Comparison of HEV RNA detection assays

Comparison of real-time reverse transcriptase (RT)-PCR assays for detection of swine hepatitis E virus in fecal samples

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Comparison of HEV RNA detection assays

Abstract

Hepatitis E virus (HEV) is a major cause of acute viral hepatitis in people in many developing countries and is also endemic in many industrialized countries. Mammalian HEV (mHEV) isolates can be divided into at least four recognized major genotypes. Several nucleic acid amplification techniques have been developed for mHEV detection with great differences in sensitivity. The aim of this study was to compare the performance of two single-plex real-time reverse transcriptase (RT)-PCR assays for broad detection of all four mHEV genotypes (assays A and B) and two duplex real-time RT-PCR assays for detection and differentiation of mHEV genotypes 3 and 4 (assay C and D). RNAs extracted from 28 fecal samples from pigs experimentally inoculated with HEV genotype 3 and 186 fecal samples from commercial pigs with unknown HEV exposure were tested by all four assays. In experimental samples, HEV RNA was detected in 96.4% (assay A), 39.2% (assay B), 14.2% (assay C), and 0% (assay D) of the samples. In field samples with unknown HEV exposure, HEV RNA was detected in 67.2% (assay A), 36.4% (assay B), 1.1% (assay C), and 0.5% (assay D) of the samples. Assays showed an overall poor agreement ($\kappa = 0.19$ to 0.03) with differences in detection rates between assays ($p < 0.01$). Assays A and B that broadly detect HEV genotypes 1-4 had significantly higher detection rates for HEV RNA than the duplex assays C and D that were both designed to detect and differentiate between HEV genotypes 3 and 4.

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Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans (1). HEV infection in pregnant women may cause particularly severe illness with a mortality rate of 10-20%, and recently there are numerous reports of persistent and chronic HEV infection in immunocompromised patients such as organ transplant recipients (2). Currently, HEV is classified in the genus *Hepevirus* in the *Hepeviridae* family (3). The virus is a non-enveloped, positive-sense, single-stranded RNA virus that encodes three open reading frames (ORFs). ORF1 encodes for non-structural proteins, ORF2 encodes the viral capsid, and ORF3, which overlaps with ORF2, encodes a multi-functional small protein (3).

HEV has been identified in several animal species including domestic pigs, chickens, deer, wild boars, mongooses, rabbits, rats, ferrets, bats, and fish (4) and, based on the host tropism, the strains genetically identified thus far can be clustered into mammalian HEV (mHEV), avian HEV (aHEV), and in piscine HEV (pHEV) strains. Within mHEV, there are at least four recognized genotypes capable of infecting humans. Genotypes 1 and 2 are associated with epidemics and restricted to humans in developing countries, whereas genotypes 3 and 4 can infect a wide variety of species including humans and pigs, and are associated with sporadic and cluster cases of human hepatitis E in both developing and industrialized countries (2). While mHEV genotype 3 has worldwide distribution (5), genotype 4 was reported in Asia (5), and more recently in Europe (6,7). In humans, infections with genotypes 1 and 2 are mainly transmitted via consumption of water contaminated with feces while infections with genotypes 3 and 4 appear to occur primarily by food-borne zoonotic transmission through the consumption of raw or undercooked meat from pigs, wild boars or deer (4).

Due to its implication in public health and pork safety, several nucleic acid amplification techniques and immunoassays have been developed for mHEV detection; however, a reliable
Comparison of HEV RNA detection assays

diagnostic procedure for mHEV is still needed (1,8). Serological studies comparing immunoassays widely used for mHEV diagnosis found 2.9- to 6.5-fold variation in anti-HEV antibodies detection rates (9-11), and only two of six commonly available IgM anti-HEV detection assays had sensitivities and specificities above 95% (11). Due to this overall low sensitivity, a combination of antibody detection and nucleic acid detection has been suggested for optimizing mHEV diagnosis (12,13).

Considering the heterogeneity of mHEV strains circulating in humans and other animal species, several conventional reverse transcription (RT)-PCR and real-time RT-PCR assays have been developed for the detection of HEV RNA in various types of samples including sera, feces and environmental samples (14-19). Comparisons of RT-PCR assays have shown a 10 to 1,000-fold variation in sensitivity when samples were tested in parallel in the same laboratory (20,21).

