged hepatitis E virions using improved strand specific RT-PCR method

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Abstract

Current hepatitis E virus (HEV) negative sense RNA detection assays have drawback of false positivity. cDNA synthesis using tag-based primer and Superscript RT-III followed by exonuclease I treatment increased the specificity. Assay could detect as low as 10 copies of negative sense RNA and could be used in detecting low levels of HEV replication in cells. Virus particles in stool samples of hepatitis E patients showed encapsidation of negative sense RNA along with HEV genomic RNA.
Hepatitis E virus (HEV) is the major etiological agent of waterborne epidemics in India and significant proportion of sporadic hepatitis in adults. The genome of HEV is a single-stranded, positive-sense RNA molecule of ~7200 bases. Due to lack of cell culture system HEV replicons are being used in understanding virus replication (4, 6, 7, 8, 10). HEV replication proceeds via a negative sense intermediate (12, 13, 14) which acts as a template for synthesis of new positive-sense genomic RNA and subgenomic RNA molecules (8, 10). Detection of negative-sense RNA has been taken as evidence for viral replication. Several studies in HEV have used strand specific assays (3, 11, 13, 14, 16, 17, 19, 20) however except one (11) none have addressed the issue of specificity and accuracy of the correct strand detection. This study deals with various factors which might be responsible for false positive results in detecting negative-sense RNA.

**Generation of synthetic transcripts:**

Partial HEV ORF1 region (2987-5698nt, 2712bp) cloned in pGEMT-EASY vector (Promega) and HEV full length cDNA clone (pGEMT-EASY-HEVT1FG) were linearized and used as templates for in vitro transcription (Ribomax in vitro transcription kit, Promega). Template DNA was removed by two rounds of RNase free DNase treatment. Concentration of purified RNA was determined using Nanodrop (ND-1000) at 260nm.

**Reverse transcription-PCR:** Primers used in the present study are enlisted in the Table 1 and 2. RNA was denatured at 65°C for 5 min in the presence of 10 pmol primer and reverse transcription (RT) reactions were performed in 20µl volume containing 625µM dNTP’s, 40 units of RNase inhibitor and either AMV-RT (Promega) or Superscript RT-III (Invitrogen) with respective buffer conditions. Wherever mentioned, after RT cDNA was incubated with 50 units of exonuclease I (USB) at
37°C, 30 min and purified (QIAquick PCR Purification Kit, Qiagen). First round and second round PCR reactions were carried out for 40 and 30 cycles respectively.

Lack of strand specificity in detecting negative-sense RNA due to false priming during cDNA synthesis:

HEV diagnostic RT-PCR (2) was initially tested for strand specificity using synthetic full genome HEV RNA (gRNA) as a template for cDNA synthesis, in two sets of reactions containing- i) forward primer (HEVF1, negative-sense RNA detection), ii) reverse primer (HEVR1, positive-sense RNA detection). For PCR, nested HEV specific primer pairs, HEVF1/HEVR1 (PCR I, 718 bp) and HEVF2/HEVR2 (PCR II, 415 bp) were used. Amplifications were seen in all reactions with RT primer HEVF1 (figure 1, lanes 1, 4, 10, 13) and with no primer (figure 1, lanes 3, 6, 12, 15), indicating self priming of RNA with both $10^6$ and $10^3$ gRNA copies. Lack of amplification in the RT reactions carried out without reverse transcriptase confirmed- i) false priming during reverse transcription step, ii) absence of carryover contamination of DNA template used for in vitro transcription.

Tag based PCR to remove false priming:

The generation of falsely primed cDNA during RT step originate mainly from opposite strand (positive-sense) as they are always in excess as compared to negative-sense RNA intermediates in the cells. The false priming events can be attributed to secondary structures of RNA (18) or to small RNA molecules present in cellular RNA (9). HEVF1 and HEVR1 primers were modified at their 5’ ends by adding 19 nt non-HEV sequence (15) to generate HEVTagF1 and HEVTagR1. The assumption is to use modified primer for RT to generate cDNA with tag at its 5’ends which can be specifically amplified with tag primer and HEV specific reverse primer by PCR. Prerequisite was to remove RT primers by exonuclease I treatment and
column purification. Synthetic gRNA, 10^6, 10^5 and 10^3 copies were reverse transcribed using either HEVTagF1 or HEVTagR1 or no primer (figure 2). Non-specific amplification was absent with 10^3 copies (figure 2C). Reactions with AMV-RT containing 10^5 and 10^6 copies and HEVTagF1 showed non-specific amplification (lane 1, figure 2A and 2B). Reactions done with AMV-RT (42°C) containing 10^6 RNA copies showed amplification due to self priming (lane 3, figure 2A). However, with Superscript RT-III (55°C), non-specific amplifications were not observed indicating better specificity of the Superscript RT-III over AMV-RT at all tested template RNA concentrations (lanes 4-6 of figure 2A, 2B and 2C).

