Genotype 3 diversity and quantification of hepatitis E virus RNA

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ABSTRACT

Genotype 3 hepatitis E viruses (HEV) are distributed across the world and are now considered to be an emerging public health concern in industrialized countries. At least 10 genotype 3 subtypes have been identified in humans and animals worldwide. It was recently reported that the sensitivities of HEV RNA assays differ greatly. We have assessed the influence of genotype 3 diversity on the performance of two HEV RNA assays: one targeting the ORF3 gene and the other targeting the ORF2 gene. We tested a panel of 5 HEV-positive reference samples, genotype 3a, 3b, 3c, 3e and 3f, at 10-fold serial dilutions. The HEV RNA concentrations obtained with both RT-PCRs were correlated, but the RT-PCR based on ORF2 under-estimated the HEV RNA concentrations. The mean [ORF3 – ORF2] differences was 1.41 log c/ml. We also tested 34 clinical specimens, genotype 3c (n=15), 3e (n=4) and 3f (n=15), representing the most prevalent subtypes in Europe. The mean [ORF3 – ORF2] differences were 1.41 log c/ml for genotype 3c, 0.96 log c/ml for genotype 3e, and 0.70 log c/ml for genotype 3f. The bias between the 2 RT-PCR assays was significantly greater for genotype 3c than for genotype 3f (p=0.007). We therefore recommend using an RT-PCR protocol based on ORF3 to quantify HEV RNA of the genotype 3 strains.

Abstract word count: 214 (<250)

KEY WORDS: hepatitis E virus, RNA quantification, one step real-time RT-PCR, sensitivity
INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of acute or fulminant hepatitis in many resource-limited regions of the world. It is transmitted primarily by the faecal-oral route (36). It is now also considered to be an emerging concern in industrialized countries (8, 41). HEV is a non-enveloped single-stranded, positive-sense RNA virus and a member of the Hepeviridae family (10). Its genome consists of a single-stranded, positive-sense RNA, approximately 7.2 kilobases (kb) long, which is capped and polyadenylated (18). It contains a short 5’ untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3), and a 3’ UTR. ORF1 encodes non-structural proteins including methyltransferase, papain-like cysteine protease, helicase and RNA-dependent RNA polymerase (25). The ORF2 encodes the viral capsid protein. The ORF3 encodes a small, phosphorylated protein, 113 or 114 amino acids long, that is involved in virion morphogenesis and release (11, 12).

Analyses of the nucleotide sequences of HEV strains have revealed extensive genomic diversity leading to the identification of 4 main genotypes and several subtypes within each same genotype (31). HEV genotypes 1 and 2 are restricted to humans and associated with epidemics in developing countries, whereas HEV genotypes 3 and 4 are zoonotic and are responsible for sporadic cases. While genotype 4 is restricted to parts of Asia, genotype 3 is present worldwide (31). However, the HEV genotype 3 subtypes each have distinct geographic distributions. 3a and 3b strains are found more frequently in North America and Japan (31, 37), while genotype 3c, 3e and 3f strains are the most prevalent subtypes in European countries (28, 31, 42).
HEV genotype 3 is an emerging cause of acute and chronic hepatitis in immunocompromised patients (21, 26) and it poses concerns for food and environmental safety worldwide (35). The optimal diagnosis of an HEV infection relies on a combination of serological tests and nucleic acid amplification techniques (NAT) (9). An accurate quantitative assay of HEV RNA is also necessary for pathophysiological studies (22) and to monitor the HEV loads of chronically infected patients on antiviral therapy (2, 19, 20).

Several in-house conventional or real-time RT-PCRs based on the amplification of the ORF2 or ORF3 genes were recently evaluated (3). The investigators found that the sensitivities of the majority of assays differed greatly. We have therefore investigated the influence of genotype 3 diversity on the performance of two quantitative real-time RT-PCR: one based on amplifying a fragment within ORF2 and the other based on amplifying a fragment within the ORF3 gene.
METHODS

HEV reference strains panel
Genotype 3a, 3b, 3c, 3e and 3f strains were selected. The genotype 3a and 3b strains were provided by the Japanese Red Cross Hokkaido Blood Center and the genotype 3c, 3e and 3f strains were blood samples collected from patients with acute or chronic infections in the Midi-Pyrenees area, France (27, 32). Each strain was characterized by sequencing a 189nt ORF2 fragment, as previously described (28). Each sample was tested undiluted or diluted 1/10 and 1/100 in HEV-negative plasma. Each dilution was quantified in duplicate in 4 distinct runs.

Clinical specimens
We tested 34 plasma samples from patients contaminated in France: 15 from patients infected with HEV genotype 3c, 15 from patients with genotype 3f and 4 from patients with genotype 3e. Each strain was characterized by sequencing a 189nt ORF2 fragment, as previously described (28).