In a blinded study to investigate the performance of conventional and real-time RT-PCR assays in 20 laboratories that performed HEV RNA detection on a regular basis, variations in sensitivity in the order of 100- to 1,000-fold were found using a standard panel of HEV genotype 3 and 4 strains (12). Currently, a real-time RT-PCR designed in 2006 (17) is the most widely used assay for detection of HEV infection in humans (12,22) primarily based on the reported high sensitivity (limit of detection of 4 genome equivalents of HEV genome) and its ability to detect all four recognized mHEV genotypes that are capable of infecting humans (23).

Although real-time PCR assays targeting conserved regions can provide accurate detection of the HEV genomes and yield results more rapidly compared to conventional RT-PCR, commonly a second molecular method such as sequencing or subtyping is required to further characterize strains. Recently, a real-time duplex RT-PCR assay for detection and identification of HEV genotype 3 and 4 in amounts as low as 50 genomic equivalents copies per
Comparison of HEV RNA detection assays

reaction has been reported (24). This assay, targeting the ORF2/ORF3 overlapping region, was
designed to allow for a sensitive and rapid detection of the zoonotic HEV genotypes to
potentially facilitate epidemiological investigations and to better understand outbreak situations.
The aim of this study was to compare the performance of two single-plex real-time RT-PCR
assays for broadly detection of all 4 recognized mHEV genotypes (assays A and B) and two
duplex real-time RT-PCR assays for detection and differentiation of mHEV genotypes 3 and 4
(assay C and D). Each single-plex and one duplex real-time RT PCR assays had been previously
described while the other single-plex assay is an in-house assay.

MATERIAL AND METHODS

Experimental samples. The experimental protocol was approved by the Virginia
Polytechnic and State University Institutional Animal Care and Use Committee and by Virginia
Polytechnic and State University Institutional Biosafety Committee. Twenty-eight serial fecal
samples were collected daily from two pigs experimentally inoculated with human HEV
genotype 3 strain US-2 (GenBank accession number AF060669) or swine HEV genotype 3 strain
Meng (GenBank accession number AF082843) from day post-inoculation (dpi) 2 to 14. The
fecal samples were suspended in saline (10% w/v), and the fecal suspensions were stored −80 °C
until use.

Field samples. A total of 186 pig fecal samples were chosen arbitrarily from routine
diagnostic cases submitted during May 2013 to the Iowa State University Veterinary Diagnostic
Laboratory (ISU-VDL). These samples originated on 86 farms located in 12 US states including
Iowa, Illinois, Indiana, Minnesota, Missouri, North Carolina, North Dakota, Nebraska, Ohio,
South Dakota, Texas, and Wisconsin. The fecal samples were obtained from different age groups
Comparison of HEV RNA detection assays

of pigs: suckling (1-2 weeks of age), nursery (3-7 weeks of age) and grow-finish pigs (8-25
weeks of age).

**Sample processing and RNA extraction.** Fecal samples of ~1 g were resuspended in
phosphate buffered saline (PBS), vigorously vortexed and centrifuged at 1500 × g for 10 min to
obtain a final 10% fecal suspension in PBS (w/v). Viral RNA extraction was carried out on 50 µl
of the fecal suspension using a MagMAX 96 Viral Isolation kit (Ambion, Foster City, CA, USA)
according to the manufacturer’s instructions on an automated extraction platform (KingFisher
Flex; Thermo Fisher Scientific). Negative controls, using DNA and RNA free, sterile water as a
sample, and positive controls, using fecal suspensions from pigs experimentally infected either
with mHEV genotype 3 or 4, were added to each extraction plate. The extracted RNA was stored
at −80°C until use.

**Primers and probes.** All primers and probes used in this study are listed in Table 1.
Primers and probes from assays B and D developed in this study were designed manually based
on a multiple sequence alignment of mHEV genotypes 1-4 in GenBank. Sequences were aligned
using CLUSTAL W within DNASTAR (Lasergene 8). A pair of primers
(HEV5606F/HEV5427DR) and a probe (HEVGenP) located in the conserved ORF2/ORF3
overlapping region broadly reactive with mHEV genotypes 1-4 were designed (assay B).
Additionally, probes specific for the detection of mHEV genotypes 3 or 4 (HEVg3 and HEVg4;
assay D) were designed in this same region. Oligonucleotide primers/probes were analyzed for
the absence of possible hairpins and dimers by Primer Express software (Version 3.0; Applied
Biosystems).

