DETECTION OF HEPATITIS E VIRUS GENOTYPES 1 TO 4 IN SWINE PRODUCTS AND HUMAN SAMPLES BY TAQMAN-BASED RT-QUANTITATIVE PCR

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Abstract. This study describes the development a rapid, specific and robust one-step RT-quantitative (q)PCR assay for detection of four major hepatitis E virus (HEV) genotypes in 120 swine products (30 each of bile, feces, liver, and pork samples) and 54 clinical human specimens (28 sera and 26 stool samples) together with 39 healthy blood donor sera. Assay sensitivity was 10^1-10^2 copies/μl HEV RNA with high specificity evidenced by absence of amplification of other virus genomes in swine products and human samples. The requirement of serum sample volume was less than 4-fold compared to commercial automated detection methods. In conclusion, the in-house RT-qPCR provided a rapid, sensitive and accurate method for HEV diagnosis in both swine products and human samples.

Keywords: hepatitis E virus, detection, humans, one-step RT-quantitative PCR, swine

INTRODUCTION

Hepatitis E virus (HEV) is endemic in developing countries with markedly poor living standards (Yugo and Meng, 2013). Recently, there has also been an increased awareness of indigenous HEV transmission in industrialized nations (Wenzel *et al*, 2011). HEV infections in immunocompromised patients and among individuals who have not traveled to an HEV endemic area have been documented (Borgen *et al*, 2008; Smith *et al*, 2013; Riveiro-Barciela *et al*, 2014). Initially, these and other sporadic HEV cases could not easily be accounted for, but a report of an HEV sequence isolated from patients who consumed raw wild boar meat provides the notion HEV could act as a zoonotic agent (Borgen *et al*, 2008).

HEV infecting humans consists of four major genotypes, namely, genotype 1 - 4 (Smith *et al*, 2016) and human-restricted HEV genotypes 1 and 2 are typically transmitted by consuming virus-contaminated water (Meng, 2013). On the other hand, HEV genotypes 3 and 4 are able to infect other hosts, such as pig and wild boar and

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infection is subsequently transmitted to humans who consume raw or undercooked meat from infected animals. This passage of pathogen is suspected in autochthonous infections arising in industrialized countries (Wenzel *et al*, 2011).

All HEV genotypes belong to a single serotype (Purcell and Emerson, 2008). Commercial enzyme-linked immunosorbent assays (ELISAs) are available, but these are only useful for diagnosis in the late stages of infection (Candido *et al*, 2012) and HEV IgM persists only for a limited time (Girón-Callejas *et al*, 2015). In addition, sensitivity of HEV ELISAs can vary between assays and cannot detect HEV infection in animals or contaminated water (Martin-Latil *et al*, 2012). On the other hand, detection of HEV RNA enables diagnosis from early to late stages of infection (Gyarmati *et al*, 2007). Various quantitative PCR methods have been applied to HEV diagnosis, with TaqMan RT-quantitative (q)PCR using a probe-based assay has been suggested as the most reliable technique, with high sensitivity and specificity (Jothikumar *et al*, 2006). However, the efficiency of the RT step plays a role in the subsequent PCR amplification process.

Thus, the requirement of an accurate detection of HEV is a highly sensitive assay with an interpolated of an RNA control in the RT step. Here we describe the development of a one-step RT-qPCR assay of HEV genotypes 1-4 (HEV-1-4). Such an assay should be helpful in the detection of hepatitis E infection from food-borne contamination of domestic pigs, a potential source of zoonotic HEV transmission to humans (Meng *et al*, 1998).

MATERIALS AND METHODS

RNA preparation

RNA from samples comprising swine products [30 each of bile, feces, liver, and pork samples (Group I)], 54 clinical samples from suspected HEV-infected patients [28 sera and 26 stool samples (Group II)] and 39 blood samples from healthy blood donors (Group III) (from the National Blood Center, Thai Red Cross Society, Bangkok) using by Ribospin™ vRD II virus RNA extraction kit (GeneAll Biotechnology, Seoul, Korea).

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB 435/58).

One-step RT-qPCR assay

Probe and primers. Assay probe and primers were designed based on multiple sequence alignment of swine and HEV-1-4 ORF2/3 regions (Fig 1).