HEV RNA extraction
HEV RNA was extracted from blood samples (850 µl) with the Total Nucleic Acid Isolation (TNAI) kit on the Cobas Ampliprep instrument according to the manufacturer’s instructions (Roche Diagnostics, France).
Real time PCR based on ORF3

One-step real-time RT-PCR was performed with the Light Cycler 480 instrument (Roche Diagnostics, France). The primers and probes targeting the ORF2/ORF3 overlapping region were used to amplify a 70nt fragment: forward primer HEVORF3-S: 5'-GGTGGTTTCTGGGGTGAC-3', reverse primer HEVORF3-AS: 5'-AGGGGGTTGGTTGGATGAA-3' and probe 5'-Fam-TGATTCTCAGCCCTTCGC-Tamra-3' (17). For RT-PCR, the 50µL reaction mix contained 1µL SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), 15µl of RNA, primers (200nM) and probes (150nM), and 40U/reaction RNase Out (Invitrogen). Reverse transcription was carried out at 50°C for 15 min, followed by denaturation at 95°C for 1 min. DNA was amplified with 50 PCR cycles at 95°C (20s) and 58°C (40s). The amplification efficiency calculated with a standard curve was 2.02. The limit of detection was 100 copies/ml.

Real-time PCR based on ORF2

One-step real-time RT-PCR was performed with the Light Cycler 480 instrument (Roche Diagnostics) as previously described (26, 34). Primers and probes targeting a 140nt fragment within the ORF2 gene were: forward primer HEVORF2-S1: 5'-GACAGAATTRATTTCGTCGGCTGG-3', reverse primer HEVORF2-A2: 5'-CCCTTRTCTGCTGNGCATTCTCGACAGA-3' and probe: HEVORF2-S2: 5'-Fam-GTYGTCTCRGCAATGGCGAGC-Tamra-3' The 50µL RT-PCR reaction mix contained 1µL SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), 15µl of RNA, primers (200nM) and probes (150nM), and 40U/reaction
RNase Out (Invitrogen). Reverse transcription was carried out at 42°C for 15 min, followed by denaturation at 95°C for 1 min. DNA was amplified with 50 PCR cycles at 95°C (20s) and 60°C (1min). The amplification efficiency calculated with a standard curve was 1.96. The limit of detection was 100 copies/ml.

RNA standards
Two transcribed RNA standards were constructed from a patient infected with genotype 3f (accession number: EU495148); one was based on the amplification of a fragment within the ORF3 gene (70 nt) and the other based on the amplification of a fragment within the ORF2 gene (140 nt). Each of the resulting cDNAs was purified and inserted and cloned into the PCR-II vector using the Topo TA cloning for sequencing (Invitrogen). The positive clone, screened by colony PCR, was confirmed by digestion with a restriction enzyme and sequencing. The vector was cut down with EcoR I and cloned into the transcriptional vector pGEM.3Z by the same enzyme sites. The pGEM.3Z was linearized with Sma I and retro-transcribed by T7 RNA polymerase to obtain a positive strand for use as RNA standard in quantitative RT-PCR. The transcribed RNA standard was titrated by measuring optical density in a spectrophotometer. A standard curve was generated from serial 10-fold dilutions of the standard.

Statistical analysis
The Spearman test was used to test the correlation between the 2 RT-PCR assays. One-way ANOVA was used to test for difference of quantification between the 2 RT-PCR assays.
RESULTS

Reference strains panel

We assayed 5 samples, containing 3a, 3b, 3c, 3e and 3f strains, undiluted or diluted, with the ORF2-based real-time RT-PCR and with the ORF3-based real-time RT-PCR. The reproducibility of each RT-PCR assay was good (Table 1). The mean standard deviation was 0.14 log c/ml (range: 0.03-0.44 log c/ml) for the ORF2-based RT-PCR and 0.13 log c/ml (range: 0.07-0.21 log c/ml) for the RT-PCR based on ORF3.

However, only one of the eight 1/100 diluted genotype 3e samples was detected with the RT-PCR based on ORF2. Similarly, only 4 of the eight 1/100 diluted genotype 3c samples were detected with the RT-PCR based on ORF2. All the other samples scored positive with both assays (Table 1).