**Construction of plasmid DNA standards for the real-time RT-PCR reactions.**
Plasmid DNA standards were constructed by amplifying a genomic region at nucleotide
Comparison of HEV RNA detection assays

positions 5311 to 5471 of a genotype 3 human HEV strain (US-2) (25) and a region at nucleotide
positions 5285 to 5445 of a genotype 4 human HEV strain (TW6196E) (26) using primers
JVHEVF and HEV5427DR described in Table 1. Conventional RT-PCR reactions were carried
out in a total volume of 20 μl using the QIAGEN® OneStep RT-PCR kit (Qiagen, Valencia, CA,
USA) according to the manufacturer’s recommendations. Purified PCR products were cloned
into the pGEM-T Vector (Promega, Madison, WI, USA) and transfected into TOP10 Escherichia
coli bacteria (Invitrogen, Foster City, CA, USA) following the instructions of the cloning kit
manual. Sequencing was performed on recombinant plasmids in both directions using the AB
3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Iowa State University
DNA Facility (Ames, IA, USA). The recombinant plasmid stocks were quantified using the
NanoDrop spectrophotometer ND-1000 according to the manufacturer’s instructions (NanoDrop
Technologies Inc., Wilmington, DE, USA) and converted into genome copy numbers. The total
numbers of genome copies in the plasmid stock was calculated as copy number = [(concentration
of linearized plasmid)/(molar mass)] × (6.023×10²³). The plasmid DNA was used to generate
standard curves using 10¹ to 10⁸ genomic equivalent (GE) copies of plasmid. The GE titers of
HEV were determined based on the standard curve.

Real-time RT-PCR assays. The real-time RT-PCRs were carried out in 96-well plates
using the TaqMan One-Step RT-PCR Master Mix Reagent (Applied Biosystems, Foster City,
CA, USA) in a 25 μl volume comprising 5 μl of extracted RNA and 20 μl of master mix
according to the manufacturers’ recommendation. All four assays (Table 1) were performed on
the same day, and the same nucleic acid extracts were utilized. Single-plex assay B, capable of
detecting mHEV genotypes 1-4, and duplex assay D, capable of detecting and differentiating of
mHEV genotypes 3 and 4, utilized both the same forward and reverse primers. The
Comparison of HEV RNA detection assays

Concentrations of the primers and probe or probes (duplex assays) were 400 and 200 nM for assay A; 800 and 200 nM for assay B; 400 and 200 nM for assay C; and 800 and 400 nM for assay D. One-step RT-PCR amplification was performed on an ABI 7500 real time PCR instrument (Applied Biosystems, Foster City, CA, USA) under the following conditions: 15 min at 50°C for the RT reaction, 10 min at 95°C followed by 45 cycles at 95°C for 15 sec for denaturation and 60°C for 45 sec for annealing and extension. A sample was considered negative if the cycle threshold (Ct) was ≥41 amplification cycles. Quality control of the real-time RT-PCR process included negative (nuclease-free water) and positive (HEV RNA and HEV plasmid DNA) controls added to each PCR plate.

Efficiency, limit of detection, intra-assay and inter-assay precision of the RT-PCR assays. Verification of assays sensitivity, specificity and precision were performed as proposed elsewhere (27). Standard curves of mHEV genotypes 3 and 4 ranging from 10^1 to 10^8 copies of HEV plasmid DNA were used to determine the efficiency, limit of detection, intra-assay and inter-assay precision of the real-time PCR assays. For assays A and B, which do not differentiate HEV genotypes, standard curves for genotypes 3 and 4 were tested separately. For assays C and B, which differentiate between HEV genotypes 3 and 4, the standard curves were tested as duplex assays or separately in single-plex assays. The amplification efficiency (E) for each assay was calculated according to the formula $E = \left[10^{-\frac{1}{S}}\right] - 1$ to determine the performance of qPCR, where S indicates the slope (S) of the regression line. The limit of detection of each assay and the intra-assay variation were assessed with the standard curves tested in triplicate. Limit of detection was specified as the lowest amount of DNA standard that could be detected with 100% probability. The inter-assay variation was determined by three independent runs of the standard curves in triplicate.
Conventional nested RT-PCR. Twenty field samples tested by all four real-time PCR assays that presented discrepant results, defined as a sample that exhibit a positive result in one assay but a negative result in another assay, were arbitrarily chosen for sequencing confirmation using a nested RT-PCR assay based on a partial HEV ORF2 fragment. Conventional nested RT-PCR were performed using primers previously described (28) (Table 1). Briefly, for the first PCR reaction 6 μM each of primers 3156N and 3157N and QIAGEN® OneStep RT-PCR kit (Qiagen, Valencia, CA, USA) were used. The thermal cycler conditions for the first reaction were as follows: 50°C for 30 min, 95°C for 15 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final elongation step at 72°C for 10 min. The second reaction was performed with 0.2 μM each of primers 3158N and 3159N and ReadyMix® Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA). The thermal cycler conditions for the second PCR reaction were as follows: 95°C for 5 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final elongation step at 72°C for 7 min. The 348 bp second round PCR products were visualized after electrophoresis on a 1% agarose gel.