**Specificity and sensitivity of the strand specific assays:**

Ten-folds diluted synthetic positive- and negative-sense partial ORF1 RNA templates (10^10-10^7 copies) were subjected to strand specific assays separately in presence of 1µg of cellular RNA (extracted from Huh-7, human hepatocarcinoma cells with Ribopure kit, Ambion).

With positive-sense RNA, positive strand specific assay was positive till 10 RNA copies (figure 3A). Similarly, negative strand specific assay done on negative-sense RNA was positive till 10 copies (figure 3C). To see the specificity of each strand specific assay, reactions were performed using only opposite-sense RNA as templates. Negative strand assay reactions showed false priming in presence of 10^10-10^7 copies of positive-sense RNA (lanes 1-4, figure 3D). However, reactions were completely negative from 10^6-10 copies. When same set of reactions were carried out using only column purified cDNA without giving exonuclease I treatment, there was 100 folds increase in the non-specific positivity (figure 3E). With positive strand specific assay, done with negative-sense RNA, false positivity was seen till 10^5 copies (figure 3B) and reactions were negative below that.
Specificity of the assay with gRNA:

Taking into account that secondary structures of 5' and 3' non-translated regions in the RNA genome may lead to self priming, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$ copies of synthetic gRNA were subjected to negative strand detection in presence of 1µg of total cellular RNA. Amplification was seen in $10^7$ copies while remaining reactions were negative (figure 4). These observations were in agreement with the results obtained with partial ORF1 RNA and also showed that non-translated regions and presence of cellular RNA are not altering the specificity.

Negative strand assay in presence of positive-sense RNA:

To check the sensitivity of negative strand detection in presence of opposite strand, assays were performed on $10^5$, $10^4$, $10^3$, $10^2$, 10 copies of negative-sense RNA, in presence $10^5$ copies of positive-sense RNA and 1µg of cellular RNA. All samples were positive indicating that the standardized assays are sensitive to detect even 10 copies of negative sense RNA (figure 5).

Screening of HEV RNA positive stool samples:

Stool samples from 20 acute phase hepatitis E cases (anti-HEV IgM positive) (2) were used to prepare 10% suspensions, processed for viral RNA isolation (Viral RNA mini kit, Qiagen) and HEV RNA copy determination using Taqman Real-time PCR assay (1). RNA positive samples, 6/20, were processed further for negative strand specific detection. Samples showing $>10^5$ copies/11.5µl were diluted appropriately so that the input copy number does not exceed $10^5$ copies/ reaction. Three of the six samples (Figure 6, lanes 2, 4, 8) were positive indicating packaging of replicative RNA intermediates in the excreted mature virus particles. To rule out the presence of detached alimentary canal cells in the stool samples, three independent HEV RNA positive stool samples were processed for virus purification.
using sucrose gradient (5) and tested. All, 3/3 samples showed presence of negative-sense RNA (Figure 6, lanes 10, 11, 12). With these results it was clear that encapsidation of negative-sense RNA is a common phenomenon during HEV replication. All samples were negative when no primer was added during cDNA synthesis (data not shown).

In conclusion, use of tag primer for cDNA synthesis, followed by exonuclease I treatment and column purification of the cDNA products were found to reduce false positive signals significantly. Assay could detect as low as 10 copies of negative-sense RNA specifically even in presence of $10^5$ copies of positive-sense RNA and cellular RNA.

References:


**Figure Legends**

**Figure 1:** Lack of strand specificity in detecting negative sense RNA due to false priming during cDNA synthesis: \(10^6\) (lane 1-9) and \(10^3\) (lane 10-18) HEVT1FG RNA copies were reverse transcribed in presence of AMV-RT (lanes: 1, 2, 3, 10, 11, 12), Superscript RT-III (lanes: 4, 5, 6, 13, 14, 15), no enzyme (lanes: 7, 8, 9, 16, 17, 18). Lanes 1, 4, 7, 10, 13, 16 represent RT reactions with primer HEVF1, lanes 2, 5, 8, 11, 14, 17 represent RT reactions with primer HEVR1 and lanes 3, 6, 9, 12, 15, 18 represent RT reactions done with no primer. Ten microliter cDNA was used for the first round PCR and 10 µl of the first PCR product was used for the nested PCR.

**Figure 2:** Tag based PCR to remove false priming: Strand specific assays were performed on \(10^6\) (A), \(10^5\) (B) and \(10^3\) (C) full genome positive sense RNA copies using two reverse transcriptase enzymes. Each gel represents: lanes 1-3 with AMV-RT (lane 1: RT with HEVTagF1 primer, lane 2: RT with HEVTagR1 primer and lane 3: no primer). Lanes 4-6 with Superscript RT-III (lane 4: RT with HEVTagF1 primer, lane 5: RT with HEVTagR1 primer and lane 6: no primer). Lanes 7-9 reactions
without any RT enzyme (lane 7: HEVTagF1 primer, lane 8: HEVTagR1 primer and lane 9: no primer). Post reverse transcription and exonuclease I treatment, cDNAs were purified by silica based column. Samples without reverse transcriptase enzyme were processed similar to samples processed with RT enzymes.

