Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Comparative analysis of different TaqMan real-time RT-PCR assays for the detection of swine Hepatitis E virus and integration of Feline calicivirus as internal control

P. Ward[1], E. Poitras[1], D. Leblanc[1], A. Letellier[2], J. Brassard[1], D. Plante[3] and A. Houde[1]

1 Agriculture and Agri-Food Canada, Food Research and Development Centre, St-Hyacinthe, QC, Canada
2 Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada
3 Health Canada, Quebec Region, 1001 St-Laurent Street West, Longueuil, QC, Canada

Keywords
Feline calicivirus, internal control, nested RT-PCR, swine Hepatitis E virus, TaqMan real-time RT-PCR.

Correspondence
Alaine Houde, Agriculture and Agri-Food Canada, Food Research and Development Centre, 3600 Casavant Blvd West, St-Hyacinthe, QC, Canada, J2S 8E3.
E-mail: houdea@agr.gc.ca

2008/1085: received 26 June 2008, revised 17 September 2008 and accepted 3 October 2008

doi:10.1111/j.1365-2672.2008.04104.x

Abstract

Aims: The aim of this study was to compare the performance of four TaqMan RT-PCR assays with a commonly used nested RT-PCR and to include the Feline calicivirus (FCV) as an internal control.
Methods and Results: RNA extracted from 87 swine faecal samples and 103 swine blood samples was subjected to different detection systems. Faecal samples naturally contaminated with Hepatitis E virus (HEV) and negative samples were artificially inoculated with $3{\cdot}2 \times 10^3$ PFU of FCV. Detection results obtained on faecal and plasma samples were 35·6% and 4·9% with the nested RT-PCR assay, 8·0% and 0%, 0% and 0%, 13·8% and 0% and 36·8% and 3·9% with TaqMan systems A, B, C and D respectively. The Ct means obtained with the multiplex TaqMan assay were 30·11 and 30·43 for the detection of FCV with HEV contaminated samples and negative samples.
Conclusions: The TaqMan system D was more suitable for the detection of swine HEV strains than the three others and FCV was integrated successfully as an internal control.
Significance and Impact of the Study: FCV was demonstrated as an efficient control to monitor the RNA extraction process and HEV amplification procedure in a multiplex HEV/FCV TaqMan assay. This control would be helpful in limiting false negative results.

Introduction

Hepatitis E virus (HEV) is a nonenveloped icosahedral virus of approximately 27–34 nm in diameter discovered in 1983 by immune electron microscopy (Balayan *et al.* 1983). The virion capsid is believed to be composed of a single structural protein (Aggarwal and Krawczynski 2000; Emerson and Purcell 2003). HEV was classified originally in the family *Caliciviridae*, but was reclassified recently as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson *et al.* 2005). The first complete genomic sequence was determined in 1991 (Tam *et al.* 1991) and the first animal strain of HEV was identified and characterized in 1997 from a pig in the United States (Meng *et al.* 1997). The genome consists of a single-stranded positive sense RNA of approximately 7·2 kb. The viral RNA contains a short 5′ untranslated region followed by three partially overlapping open reading frames (ORF1, ORF2, ORF3) and a 3′ untranslated region that is terminated by a poly(A) tract (Aggarwal and Krawczynski 2000; Emerson and Purcell 2003; Lu *et al.* 2006). Mammalian HEV isolates are subdivided into 4 genotypes (1, 2, 3, and 4) based on complete genome sequence. Genotype 1 regroups strains from Asia and Africa, genotype 2 consists of Mexican and African strains, genotype 3 contains strains from industrialized countries and genotype 4 contains strains from sporadic cases in Asia (Lu *et al.* 2006; Okamoto 2007).

HEV is transmitted by the faecal-oral route or contaminated water. It may occur in three different forms: large epidemics, smaller outbreaks or sporadic infections (Okamoto 2007). Symptoms of HEV infection are fever,

nausea and vomiting, fatigue and anorexia, abdominal pain, jaundice, dark urine and elevated liver enzymes. Most of the time, it is difficult to differentiate between HEV and Hepatitis A virus (HAV) infections based only on symptom analysis (Aggarwal and Krawczynski 2000; Smith 2001).