Preparation of HEV-1, -2, -3, and -4 positive controls. Primers for generation of HEV-1, -2 and -4 oligonucleotide positive controls are shown in Table 1. Reaction mixture (25 μl) consisted of 10 μl of 2.5X 5 PRIME MasterMix (5 PRIME GmbH, Hamburg, Germany), 0.5 μl of 10 μM forward and reverse primers as template (Table 1) and sterile distilled water. For HEV-3 positive control, RNA was extracted from 200 μl of serum from a confirmed HEV-3-infected patient as described above, dissolved in 30 μl of nuclease-free water, converted to cDNA using ImProm-II™ Reverse Transcription kit (Promega, Madison, WI), and 4 μl aliquot of cDNA was used as template for PCR amplification using 0.5 μl of 10 μM HEV_ORF2_F and HEV_ORF2_R primers (Table 1). Thermocycling was performed in a Mastercycler® pro (Eppendorf, Hamburg, Germany) as follows: 94°C for 3 minutes; 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds; with a final step of 72°C for 7 minutes. Amplicons (89 bp of HEV-1,

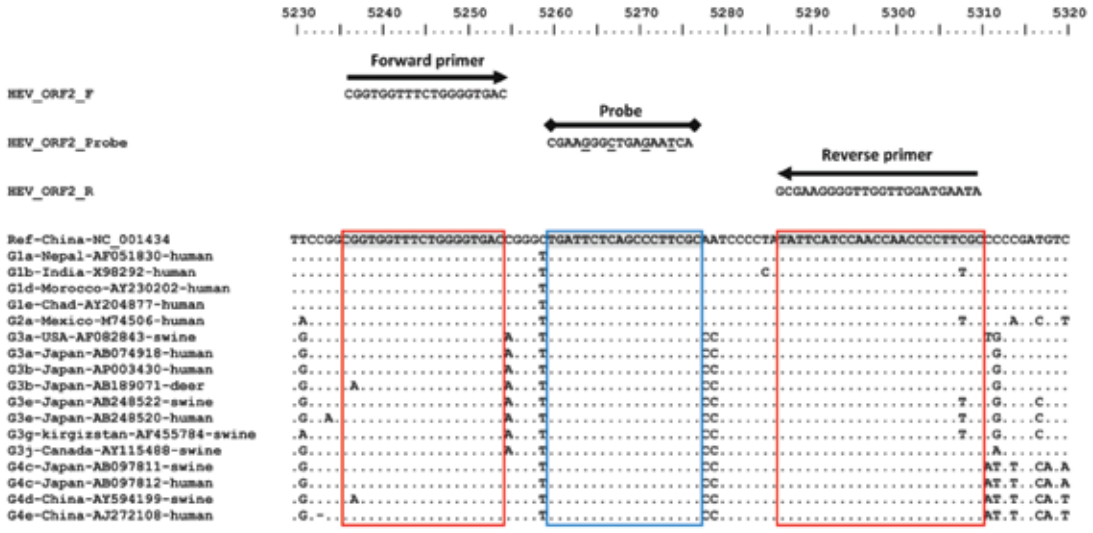

Fig 1-Alignment of hepatitis E virus (HEV) probe and primers sequences in ORF2/3 region. The name of each strain is represented as Subgenotype-Isolation country-GenBank accession no.-Host. Sequence regions that are complementary with probe and primers are represented in gray. Blue and red squares represent alignment of HEV sequences with probe and primers, respectively. Underlined letters in probe sequence indicate locked nucleic acid residues. Positions are numbered on the top line according to the complete sequence of HEV genotype 1 Chinese strain (GenBank accession no. NC_001434).

-2 and -4 and 75 bp of HEV-3) were separated by 2% agarose gel-electrophoresis, stained with ethidium bromide, purified using GeneAllExpin™ Combo (GeneAll Biotechnology, Seoul, Korea) and directly sequenced (First BASE Lab, Selangor, Malaysia).

After checking correctness of amplicon sequences, HEV-1, -2, -3 and -4 oligonucleotides were inserted into pGEM®-T Easy Vector (Promega) and used to transformed *E. coli* DH5α competent cells. Recombinant plasmids were extracted from transformed *E. coli* cells using FastPlasmid® Mini extraction kit (Eppendorf) and direction of insertion determined (First BASE Lab). Plasmid inserts were amplified using primers M13F (5′-GGGTTTTCCCAGTCACGAC-3′) and M13R (5′-AGGAAACAGCTATGAC-CATG-3′) and purified as described above. Subsequently, amplicons were subjected *in vitro* transcription using RiboMAX™ Large Scale RNA Production Systems-SP6 or T7 (Promega). RNA transcripts were purified using a virus RNA purification kit (Ribospin™ vRD II, GeneAll Biotechnology, Seoul, Korea), concentration measured using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and stored at -70°C until used.