**Figure 3: Specificity and sensitivity of the strand specific assays:** Synthetic positive sense and negative sense RNA were diluted 10 folds and RNA copies ranging from $10^{10}$ - 10 were subjected to strand specific PCR separately. The cDNAs were synthesized in presence of 1µg of total cellular RNA with Superscript Rt-III, treated with exonuclease I (whereever mentioned) for 30 min followed by silica based column purification. Ten microliter of cDNA was used for first round of PCR and 5µl of the first PCR product was further subjected to nested PCR. Lanes in all four gels represent, 1: $10^{10}$ RNA copies, 2: $10^{9}$ RNA copies, 3: $10^{8}$ RNA copies, 4: $10^{7}$ RNA copies, 5: $10^{6}$ RNA copies: 6: 100 bp Ladder, 7: $10^{5}$ RNA copies, 8: $10^{4}$ RNA copies, 9: $10^{3}$ RNA copies, 10: $10^{2}$ RNA copies, 11: 10 RNA copies, 12: No RNA

3A: Positive strand assay on synthetic positive strand with exonuclease

3B Positive strand assay on synthetic negative strand with exonuclease

3C Negative strand assay on synthetic negative strand with exonuclease

3D Negative strand assay on synthetic positive strand with exonuclease

3E Negative strand assay on synthetic positive strand without exonuclease

**Figure 4: Specificity of the assay with full length positive strand RNA:** Negative strand assay was performed on $10^{7}$, $10^{6}$, $10^{5}$, $10^{4}$, $10^{3}$ (lanes 1, 2, 3, 5, 6,
respectively) copies of HEVT1FG positive sense synthetic RNA in presence of 1µg of total cellular RNA. Nuclease free water served as negative control (lane 7).

Figure 5: Negative strand assay in presence of positive sense RNA: Negative strand assay was performed on $10^5$, $10^4$, $10^3$, $10^2$, 10 (lanes 1, 2, 3, 5, 6, respectively) copies of negative sense synthetic RNA in presence of $10^5$ copies of positive sense synthetic RNA and 1µg of total cellular RNA. Nuclease free water served as negative control (lane 7).

Figure 6: Screening of HEV RNA positive stool samples from acute hepatitis E cases: Negative strand detection was carried out on RNA isolated from- lane 1: stool sample 1 (539 copies of HEV positive sense genomic RNA/ reaction), lane 2 stool sample 2 (1x $10^5$ copies), lane 3: negative control, lane 4: stool sample 3 (1x$10^5$ copies), lane 5: stool sample 4 (619 copies), lane 6: negative control, lane 7: stool sample 5 (9591 copies), lane 8: stool sample 6 (1x$10^5$ copies), lane 9: 100bp ladder, lane 10: purified virus stock 1 (1.4x$10^4$ copies), lane 11: virus stock 2 (2653 copies), lane 12: virus stock 3 (1782 copies).
Table 1: Primers used for reverse transcription

<table>
<thead>
<tr>
<th>Primer</th>
<th>Negative strand assay</th>
<th>Positive strand assay</th>
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<tbody>
<tr>
<td>cDNA</td>
<td>HEVTagF1:</td>
<td>HEVTagR1</td>
</tr>
<tr>
<td>First PCR</td>
<td>Tag as forward primer</td>
<td>F1 as forward primer</td>
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<tr>
<td></td>
<td>R1 as reverse primer</td>
<td>Tag as reverse primer</td>
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<tr>
<td></td>
<td>R2 as reverse primer</td>
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Table 2: Sequence and locations of primers:

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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position (nt)</th>
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<tbody>
<tr>
<td>HEVF1</td>
<td>5'TGAGAATGATTCTCTGAGTTTG3'</td>
<td>4398-4420</td>
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<tr>
<td>HEVF2</td>
<td>5'ATACCGTCTGGAAACATGGC3'</td>
<td>4604-4622</td>
</tr>
<tr>
<td>HEVR1</td>
<td>5'ATGTTATTCACTCCACCCG3'</td>
<td>5116-5098</td>
</tr>
<tr>
<td>HEVR2</td>
<td>5'AGCATCCCAATCAGGTATG3'</td>
<td>5018-4999</td>
</tr>
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</table>
| HEVTagF1*   | 5'CGGTCATGGTGCGAATAG  
             | AGAATGTTTCTGATTTG3'            | 4398-4420     |
| HEVTagR1*   | 5'CGGTCATGGTGCGAATAG  
             | ATTCTGAGTTTG3'                 | 5116-5098     |
| Tag         | 5'CGGTCATGGTGCGAATAG3'         |               |

*Underlined sequence in HEVTagF1 and HEVTag R1 are non HEV sequences