There is increasing evidence that animals (such as pigs) could serve as reservoir for HEV (Meng *et al.* 1997, 1998, 1999, 2002; Yoo *et al.* 2001; Renou *et al.* 2007). Swine HEV isolates have been identified now in many countries worldwide including Canada (Meng *et al.* 1997; Choi *et al.* 2003; Emerson and Purcell 2003; Takahashi *et al.* 2003; Cooper *et al.* 2005; Caron *et al.* 2006; Chobe *et al.* 2006; Lu *et al.* 2006; Okamoto 2007; Ward *et al.* 2008) and high genetic relatedness between HEV isolates obtained from humans and those obtained from swine in the same geographical region (USA, Taiwan, Spain, China and Japan) was observed in different studies (Huang *et al.* 2002; Nishizawa *et al.* 2003; Takahashi *et al.* 2003; Lu *et al.* 2006). The transmission of HEV through food, such as pig liver, wild boar and deer meat was also reported in different studies (Tei *et al.* 2003; Yazaki *et al.* 2003; Li *et al.* 2005; Mizuo *et al.* 2005; Feagins *et al.* 2007).

Serological and nucleic acid amplification tests have been developed for epidemiological and diagnostic purposes. The serological tests were designed for the detection of serum antibodies to HEV and the nucleic acid assays were used mostly for the detection of HEV RNA in serum, bile or faecal samples (Mushahwar 2008). In the last 10 years, many conventional RT-PCR and quantitative real-time RT-PCR tests using SYBR Green or TaqMan probes targeting the ORF2 or ORF3 gene were developed for the detection of HEV RNA (Meng *et al.* 1997; Jothikumar *et al.* 2000, 2006; Williams *et al.* 2001; Huang *et al.* 2002; Mansuy *et al.* 2004; Orru *et al.* 2004; Ahn *et al.* 2006; Enouf *et al.* 2006; Inoue *et al.* 2006; Gyarmati *et al.* 2007). The sensitivity of these detection assays can be affected by the quality of extracted RNA, RNase contamination or RT-PCR inhibitors in environmental and clinical samples, especially in faecal material (Escobar-Herrera *et al.* 2006; Rutjes *et al.* 2007; Scipioni *et al.* 2008). Failure to amplify the viral RNA due to these factors would result in false negative results. The use of an internal control artificially added to the samples before the concentration of the viral particles and RNA extraction would be extremely useful in monitoring the quality of the extraction procedure and for identifying the presence of possible RT-PCR inhibitors interfering with amplification reactions. In this study, the Feline calicivirus (FCV) was used as internal quality control. FCV is not a hazard for humans and presents some physical similarities with HEV and other food-borne viruses such as HAV and Norovirus (Bidawid *et al.* 2003).

The aim of this study was to evaluate and determine the most efficient and sensitive swine HEV molecular detection system by comparing the performance of four previously published TaqMan real-time RT-PCR assays with a commonly used conventional nested RT-PCR test (Huang *et al.* 2002) and to include the FCV as an internal control to monitor the isolation and amplification of viral RNA.

Materials and methods

Faecal and blood samples

A total of 87 swine faecal samples and 103 swine blood samples, randomly collected from different fattening farms located in Quebec (Canada), were used in this study. Faecal material included a set of archived and new samples and was unrelated to blood samples. Faecal samples were collected directly from the pen's floor (1 g of faecal material from five sites) of each farm, diluted in Minimum Essential Medium or Phosphate Buffered Saline (PBS), pH 7·4 (Invitrogen Canada Inc, Burlington, ON, Canada) to obtain a final 20% suspension (w/v) and stored at −80°C. All these swine faecal samples were tested previously for HEV by conventional nested RT-PCR, sequenced for confirmation and were associated with genotype 3 (Ward *et al.* 2008). Plasma was extracted from blood samples by sedimentation using Ficoll-Plaque (GE Healthcare Bio-Sciences Inc, QC, Canada) according to the manufacturer's instructions and was stored at −80°C until use.

RNA extraction from faecal material and plasma

Viral RNA was extracted from faecal clarified suspensions and plasma using the QIAamp® Viral RNA mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Recovered RNA was frozen at −80°C until further use.

Primers and probes

All primers and TaqMan probes (IDT, Coralville, IA, USA) used in this study are listed in Table 1.

Conventional nested RT-PCR

HEV RNA was detected by conventional nested RT-PCR using the primers developed by Huang *et al.* (2002) and according to the procedures previously described by Leblanc *et al.* (2007).