Assay protocol. Assay was performed using a SensiFast™ Probe No-ROX One-Step kit (Bioline US, Taunton, MA). In brief, reaction mixture (10 μl) comprised of 0.4 μl of 10 μM HEV_ORF2_F and 10 μM HEV_ORF2_R primers, 5 μl of 2X SensiFast™ Probe No-ROX One-Step Mix, 0.1 μl of 10 μM HEV_ORF2_Probe (5′ 6-FAM™

Table 1
Probe and primers used to construct hepatitis E virus (HEV) positive controls.

HEV	Primer name	Sequence (5′ to 3′)	Amplicon size (bp)
Genotype 1	HEV_G1_F	GGTTCCGGCGGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTCGCAATCC	89
	HEV_G1_R	TCGGGGGCGAAGGGGTTGGTTGGATGAATATAGGGGATTGCGAAGGGCTGAGAAT	
Genotype 2	HEV_G2_F	GGTACCGGCGGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTCGCAATCC	89
	HEV_G2_R	TCTGGGGCAAAGGGGTTGGTTGGATGAATATAGGGGATTGCGAAGGGCTGAGAAT	
Genotype 4	HEV_G4_F	GGTGCCGGCGGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTCGCCCTCC	89
	HEV_G4_R	TCAGATGCGAAGGGGTTGGTTGGATGAATATAGGGGAGGGCGAAGGGCTGAGAAT	
Genotype 3	HEV_ORF2_F	CGGTGGTTTCTGGGGTGAC	75
	HEV_ORF2_R	GCGAAGGGGTTGGTTGGATGAATA	
Probe	HEV_ORF2_Probe	6-FAM™-CGAA**G**GG**C**TGA**G**AA**T**CA-BHQ®-1	

Letter in bold and underlined is locked nucleic acid base. BHQ®-1, (Black Hole Quencher-1); 6-FAM™, (6-carboxyfluorescein).

(6-carboxyfluorescein)/3′ BHQ®-1 (Black Hole Quencher-1); Integrated DNA Technologies P/L, Singapore), 0.1 U reverse transcriptase (Bioline US), 0.2 μl of RiboSafe RNase Inhibitor (Bioline US), 2 μl of sample RNA, and 1.8 μl of DEPC-H_2O. RT-qPCR was performed in a ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific) as follows: 45°C for 10 minutes; 95°C for 2 minutes; 45 cycles of 95°C for 5 seconds and 61°C for 20 seconds. Fluorescence of FAM (518 nm) was measured at each completion of annealing/extension. Data were analyzed using QuantStudio™ Real-Time PCR Software version 1.2 (Life Technologies, Carlsbad, CA). Each experiment included HEV-1-4 RNA positive controls (10^1 to 10^6 copies/μl) and negative control (without template).

Sensitivity determination

Control RNA transcript copy number of each genotype was calculated using the formula: transcript copy (copies/μl) = [RNA concentration (g/μl) x 6.02 x 10^{23}]/[length of *in vitro* transcribed RNA (bases) x 340]. Ten-fold RNA dilutions were serially prepared (10^6 to 10^0 copies/μl RNase-free water) and subjected to the one-step RT-qPCR as described above. A standard curve was produced by plotting threshold cycle (C_T) against RNA copy number (log scale). QuantiStudio™ Real-Time PCR Software version 1.2 (Life Technologies) was employed to determine y-axis intercept, correlation coefficient (R^2), slope, and PCR amplification efficiency (E).

Limit of detection (LOD) is defined as the lowest amount of RNA standard detected from experiment conducted in triplicate. Intra-assay reproducibility was determined from three repeats with newly prepared dilutions of RNA standards, and inter-assay reproducibility was assessed from three separate experiments. Both intra- and inter-assay reproducibility were calculated by determining mean copy number and standard deviation (SD) to find the coefficient of variation (CV). Cut-off value of each genotype was calculated using C_T of control at LOD.