The virus loads in the 109 positive samples measured by each assay are shown in Figure 1. The ORF3 RT-PCR and ORF2 RT-PCR results were linearly associated ($R^2=0.52$) and correlated ($\rho=0.69$, $p<0.001$) (Figure 1). The ORF2 RT-PCR gave a mean HEV RNA concentration of 3.15 log c/ml and the ORF3 RT-PCR assay gave a mean of 4.56 log c/ml. The mean deviation between the ORF3 and the ORF2 RT-PCR results was 1.41 log c/ml. Bland-Altman analysis showed that differences in virus load were independent of the concentration of HEV RNA (Figure 2). However, the average deviation varied with HEV subtype. The mean [ORF3 – ORF2] differences was 2.54 log c/ml for the genotype 3e samples, 2.03 log c/ml for the genotype 3c samples, 1.09 log c/ml for the genotype 3f samples, 1.05 log c/ml for the genotype 3a samples and 0.34 log c/ml for the genotype 3b samples. HEV genetic polymorphism may generate mismatches between HEV RNA and primers or probes, which could contribute to under-
quantification of the RT-PCR based on ORF2 (Figure 3).

Clinical specimens

We also tested 34 clinical samples with both assays in order to eliminate any potential bias due to assaying only the 5 reference strains. They included the genotypes 3c (n=15), 3e (n=4) and 3f (n=15) that are the most prevalent genotype 3 subtypes found in Europe. The ORF3 RT-PCR and ORF2 RT-PCR results were correlated ($\rho=0.82$, $p<0.001$). The ORF3 RT-PCR gave a mean HEV RNA concentration of 5.40 log c/ml and the ORF2 RT-PCR assay a mean of 4.36 log c/ml. The mean deviation between the ORF3 and the ORF2 RT-PCR results was 1.04 log c/ml. The Bland-Altman analysis is shown in Figure 4. Again, the average deviation varied with the HEV subtype. The mean [ORF3 – ORF2] differences was 1.41 log c/ml for genotype 3c, 0.96 log c/ml for genotype 3e, and 0.70 log c/ml for genotype 3f. The average deviation between the 3 subtypes was significantly different ($p=0.009$). The bias between the 2 RT-PCRs was significantly greater for the genotype 3c samples than for the genotype 3f samples ($p=0.007$).
DISCUSSION

We have assessed the influence of HEV genotype 3 diversity on the performance of two HEV RNA quantitative assays. The RT-PCR assays were correlated, but we found substantial differences in the quantities of RNA of the main genotype 3 subtypes detected by the two real-time RT-PCRs.

Several real-time PCR protocols targeting the ORF3 region (13, 17, 40) or the ORF2 region (1, 16, 34, 47) have been developed over the past ten years for detecting HEV RNA. The performance of assays based on amplifying HEV RNA nucleic acid was recently investigated using a panel of HEV-containing plasmas (3). The panel comprised 22 HEV-positive plasma samples representing 10-fold serial dilutions of HEV subtypes 3a, 3b, 3f and 4c obtained from blood donors. Only two of the 20 laboratories that tested the panel used an RT-PCR protocol that targeted the ORF1 region of the HEV genome; the others targeted the ORF2 and ORF3 regions. The study demonstrated that real-time RT-PCRs are more sensitive than nested-PCRs, but the sensitivities of the majority of assays differed enormously (100-fold to 1,000-fold), independent of the virus strains (3).

Among protocols targeting ORF3, we selected the one developed by Jothikumar, because it is the most frequently used in the recent evaluation performed by Baylis et al. (17). Among protocols targeting ORF2, we selected the method developed by Mansuy et al., because it was previously used in our laboratory for HEV RNA detection and quantification (26, 34).

The great genetic diversity of RNA viruses makes it very difficult to design appropriate primers and probes for use in developing molecular diagnostic assays. The performance of quantitative assays for RNA viruses is influenced by their genetic...
diversity (15, 23, 43). For the 34 clinical samples, the sequence identity ranges from 83% to 90.4% between the different subtypes in the ORF2 region (data not shown).

Based on the 3c, 3e and 3f complete genome sequences available in Genbank, the sequence identity between the different subtypes ranges from 82.4% to 90% in the ORF2 region and ranges from 89.5% to 95.2% in the ORF3 region. This variability between subtypes has prompted us to assess its influence on HEV RNA quantification.

Our sequence alignments have shown several mismatches, mainly for the primers and the probe targeting ORF2. Nevertheless, ORF2 assay showed a similar sensitivity in detecting genotype 3a and 3f standard preparations despite a higher numbers of mismatches between the ORF2 primers and probe and the 3a strain than between the ORF2 primers and probe and the 3f strain. This may be because other critical parameters such as RNA conformation could be similar for the two Taqman detection systems.