Sequencing and phylogenetic analysis. Sequencing of PCR products from HEV RNA positive samples was performed directly on both strands at the Iowa State University DNA Facility, Ames, Iowa, USA. Sequences were aligned with published data using BLAST at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Sequences were compiled using the Lasergene software and the Clustal W alignment algorithm (DNAStar, Madison, WI, USA). The nucleotide distance of the sequences was evaluated by neighbor-joining (NJ) using Lasergene MegAlign. Confidence in the NJ tree was estimated by
bootstrap replicates. Sequences reported in this paper have been deposited in the GenBank database under the accession numbers KF719308 to KF719310.

**Statistical analysis.** Inter and intra-assay variances were computed using the Ct values, standard deviations and coefficient of variation of the standard curves. The variance was analyzed by a one-way repeated measures analysis of variance followed by Bonferroni’s test for pairwise comparison. Cochran’s Q test for matched data, followed by McNemar’s test for pairwise comparisons were used to determine whether the proportions of RT-PCR positive samples were significantly different between assays. Differences between groups were considered significant if \( p < 0.05 \). A Kappa index was performed to determine the agreement of positive and negative results between assays. The strength of agreement was considered \( \leq 0 \) = poor, \( 0.01-0.2 \) slight, \( 0.21-0.4 \) = fair, \( 0.41-0.60 \) = moderate, \( 0.61-0.80 \) = substantial, and \( 0.81-1 \) = almost perfect as previously described (14). Statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA).

**RESULTS**

For assays C and B, which differentiate between HEV genotypes 3 and 4, there was no difference between the standard curves tested as duplex assays or single-plex regarding PCR efficiency, limit of detection and intra- inter-assay precision, therefore only results of the duplex assays were presented. A purine-pyrimidine mismatch was identified in the probes used to detect HEV genotype 4 for both assays C (base 19) and D (base 17) compared to the strain used as control (5389 C/A in the virus/probes).

**Evaluation of real-time RT-PCR assays.** Standard curves were established for each real-time PCR assay using the HEV genotypes 3 and 4 DNA controls serially diluted from \( 1 \times 10^4 \) to
Comparison of HEV RNA detection assays

1 × 10^1 copies and amplified in triplicate. Efficiency, regression coefficient, slope and intercept for each assay are shown in Table 2. Assays A and B had a similar performance regardless of the HEV genotype used while assays C and D presented a decrease in efficiency of at least 11% when HEV genotype 4 was used (Table 2).

Limit of detection and inter- and intra-assay precision of the four real-time PCR assays. For each assay, inter-assay precision was assessed by calculating the standard deviation and coefficient of variation of Ct obtained for each standard dilution tested in three independent runs, the coefficient of variation was found to be < 7% for all assays (data not shown). Intra-assay precision was assessed by calculating the standard deviation and coefficient of variation of Ct obtained for each standard dilution tested in triplicate, the coefficient of variation was found to be < 4% (Table 3). Limit of detection of each assay was specified as the lowest recognized concentration of genotypes 3 or 4 mHEV DNA control serially diluted from 1×10^5 to 1×10^1 in triplicate (Table 3). However, the GE copy numbers do not reflect the number of RNA molecules, since the efficiency of the RT reaction was not directly determined.