Specificity determination

The one-step RT-qPCR assay was used on samples positive for group A porcine rotavirus (RVA), porcine epidemic diarrhea virus (PEDV), porcine reproductive and respiratory syndrome virus (PRRSV) US strain, and African swine fever virus (ASFV), and on human samples positive for hepatitis A virus (HAV) and hepatitis B virus (HBV). All positive samples were previously confirmed by direct sequencing of the respective PCR amplicons (Cherdpong Phupolphan and Thanunrat Thongmee, personal communication, 2016).

HEV RNA screening of samples

RNA extracted from swine (Group I) and human clinical samples (Group II) previously giving both positive and negative HEV results using semi-nested RT-PCR of ORF2 region (Test A) (Intharasongkroh *et al*, 2016) were tested using the one-step RT-qPCR assay (Test B). A number of positive samples by Test A had already been confirmed by direct sequencing of the amplicons (Intharasongkroh *et al*, 2016). All sequences obtained from the swine samples in Table 2 were deposited in GenBank database (accession nos. KU550422-KU550426, KU550430-KU550439, KU550448-KU550462 and KU550468-KU550470). RNA of 39 healthy donor sera (Group III) previously analyzed for HEV by an automated method (cobas® HEV; Roche Molecular Diagnostics, Pleasanton, CA) (Test C) were further tested by Test B carried out in VersiPlate 96-well Low Profile PCR strips (Thermo

Table 2
Swine and human clinical test samples.

Source	Sample type	Number of samples	Semi-nested RT-PCR[a] (Test A)		One-step RT-quantitative PCR (Test B)		C_T of positive samples (mean ± SD)	
			Negative	Positive	Negative	Positive	Unmatched[b]	Matched[b]
Swine	Feces	30	15	15	14	16	34 ± 0	29 ± 6
	Bile	30	15	15	8	22	31 ± 5	22 ± 5
	Pork	30	29	1	15	15	33 ± 3	32 ± 0
	Liver	30	28	2	24	6	35 ± 1	25 ± 1
Human	Serum	28	24	4	21	7	24 ± 2	27 ± 4
	Stool	26	22	4	21	5	33 ± 0	28 ± 5
	Total	174	133	41	103	71	32 ± 4	26 ± 6

[a]Intharasongkroh *et al* (2016). [b]Compared between Test A and Test B.

Scientific, Loughborough, UK) with each plate containing positive controls of HEV-1-4 and one non-template H_2O control (NTC). Results with C_T values above that of LOD are considered negative. All swine and human samples were also tested using a previously published in-house one-step RT-qPCR (Test D) (Son *et al*, 2014).

In order to avoid cross-contamination, all assay steps including sample collection, RNA extraction, reaction solution preparation, standard RNA solution preparation, template addition, and thermocycling were conducted in areas isolated from one another.

RESULTS

Sensitivity of HEV one-step RT-qPCR assay

Minimum detection of HEV-1, -2, -3, and -4 was 10^1 copy/μl (mean ± SD = 36 ± 1), 10^1 copy/μl (36 ± 2), 10^2 copies/μl (36 ± 1), and 10^2 copies/μl (33 ± 1), respectively (Fig 2A). Regression analysis of standard curves showed linearity for all genotypes, with R^2 values approaching 1 and y-axis intercept of HEV-1 to 4 of 40.4, 38.8, 42.5, and 39.7, respectively, E values, a test performance indicator, between 91.2% and 94.8%, and slope of each HEV genotype standard curve within the accepted range (from -3.1 to -3.6) (Fig 2B).

LOD was calculated from experiments conducted in triplicate at 20, 20, 200, and 200 copies of RNA standard per reaction for HEV-1, -2, -3, and -4, respectively (Table 3). CV of each genotype assay was <2, demonstrating good reproducibility and reliability of the method for both intra- and inter-assays. C_T cut-off value, established from the relationship between log-transformed copy number and mean C_T of LOD of each genotype was 38, 38, 38, and 25 for HEV-1, -2, -3, and -4, respectively.

Specificity of HEV one-step RT-qPCR assay

There were no DNA or RNA amplification fluorescence signals detected in RVA, PEDV, PRRSV, ASFV, HAV, or HBV samples, whereas samples positive for HEV-1 to -4 showed acceptable amplification signals (data not shown).