We evaluated the capacities of two different TaqMan real-time RT-PCRs to detect and quantify HEV genotype 3 subtypes. Our assays of serial 10-fold dilutions of genotypes 3a, 3b, 3c, 3e, and 3f reference samples indicated that the ORF2-based RT-PCR was less sensitive than the ORF3-based RT-PCR. The RT-PCR based on ORF2 rarely detected the 100-fold diluted 3c and 3e samples. And our assays of several clinical plasma samples showed that the RT-PCR based on ORF2 significantly underestimated the HEV RNA concentration in genotype 3c samples compared to the genotype 3f samples. These data agree well with the findings of Ward et al. (46), who studied the performance of 4 real-time RT-PCRs based on ORF3 and ORF2 by testing Canadian swine genotype 3 samples. They compared the Ct values obtained with the real-time RT-PCR tests and found that the RT-PCR test based on ORF3 was the most...
sensitive for detecting swine HEV strains (46). Unfortunately, they did not determine the
HEV subtypes and their assays were only semi-quantitative.

Our study comparison of two HEV RNA assays was focused on HEV genotype 3, the most prevalent genotype in industrialized countries. However, the primers and the probe of the RT-PCR based on the ORF2 region, adapted from our previous in-house protocol (26, 34), was designed in order to detect the 4 main genotypes of HEV. They were used to conducted several studies on HEV infections (19-21, 26, 32-34) and it allows us to detect HEV genotype 1 and 4 infections (32). Similarly, we have used the primers and the probes targeting the ORF3 region designed by Jothikumar et al. (17). They have validated on several samples the capacity of these primers to detect HEV isolates representing genotypes 1 to 4. However, further studies are needed to address if the quantitative HEV RNA assays are influence by the HEV genetic diversity for all the genotypes.

The extraction protocol can affect the sensitivity of molecular tests (4, 14), but we used automated extraction on the Cobas Amplicrep instrument to extract the HEV RNA used for both assays. This automated extraction is more reliable, standardized, reproducible, and time-saving than manual extraction for preparing nucleic acids (24, 44). This procedure also limits the risk of cross-contamination in the laboratory.

The diagnosis of an HEV infection in a patient requires accurate and sensitive tools. Because serological tests may lack sensitivity (9, 29), detecting virus genomic RNA in the serum or stool by RT-PCR is a crucial marker of an acute or a chronic HEV infection. The ubiquitous nature of HEV genotype 3 in domestic pigs and wild boar also raises public health concerns for zoonotic infection through direct contacts with infected animals (5, 7, 38), or the consumption of contaminated animal products (6, 30, 45). In
addition, genotype 3 HEV-contaminated coastal, drinking, irrigation and sewage water could all be sources for human infections (35). This reinforces the need for a highly sensitive RT-PCR protocol for detecting HEV in food or water samples. The development of sensitive assays may reveal that HEV is far more common in industrialized countries than was previously thought (39). Additionally, an underestimation of HEV RNA quantification could result in false negative results in treated immunocompromised patients (2, 19, 20), and thus may be the cause of a relapse if the treatment is stopped too early.

In conclusion, our findings indicate that an RT-PCR protocol based on ORF3 provides the most suitable tool for assaying the HEV RNA of the genotype 3 strains.
REFERENCES


Figure 1: HEV RNA concentrations in the 3a, 3b, 3c, 3e and 3f reference strains measured by the ORF3 and ORF2 RT-PCRs.

Figure 2: Bland-Altman plot for bias analysis between RT-PCRs based on ORF3 and ORF2 for the 3a, 3b, 3c, 3e and 3f reference strains panel.

Figure 3: Alignment of reference sequences showing the positions of the primers and probes in the HEV ORF2 (A) and HEV ORF3 regions (B). Nucleotides in grey indicate mismatches with the primer or probe. Numbers refer to the corresponding nucleotide position of the HEV virus (GenBank accession number M73218).

Figure 4: Bland-Altman plot for bias analysis between the RT-PCRs based on ORF3 and ORF2 for the 34 clinical samples.
Table 1: HEV RNA results for the 3a, 3b, 3c, 3e and 3f strains panel

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Note: SD, standard deviation; CV, coefficient of variation; * Mean standard deviation; ** mean coefficient of variation; †mean difference
Figure 1: HEV RNA concentrations in the 3a, 3b, 3c, 3e and 3f reference strains measured by the ORF3 and ORF2 RT-PCRs.
Figure 2: Bland-Altman plot for bias analysis between RT-PCRs based on ORF3 and ORF2 for the 3a, 3b, 3c, 3e and 3f reference strains panel.
Figure 3

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3b-AB630971  
3c-JN398372  
3e-JN398371  
3f-JN398370

3b-JN398371  
3c-JN398372  
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3f-JN398370

Figure 3: Alignment of reference sequences showing the positions of the primers and probes in the HEV ORF2 (A) and HEV ORF3 regions (B). Nucleotides in grey indicate mismatches with the primer or probe. Numbers refer to the corresponding nucleotide position of the HEV virus (GenBank accession number M73218).
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