Construction of plasmid DNA standards for real-time RT-PCR reactions

Conventional RT-PCR reactions were carried out in a total volume of 20 μl using the Qiagen one-step RT-PCR

Table 1 Primers and probes used in this study

Molecular method	Primer or probe	Sequence 5′–3′	Temperature (°C)	Polarity	Location	Reference
Nested RT-PCR	Primer 3156N	AATTATGCYCAGTAYCGRGTTG	53·8	+	5663–5684*	Huang *et al.* (2002)
HEV detection	Primer 3157N	CCCTTRTCYTGCTGMGCATTCTC	59·1	–	6393–6371*	
	Primer 3158N	GTWATGCTYTGCATWCATGGCT	54·9	+	5948–5969*	
	Primer 3159N	AGCCGACGAAATCAATTCTGTC	55·5	–	6295–6274*	
Real-time RT-PCR	Sense primer	GACAGAATTRATTCGTCGGCTGG	57·3	+	6274–6296*	Mansuy *et al.* (2004)
HEV detection (system A)	Anti-sense primer	TGYTGGTTRTCATAATCCTG	49·8	–	6462–6443*	
	Probe	FAM-GTYGTCTCRGCCAATGGCGAGCNT-IBFQ	64·7	+	6323–6246*	
Real-time RT-PCR	TaqHEV-F	GCCCGGTCAGCCGTCTGG	64·3	+	5207–5224†	Enouf *et al.* (2006)
HEV detection	TaqHEV-R	CTGAGAATCAACCCGGTCAC	55·5	–	5292–5273†	
(system B)	TaqHEV-S	FAM-CGGTTCCGGCGGTGGTTTCT-IBFQ	62·9	+	5250–5269†	
Real-time RT-PCR	HEV-forward	TTACTACCACAGCAGCCACAC	57·2	+	6145–6165*	Ahn *et al.* (2006)
HEV detection	HEV-reverse	TCAGCAAGATTAAACAGTGTCAGG	55·2	–	6252–6229*	
(system C)	HEV-TaqMan	FAM-CCACGACCCACCTCACCAACGCC-IBFQ	66·5	–	6222–6200*	
Real-time RT-PCR	JVHEVF	GGTGGTTTCTGGGGTGAC	55·9	+	5261–5278†	Jothikumar *et al.* (2006)
HEV detection	JVHEVR	AGGGGTTGGTTGGATGAA	53·8	–	5330–5313†	
(system D)	JVHEVP	FAM-TGATTCTCAGCCCTTCGC-IBFQ	55·0	+	5284–5301†	
Real-time RT-PCR	FCV3-Q-A	GACACCTCCGACGAGTTATGC	57·6	+	299–319‡	Mattison *et al.* (2007)
FCV detection	FCV3-Q-1	CCGGGTGGGACTGAGTGG	60·6	–	383–366‡	
	FCV3-Q	Cy5-CGCCTTACGGATATGAGCAGCCACATTAAC-IBRQ	62·2	–	361–332‡	

*Number refers to the corresponding nucleotide position of HEV virus (GenBank accession number NC_001434).
†Number refers to the corresponding nucleotide position of HEV virus (Burna) (GenBank accession number M73218).
‡Number refers to the corresponding nucleotide position of Feline calicivirus (GenBank accession number M863679).
Y = C, T; R = A, G; M = A, C; W = A, T; Y = C, T; N = A, C, G, T.

kit according to the manufacturer's recommendations in an Eppendorf Mastercycler gradient system (Brinkman Instruments Canada Ltd., Mississauga, ON, Canada). Amplifications were performed using HEV strain STHY-233 as positive control and the different primer sets described in Table 1. RT-PCR fragments of 189, 89, 108 and 70 bp corresponding to TaqMan amplification primer system A (Mansuy *et al.* 2004), B (Enouf *et al.* 2006), C (Ahn *et al.* 2006) and D (Jothikumar *et al.* 2006) were excised from the gel and purified using the QIAquick Gel Extraction kit (Qiagen). Purified PCR products were cloned into pCR 2·1 TOPO vector using TOPO TA Cloning kit (Invitrogen) with TOP10 electrocompetent cells in accordance with the manufacturer's recommendations. Sequencing was performed on recombinant plasmids in both directions using a CEQ^{TM} 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) and a CEQ Dye Terminator Cycle sequencing kit (Beckman Coulter) with M13 forward and reverse primers, to confirm the identity of the target sequences amplified. 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 copy number. The copy number of plasmid was calculated as copy number = [(concentration of linearized plasmid)/(molar mass)] $\times$ ($6{\cdot}023 \times 10^{23}$). These DNA plasmids were used for optimization of TaqMan real-time RT-PCR assays (concentration of primers, probe and $MgCl_2$), generation of standard curves and as positive controls.