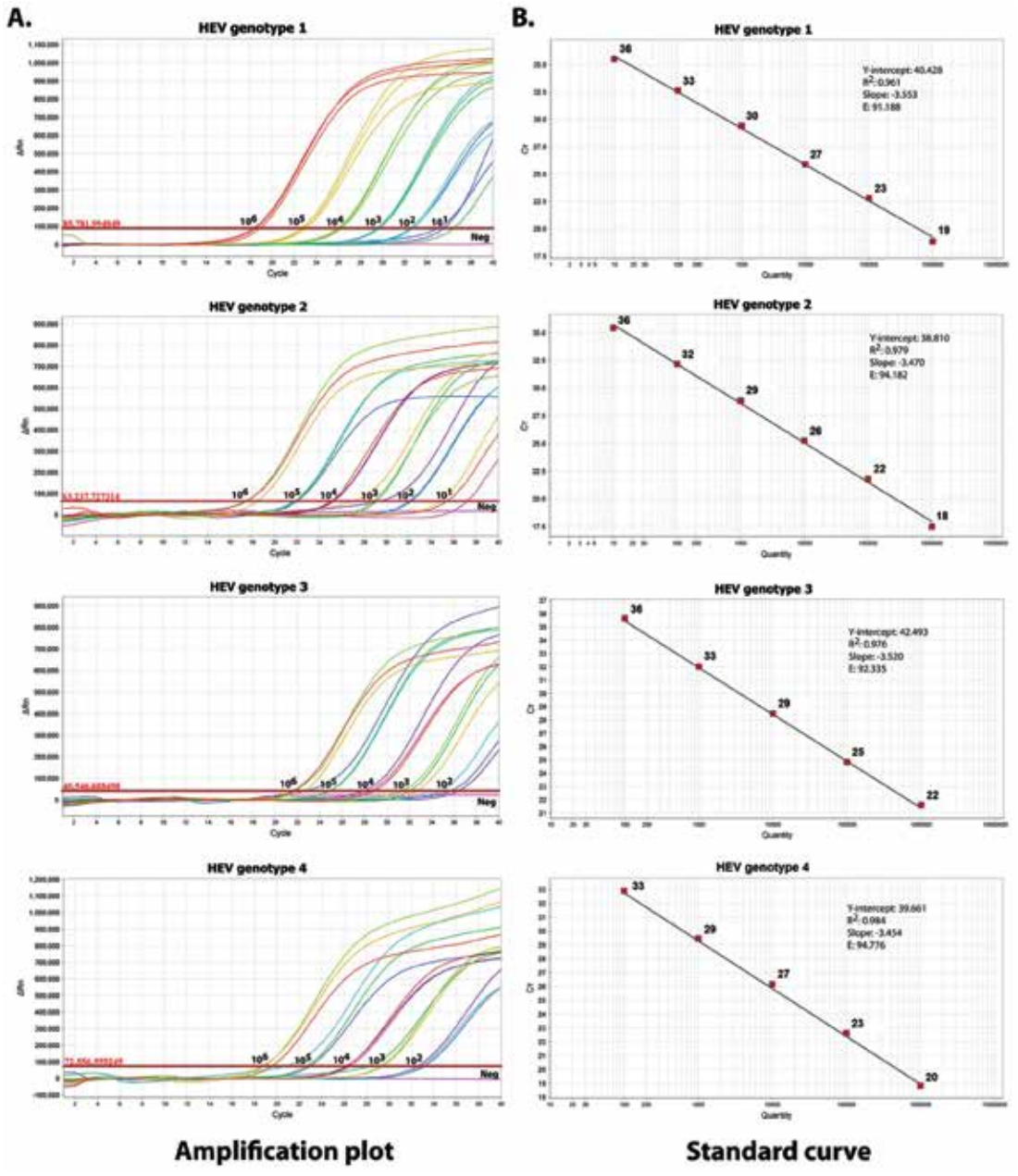

Fig 2- Amplification profiles of ORF2/3 region (A) and standard curves (B). Ten-fold serial dilution of hepatitis E virus genotypes 1 to 4 RNA were detected using a TaqMan detection system employing 6-FAM™ (6-carboxyfluorescein) fluorescence. Neg, negative control (water).

Table 3
Intra- and inter-assay reproducibility of hepatitis E virus (HEV) one-step RT-quantitative PCR.