Variation of detection limits was in order of 10 to 1,000-fold among assays. Assay A was able to detect 10^1 GE copies of the plasmid HEV DNA per reaction (4×10^3 copies ml^-1), assay B detected all 10^2 GE copy dilutions (4×10^4 copies ml^-1) and occasionally the 10^1 GE copies dilution, and assays C and D were able to detect all HEV DNA standards down to the 10^4 GE copy dilutions (4×10^6 copies ml^-1) (Table 3). Impact of HEV genotype was observed for assays C and D that could detect all 10^2 GE copy dilutions of genotype 3, but only 1/3 10^3 GE copy dilutions of genotype 4. Assays A and B detection was genotype independent.
Detection of HEV RNA in experimental samples with known mHEV exposure by the four real-time RT-PCR assays. Detection of mHEV RNA in experimental samples evaluated in parallel using the same RNA extracts are shown in Fig. 1. The GE titers of mHEV were determined from the HEV genotype 3 standard curve included in each run and for each assay. Considering RNA detection over time, assay A presented the highest rate of cumulative positive detection (96.4%, 27/28) ($p < 0.05$), followed by assay B (39.2%, 11/28), which presented a positive detection rate higher than assays C (14.2%, 4/28) and D (0/28) ($p < 0.05$). The detected viral RNA loads ranged from 3.62 to 7.16 log$_{10}$ HEV GE copies ml$^{-1}$ 10% fecal samples for assay A; from 3.22 to 5.26 log$_{10}$ HEV GE copies ml$^{-1}$ in 10% fecal samples for assay B, and from 4.68 to 4.81 log$_{10}$ HEV GE copies ml$^{-1}$ for assay C (Fig. 1). In order to further investigate the reason of the low detection rates found with assay C and the lack of detection of any positive sample with assay D, the primer and probe sequences from each assay were compared to the genome sequence of each of the HEV strains used. Mismatches were not identified for any primer or probe (data not shown) indicating that the detection rates achieved were due to intrinsic differences in the limit of detection for each assay.

Detection of HEV RNA in field samples with unknown mHEV exposure by each of the four real-time RT-PCR assays. The rates of HEV RNA detection with the four assays on field samples are summarized in Table 4. The overall detection rate of HEV RNA positive samples regardless of age was 67.2% (125/186) for assay A, 36.4% (68/186) for assay B, 1.1% (2/186) for assay C and 0.5% (1/186) for assay D. Assays showed an overall poor agreement ($\kappa = 0.19$ to 0.03) with difference in detection rates between assays ($p < 0.01$). Assay A presented the highest HEV RNA detection rate ($p < 0.01$). All positive
samples with assays C and D were also positive with assays A and B. Regarding positive samples with assay B, 80.8% (55/68) were also positive by assay A, indicating that assay B identified additional 7.0% (13/186) positive samples that were not identified by assay A. However, assay A identified an additional 37.6% (70/186) positive samples that were not identified by assay B ($\kappa = 0.19, p < 0.01$).

Further evaluation of 20 field samples with discrepant results among real-time RT-PCR assays. Twenty samples with Ct values lower than 36 on the real-time RT-PCR assay were arbitrarily selected for amplification with a conventional nested RT-PCR assay followed by sequencing to further verify the results (Table 5). Twelve of 20 (60%) samples positive in at least one real-time RT-PCR assay were also positive in the nested RT-PCR, and 3/12 samples that were successfully sequenced were determined to be mHEV genotype 3 (Table 5).