TaqMan real-time RT-PCR assays

The TaqMan RT-PCR assays were carried out in 25 μl of a reaction mixture comprising 2·5 μl of extracted RNA and 22·5 μl of master mix. Master mix was made with the Brillant QRT-PCR core reagent kit, 1-step (Stratagene, La Jolla, CA, USA) and contained 5·0 mmol l^{-1} of $MgCl_2$, 500 nmol l^{-1} of both forward and reverse primers and 300 nmol l^{-1} of TaqMan probe for system A (Mansuy *et al.* 2004); or 5·0 mmol l^{-1} of $MgCl_2$, 600 nmol l^{-1} of both forward and reverse primers and 250 nmol l^{-1} of TaqMan probe for system B (Enouf *et al.* 2006); or 4·0 mmol l^{-1} of $MgCl_2$, 300 nmol l^{-1} of forward primer, 600 nmol l^{-1} reverse primer and 150 nmol l^{-1} of TaqMan probe for system C (Ahn *et al.* 2006); or 3·0 mol l^{-1} of $MgCl_2$, 150 nmol l^{-1} of forward primer, 400 nmol l^{-1} reverse primer and 200 nmol l^{-1} of TaqMan probe for system D (Jothikumar *et al.* 2006). RT-PCR amplifications were run in a Stratagene Mx3005P system (Stratagene, La Jolla, CA, USA) in a 96-well format under

the following conditions: 30 min at 50°C for reverse transcription, 95°C for 10 min for initial denaturation then followed by 45 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. A standard curve for each system was generated using 10-fold serial dilution (10^8–10^0 genomic equivalents) in a 5 ng ml^{-1} salmon sperm DNA solution of appropriate purified DNA plasmid.

FCV stock production

FCV strain F9 (ATCC VR-782) was previously propagated in CrFK cells, aliquoted in 2 ml cryogenic vials and stored at −80 C. Stock production was titrated by plaque assay (Bidawid *et al.* 2003) and $3{\cdot}2 \times 10^3$ plaque forming units (PFU) of FCV were artificially inoculated in 140 μl of clarified faecal suspensions before RNA extraction with QIAamp® Viral RNA mini kit (Qiagen). Both FCV and HEV RNAs were detected from the same reaction well by multiplex TaqMan RT-PCR assay.

Multiplex TaqMan real-time RT-PCR assay for the detection of FCV and HEV

The multiplex TaqMan assay for the simultaneous detection of FCV and HEV was carried out in 25 μl of a reaction mixture comprising 2·5 μl of extracted RNA and 22·5 μl of master mix. Master mix was made with the Brillant QRT-PCR core reagent kit, 1-step (Stratagene, La Jolla, CA, USA) and contained 3·0 mmol l^{-1} of $MgCl_2$, 150 nmol l^{-1} of forward primer, 400 nmol l^{-1} of reverse primer and 200 nmol l^{-1} of TaqMan probe for the detection of HEV using the detection system D (Jothikumar *et al.* 2006) and 300 nmol l^{-1} of forward and reverse primers and 200 nmol l^{-1} of TaqMan probe for the detection of FCV. Multiplex RT-PCR amplifications were performed with a Stratagene Mx3005P system (Stratagene, La Jolla, CA, USA) in a 96-well format under the following conditions: 30 min at 50°C for reverse transcription, 95°C for 10 min for initial denaturation then followed by 45 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. A standard curve was generated for each system individually and in multiplex using 10-fold serial dilution (10^8–10^0 genomic equivalents) in a 5 ng ml^{-1} salmon sperm DNA solution of respective purified DNA plasmids.

Statistical analyses

One-way ANOVA was used to test for differences in the overall performance of the various TaqMan real-time RT-PCR systems. Differences between individual pairs were tested with the Tukey's multiple comparison test. The *t*-test with Welsh's correction for unequal variances was used to analyse the effect of the presence of HEV on the Ct values of the internal control (FCV). The PRISM 5 statistical package (GraphPad Software, La Jolla, CA, USA) was used throughout.