Standard RNA	RNA copy number[a]	Intra-assay			Inter-assay		
		Copy number determined[b]		CV[d]	Copy number determined[b]		CV[d]
		Average	SD[c]		Average	SD[c]	
HEV-1	2,000,000	1,312,126	254,946	0.194	2,967,763	1,469,649	0.495
	200,000	156,487	18,925	0.121	148,908	39,834	0.268
	20,000	10,157	1,038	0.102	14,204	4,226	0.298
	2,000	1,027	85	0.083	1,335	426	0.319
	200	149	11	0.074	192	68	0.353
	20	28	12	0.429	32	9	0.281
HEV-2	2,000,000	2,900,032	976,387	0.337	2,233,719	1,386,434	0.620
	200,000	142,294	9,374	0.066	195,197	84,732	0.434
	20,000	17,365	1,440	0.083	10,884	5,644	0.519
	2,000	2,586	2,032	0.786	4,786	2,818	0.589
	200	543	575	1.058	1,420	2,114	1.489
	20	13	8	0.650	12.8	15.372	1.201
HEV-3	2,000,000	898,017	238,084	0.265	3,455,986	3,471,874	1.005
	200,000	549,798	515,097	0.937	249,479	180,092	0.722
	20,000	20,326	6,406	0.315	16,925	4,206	0.249
	2,000	1,995	851	0.426	2,060	191	0.092
	200	177	94	0.529	241	103	0.428
HEV-4	2,000,000	1,815,534	519,401	0.286	3,582,802	3,047,034	0.850
	200,000	330,547	281,497	0.852	124,165	65,730	0.529
	20,000	13,856	1,478	0.107	13,090	3,553	0.271
	2,000	2,976	1,513	0.508	1,186	992	0.837
	200	170	33	0.195	394	295	0.750

[a]Determined by spectrophotometry. [b]Calculated from C_T value according to standard curve. [c]Standard deviation. [d]Coefficient of variation (standard deviation/average of copy number determined).

HEV one-step RT-qPCR detection of test swine and human samples

There are no significant differences in the results of positive samples between Test A (semi-nested RT-PCR) and Test B (this study); however, 30/133 (23%) samples negative in Test A were positive in Test B (Table 2). Swine samples negative in Test A had an average C_T value of 33 (range of 24 to 37) and human samples an average C_T value of 26 (range of 22 to 33). Three unmatched human serum samples (Group II) between Tests A and B, when tested for presence of anti-HEV

IgG and IgM (Euroimmun ELISA, Lübeck, Germany), showed all three positive for anti-HEV IgM and two negative for anti-HEV IgG.

Of the 39 healthy blood donor samples (Group II), six positive samples from a complete analysis by Test C (cobas® HEV assay) were confirmed by direct sequencing (GenBank accession nos. KY399948-KY399958) (Table 4). Four samples tested negative in Tests A, C and D gave positive results in Test B, demonstrating C_T values in the high range (>26; average 29 ± 3).

DISCUSSION

Anti-HEV IgM may persist for 3-5 months post-symptom onset (Aggarwal *et al*, 2000). Serological diagnosis through detection of anti-HEV IgM has proven useful in screening large numbers of human sera samples (Gyarmati *et al*, 2007). However, there are limitations to such detection due to the absence of serological markers during the window period before seroconversion to anti-HEV in immunocompromised patients. In the latter cases diagnosis must be confirmed by HEV RNA detection (Girón-Callejas *et al*, 2015), which can also be used in different types of samples from various test host species (Gyarmati *et al*, 2007). However, PCR inhibitors in samples such as blood or fat affect RT-PCR technique (Deng *et al*, 2005). In addition, RT-PCR assay is a time-consuming procedure producing qualitative results (Vijgen *et al*, 2005).

RT-qPCR shows more resistance to PCR inhibitors, especially in assays of RNA viruses with highly variable genomes (Oleksiewicz *et al*, 2001; Qiu *et al*, 2014). The first developed test employed a probe to detect all HEV genotypes in sera and stool (Jothikumar *et al*, 2006). However, this assay used plasmids to generate standard curves and thus could not take into account possible variations in the efficiencies of the RT step. Adopting serial concentrations of *in vitro* transcribed HEV RNA for construction of standard curves provided a procedure that improved the reliability and sensitivity of detection by RT-qPCR (Zhang *et al*, 2013).