DISCUSSION

In this study, two single-plex real-time RT-PCR assays for detection of all four known mammalian HEV genotypes without differentiation and two duplex real-time RT-PCR assays for detection and differentiation of HEV genotypes 3 and 4 were evaluated. All assays were compared on the same real-time RT-PCR instrument at the same day, using the same RT-PCR enzymes, standard curves and nucleic acid extracts. Under these conditions, single-plex assays A and B designed to broadly detect HEV genotypes 1-4 showed a significantly better performance ($p < 0.01$) than duplex assays C and D which both allow detection and differentiation of HEV genotypes 3 and 4. On field samples the single-plex real-time RT-PCR assays detected at least 34-fold more positive samples than the duplex real-time RT-PCR assays.
Design of a broadly reactive assay for detection of mHEV genotypes is a complex and challenging task due to the heterogeneity among the various HEV strains (5,29). Sensitivity of real-time assays can vary widely depending on target region and HEV genotype (20,21). Previous comparison of conventional and real-time RT-PCR assays to detect HEV RNA have shown that targeting a more conserved region as ORF2/3 appears more reliable than the use of degenerate primers and probes targeting a less conserved region such as ORF2 (20,21,30). In this study, all the real-time RT-PCR assays used target the overlapping region of ORF2 and ORF3; however, with the exception of assay A, the assays B, C and D used degenerate primers and probes. Degeneracies may reduce the sensitivity and specificity of an assay due to factors such as a lower effective concentration of each primer or difficulties in estimating the suitable annealing temperatures and primer lengths (31), which could partially explain the poor results achieved with assays B, C and D when compared with assay A in the present study. Analysis of full-length genomes of various human and animal mHEV strains revealed that the HEV genomes vary even in the conserved regions (5,29) and this genetic variability complicates a reliable detection of different mHEV genotype and subtypes. In fact, recent studies found polymorphisms in the probe-binding site region of the most widely used real-time RT-PCR assay for HEV detection (assay A) (17), and a modification of the probe and subsequent increase of the melt temperature, restored detection of the polymorphic strains (22,23). Employment of more than one set of primers targeting different regions of the HEV genome could increase the likelihood of HEV detection (30). The same strategy has been used for detection of other highly variable RNA viruses such as influenza A virus (32) and porcine reproductive and respiratory syndrome virus (PRRSV) (33,34).
The best PCR performance on experimental samples was observed for assay A (27/28), followed by assay B (11/28) and C (4/28). Assay D could not detect a single positive sample. In similar PCR comparisons as presented here, assay A was determined to be the most suitable, reproducible and reliable assay for the detection of HEV RNA \((12,20,21)\). It is well recognized that fecal samples as used in this study could contain metabolic compounds that possibly interfere with the RT-PCR reactions. The addition of an internal control to monitor the presence of such inhibitors would ensure reliability of negative results. Although such a control was not included in this study, all assays used the same nucleic acid extracts and issues with the viral RNA extraction recovery can therefore be excluded. No mismatch could be found for any primer or probe when compared to the mHEV strains used to infect the pigs, indicating that the detection rates achieved were due to intrinsic differences in the limit of detection for each assay. Moreover, it is worth noting that the single-plex assay B and the duplex assay D developed in the present study used the same primer pair and the difference in the positive detection rates between them \((11/28 \text{ vs. } 0/28, p < 0.01)\) are likely due to the differences in the nucleotide composition of the targeted region for the probes.

HEV genome variability may also influence the quantification of its RNA. Comparison of analytical sensitivities of the assays based on the detection of the plasmid DNA standards showed that the sensitivities of assay A and B were independent of HEV genotypes \((3 \text{ or } 4)\), and assay A was 10-fold more sensitive compared to assays B, C and D based on the genotype 3 standard curve detection. However, the sensitivities of assays C and D for HEV genotype 4 were 100-fold lower than the sensitivities yielded by using HEV genotype 3 within the same assay which could be partially explained by a single mismatch in the probe region. Although a study has reported that probes with up to two mismatches showed little variation in the PCR efficiency.
Comparison of HEV RNA detection assays

and nucleic acid quantification compared to probes that were fully complementary (35), another study has shown that a single mismatch in the probe binding region can result in a quantification error up to 33% (36). Due to the usage of plasmid DNA for quantification, reverse transcription reaction, as one of the crucial steps of RT-PCR was dismissed and the limit of detection presented here cannot be fully compared to assays in which there is usage of RNA standards.

In summary, real-time RT-PCR assays A and B that broadly detect HEV genotypes 1-4 showed better results for RNA detection than the duplex assays C and D that were both designed to detect and differentiate between HEV genotypes 3 and 4. Assay A presented the overall best performance among the tested assays.

References


Comparison of HEV RNA detection assays


Comparison of HEV RNA detection assays


Comparison of HEV RNA detection assays

Figure Legend

Figure 1

Comparison of four real-time RT-PCR assays (A, B, C and D) in detecting and quantifying HEV RNA on fecal samples after experimental inoculation of a pig with HEV genotype 3 strains US-2 (A) or Meng (B).
Table 1

Primers and probes used in this study.

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<th>Annealing temperature</th>
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<td>Conventional</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nested RT-PCR</td>
<td>ORF2</td>
<td>AATTATGCYCAGTAYCGRGTTG</td>
<td>55°C</td>
<td>5737-5732</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3156N</td>
<td>CCCTTCTCTGCTGCMCATTTC</td>
<td>6445-6467</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>3157N</td>
<td>GTWATGCTYTGATWCATGGCT</td>
<td>6022-6043</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3158N</td>
<td>AGCCGACGAAATCAATTCGTC</td>
<td>6348-6369</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Nucleotide positions are in accordance with GenBank accession number AF60669, except for the HEV genotype 4 probes that are in accordance with GenBank accession number HQ634346.
Table 2

Efficiency, regression coefficient, slope and intercept for real-time PCR assays A, B, C and D obtained by quantification of serially diluted plasmid DNA containing HEV genotypes 3 or 4 ORF2 and ORF3 overlapping region from $1 \times 10^8$ to $1 \times 10^1$ copies of genome equivalents per reaction.