This study improves on the previous RT-qPCR technique by constructing primers and a probe containing invariant nucleic acid residues that has more sensitivity and specificity compared to previous traditional minor grove binding assay (Letertre *et al*, 2003), targeting the most conserved regions (ORF2/3) for HEV-1 to 4. This assay allowed quantification of viral RNA in both human and swine and required only a short turn-around time.

Specificity and intra- and inter-assay reproducibility were within acceptable range. LOD results demonstrated that the assay was sensitive (20 RNA copies per reaction). In the discordant results, test clinical samples could possibly contain polymerase inhibitor(s), but the main factor was the low amounts of viral RNA. The TaqMan detection assay developed in this study will be a valuable tool to overcome this latter problem as it provides maximum sensitivity compared to previous assay formats (Petitjean *et al*, 2006). Three clinical serum samples negative by semi-nested RT-PCR assay (Intharasongkroh *et al*, 2016), but positive in our assay contained anti-HEV IgM and two of which anti-HEV IgG, indicating the HEV one-step RT-qPCR assay could detect early stages of HEV infection.

Among the semi-nested RT-PCR (Intharasongkroh *et al*, 2016), previous published one-step RT-qPCR (Son *et al*, 2014), and commercial one-step RT-qPCR (cobas® HEV) assays, our one-step RT-

qPCR assay showed good correlation to the results of Son *et al* (2014) as evidenced by the minimal number of discordance. The cause of the discrepancies between in-house one-step RT-qPCR assays and conventional semi-nested RT-PCR and automated assays might arise from intrinsic differences in the PCR processes. Variables, such as differences in reagents, standardization procedures, extraction

Table 4
Comparison of four hepatitis E virus (HEV) RNA detection assays of sera from healthy blood donors.

Donor[a]	Result						
	In-house					Automated	
	Semi-nested RT-PCR[b] (Test A)	This study (Test B)		Previous study[c] (Test D)		commercial assay[d] (Test C)	
		CT value	Interpretation	CT value	Interpretation	CT value	Interpretation
1	+	27	+	34	+	27.63	+
2	–	35	+	35	+	–	–
3	+	–	–	–	–	40.38	+
6	–	27	+	–	–	–	–
7	+	38	+	32	+	24.7	+
8	–	26	+	–	–	–	–
10	–	34	+	36	+	–	–
11	–	34	+	34	+	–	–
13	+	33	+	34	+	34.2	+
17	–	35	+	34	+	–	–
19	–	33	+	–	–	–	–
21	–	34	+	32	+	–	–
26	–	37	+	36	+	–	–
28	+	33	+	36	+	34.95	+
34	+	29	+	34	+	26.69	+
36	–	28	+	–	–	–	–
39	–	–	–	36	+	–	–
Total positive	6		15		12		6
Average CT value		32		35		32	

[a]Donors with negative results for all tests were not included. [b]Intharasongkroh *et al* (2016). [c]Son *et al* (2014). [d]cobas® HEV (Roche Molecular Diagnostics, Pleasanton, CA).

methods, sample volumes, template amounts, and final reaction volumes, could affect the results.

Interestingly, only the commercial (cobas® HEV) assay was able to detect HEV RNA in "donor 3" serum sample (Table 4). The C_T of this sample was 41 while our assay and that of Son *et al* (2014) had an upper of 40 cycles, and if the upper cycle limit were extended, the three assays would have concordant results for "donor 3" sample.

For research use only, our in-house one-step RT-qPCR assay provides a powerful tool for quantifying HEV RNA in both swine and human samples. However, applying the assay for reliable and accurate detection of HEV RNA in patients and/or healthy blood donors remains a challenge as the assay has only been applied to swine and human clinical HEV-3 samples due to the lack of other HEV genotypes. Thus, further experiments on samples containing other HEV genotypes are needed. Also, positive results from our study could not indicate the number of infectious HEV virus particles because the technique cannot distinguish between infectious and non-infectious particles.

In conclusion, the in-house hepatitis E virus one-step RT-qPCR assay employing a TaqMan probe developed in this study was able to detect a smaller number of hepatitis E virus RNA copies compared to other similar in-house assays, and produced more reliable results and used less sample volume compared to a commercial automated detection instrument. This simple, rapid and reliable method should be useful for measuring viral loads of the four genotypes of hepatitis E virus.

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