<table>
<thead>
<tr>
<th>Variable</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficiency (%)</td>
<td>HEV-3</td>
<td>HEV-4</td>
<td>HEV-3</td>
<td>HEV-4</td>
</tr>
<tr>
<td></td>
<td>95.9</td>
<td>98.7</td>
<td>88.2</td>
<td>90.7</td>
</tr>
<tr>
<td></td>
<td>0.992</td>
<td>0.99</td>
<td>0.991</td>
<td>0.992</td>
</tr>
<tr>
<td>Slope</td>
<td>42.7</td>
<td>42.1</td>
<td>43.0</td>
<td>42.4</td>
</tr>
<tr>
<td>Intercept</td>
<td>47.82</td>
<td>50.88</td>
<td>43.6</td>
<td>48.7</td>
</tr>
</tbody>
</table>
Table 3

Limit of detection of four real-time PCR assays and intra-assay precision results of 10-fold dilutions of HEV genotype 3 or HEV genotype 4 DNA plasmid controls tested in triplicate.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Detected samples</th>
<th>Mean Ct</th>
<th>SD</th>
<th>CV%</th>
<th>Detected samples</th>
<th>Mean Ct</th>
<th>SD</th>
<th>CV%</th>
<th>Detected samples</th>
<th>Mean Ct</th>
<th>SD</th>
<th>CV%</th>
<th>Detected samples</th>
<th>Mean Ct</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEV-3*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100,000</td>
<td>3/3</td>
<td>24.55</td>
<td>0.47</td>
<td>1.86</td>
<td>3/3</td>
<td>25.30</td>
<td>0.94</td>
<td>3.56</td>
<td>3/3</td>
<td>30.28</td>
<td>0.85</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>10,000</td>
<td>3/3</td>
<td>28.58</td>
<td>0.37</td>
<td>1.24</td>
<td>3/3</td>
<td>28.09</td>
<td>0.64</td>
<td>2.05</td>
<td>3/3</td>
<td>33.48</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>1,000</td>
<td>3/3</td>
<td>31.37</td>
<td>0.34</td>
<td>1.09</td>
<td>3/3</td>
<td>32.77</td>
<td>0.16</td>
<td>0.48</td>
<td>3/3</td>
<td>37.34</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>3/3</td>
<td>34.91</td>
<td>0.23</td>
<td>0.65</td>
<td>3/3</td>
<td>36.35</td>
<td>1.32</td>
<td>3.53</td>
<td>3/3</td>
<td>40.78</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>3/3</td>
<td>37.16</td>
<td>0.73</td>
<td>1.95</td>
<td>2/3</td>
<td>39.31</td>
<td>1.81</td>
<td>4.60</td>
<td>0/3</td>
<td>39.92</td>
<td>0/3</td>
</tr>
<tr>
<td>HEV-4*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100,000</td>
<td>3/3</td>
<td>23.78</td>
<td>0.66</td>
<td>2.77</td>
<td>3/3</td>
<td>25.86</td>
<td>0.18</td>
<td>0.69</td>
<td>3/3</td>
<td>31.85</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10,000</td>
<td>3/3</td>
<td>28.44</td>
<td>0.39</td>
<td>1.36</td>
<td>3/3</td>
<td>29.25</td>
<td>0.72</td>
<td>2.31</td>
<td>3/3</td>
<td>35.86</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,000</td>
<td>3/3</td>
<td>31.59</td>
<td>0.68</td>
<td>2.16</td>
<td>3/3</td>
<td>32.81</td>
<td>0.09</td>
<td>0.24</td>
<td>1/3</td>
<td>39.54</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>3/3</td>
<td>34.53</td>
<td>0.56</td>
<td>1.64</td>
<td>3/3</td>
<td>36.24</td>
<td>0.58</td>
<td>1.53</td>
<td>0/3</td>
<td>36.24</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>10</td>
<td>3/3</td>
<td>37.36</td>
<td>0.13</td>
<td>0.35</td>
<td>2/3</td>
<td>40.22</td>
<td>0.51</td>
<td>1.26</td>
<td>0/3</td>
<td>40.22</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* Number of plasmid DNA copies per reaction

Ct, cycle threshold; SD, standard deviation; CV%, coefficient of variation
Table 4

Detection rates for HEV RNA in fecal samples collected from pigs of unknown HEV status by real-time RT-PCR assays A, B, C and D.

<table>
<thead>
<tr>
<th>Age-group</th>
<th>N. tested</th>
<th>A + (%)</th>
<th>B + (%)</th>
<th>C + (%)</th>
<th>D + (%)</th>
<th>HEV-3</th>
<th>HEV-4</th>
<th>HEV-3</th>
<th>HEV-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling</td>
<td>46</td>
<td>25 (54.3)</td>
<td>17 (36.9)</td>
<td>0$^a$</td>
<td>0$^b$</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
</tr>
<tr>
<td>Nursery</td>
<td>86</td>
<td>62 (72.0)</td>
<td>24 (27.9)</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
</tr>
<tr>
<td>Grow-finish</td>
<td>54</td>
<td>38 (70.3)</td>
<td>27 (49.1)</td>
<td>2 (3.6)</td>
<td>0$^c$</td>
<td>1 (1.8)</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
</tr>
<tr>
<td>Total</td>
<td>186</td>
<td>125 (67.2)</td>
<td>68 (36.4)</td>
<td>2 (1.1)</td>
<td>0$^b$</td>
<td>1 (0.5)</td>
<td>0$^c$</td>
<td>0$^c$</td>
<td>0$^c$</td>
</tr>
</tbody>
</table>

$^a$Different superscripts (A,B,C) within each column indicate significant differences ($p < 0.05$) within assays.
Table 5

Detection of HEV RNA by conventional nested RT-PCR and real-time RT-PCR assays in 20 swine field samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Real-time RT-PCR assay</th>
<th>Conventional nested RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>19482</td>
<td>- 35.69 - -</td>
<td>+ (w)</td>
</tr>
<tr>
<td>19702-C</td>
<td>- 36.18 - -</td>
<td>+ (w)</td>
</tr>
<tr>
<td>19714</td>
<td>- 36.00 - -</td>
<td>+ (w)</td>
</tr>
<tr>
<td>19762-B</td>
<td>33.02 34.46 - -</td>
<td>+*</td>
</tr>
<tr>
<td>19775</td>
<td>36.97 - - -</td>
<td>-</td>
</tr>
<tr>
<td>19913</td>
<td>33.04 35.51 33.62 -</td>
<td>+*</td>
</tr>
<tr>
<td>19903-A</td>
<td>- 35.63 - -</td>
<td>-</td>
</tr>
<tr>
<td>19912-B</td>
<td>36.06 - - -</td>
<td>+ (w)</td>
</tr>
<tr>
<td>19955</td>
<td>37.41 36.06 - -</td>
<td>-</td>
</tr>
<tr>
<td>20354-A</td>
<td>35.99 38.99 - -</td>
<td>-</td>
</tr>
<tr>
<td>20361-A</td>
<td>36.40 - - -</td>
<td>+</td>
</tr>
<tr>
<td>20383-B</td>
<td>36.39 33.32 - -</td>
<td>-</td>
</tr>
<tr>
<td>20513</td>
<td>36.13 - - -</td>
<td>-</td>
</tr>
<tr>
<td>20517</td>
<td>36.88 - - -</td>
<td>+</td>
</tr>
<tr>
<td>20613</td>
<td>- 36.78 - -</td>
<td>+ (w)</td>
</tr>
<tr>
<td>20467-E</td>
<td>36.59 38.05 - -</td>
<td>+</td>
</tr>
<tr>
<td>20468-D</td>
<td>36.31 37.84 - -</td>
<td>+*</td>
</tr>
<tr>
<td>20777-B</td>
<td>36.52 35.86 - -</td>
<td>-</td>
</tr>
<tr>
<td>20792-B</td>
<td>37.23 36.46 - -</td>
<td>+ (w)</td>
</tr>
<tr>
<td>20855</td>
<td>35.93 - - -</td>
<td>-</td>
</tr>
</tbody>
</table>

-, HEV RNA negative sample; +, HEV RNA positive sample; w, weak reaction; * HEV genotype 3 by sequencing