Results

Evaluation of the different TaqMan real-time RT-PCR systems

For each TaqMan amplification system used in this study, RT-PCR fragments obtained after a conventional RT-PCR amplification were cloned and sequenced for confirmation.

A defined amount of 1×10^4 copies of purified plasmid containing the appropriate cloned amplicon was used to optimize the primers, TaqMan probe and $MgCl_2$ concentrations for each TaqMan assay. Standard curve was established for each system using the corresponding cloned amplicon that was serially diluted from 1×10^8 to 1×10^0 copies and amplified in triplicate. The threshold cycle number values (Ct) were plotted against genomic equivalent copies. Standard curves obtained showed an efficiency of 97·2%, a regression coefficient of 0·993, a slope of −3·390 and an intercept of 41·29 with the primer and probe system A, an efficiency of 85·3%, a regression coefficient of 0·992, a slope of −3·732 and an intercept of 44·14 with the primer and probe system B, an efficiency of 104·3%, a regression coefficient of 0·995, a slope of −3·224 and an intercept of 35·96 with the primer and probe system C, and an efficiency of 94·4%, a regression coefficient of 0·997, a slope of −3·464 and an intercept of 40·15 with the primer and probe system D.

Detection of HEV RNA by conventional nested RT-PCR and TaqMan real-time assays

The different HEV molecular detection assays were evaluated and compared in parallel using the same RNA extracts. Each molecular assay included a negative control (NTC: RNAse free water) and a positive control (cloned amplicon). All nested RT-PCR products had the appropriate size on ethidium bromide stained agarose gel with nothing showing on with negative controls. The detection results for HEV obtained with the conventional RT-PCR and the four different TaqMan assays on 87 faecal samples and 103 plasma samples are presented in Table 2. The nested RT-PCR detected swine HEV RNA in 31 faecal and 5 plasma samples compared to 7 and 0, 0 and 0, 12 and 0 and 32 and 4 for TaqMan systems A, B, C and D respectively. In this experiment, the TaqMan system D showed higher detection performance for swine HEV

Table 2 Detection of HEV RNA by conventional nested RT-PCR and four different TaqMan real-time RT-PCR systems in swine faecal and plasma samples

	Faecal samples (n = 87)	Plasma samples (n = 103)
Conventional nested RT-PCR (Huang *et al.* 2002)	31 (35·6%)	5 (4·9%)
Real-time RT-PCR system A (Mansuy *et al.* 2004)	7 (8·0%)	0 (0%)
Real-time RT-PCR system B (Enouf *et al.* 2006)	0 (0%)	0 (0%)
Real-time RT-PCR system C (Ahn *et al.* 2006)	12 (13·8%)	0 (0%)
Real-time RT-PCR system D (Jothikumar *et al.* 2006)	32 (36·8%)	4 (3·9%)

RNA than the three other TaqMan systems tested in both faecal and plasma samples. Thirty of the 31 feacal samples and 2 of the 5 plasma samples found positive with the nested RT-PCR system were also found positive with the primers and probe D. The real-time system D also detected two additional faecal and plasma samples, which could not be confirmed by nested RT-PCR. In addition, the detection results obtained with this system are comparable to those obtained with the conventional nested RT-PCR which had all previously been confirmed as HEV genotype 3 by sequencing. As the TaqMan system B developed by Enouf *et al.* (2006) was unable to detect any of HEV RNA in all the swine samples tested, this TaqMan system was discarded for other experiments.

Sensitivity of TaqMan systems

The analytical sensitivity of the TaqMan detection systems A, C and D was evaluated by comparing the Ct values obtained from triplicate RNA extractions of 3 different HEV positive faecal samples (Table 3). The detection results were reproducible for each RNA extraction. The system D showed a higher sensitivity than the two other systems with Ct average of 2·08 to 4·06 lower than system A and 9·88 to 10·45 lower than system C resulting in a difference of approximately 1 and 3 logs respectively. There was a significant difference among the three systems ($P < 0{\cdot}05$) based on Tukey's Multiple Comparison Test.

Limit of detection of conventional nested RT-PCR *vs* TaqMan system D

The analytical sensitivities of the conventional nested RT-PCR and the TaqMan system D were evaluated using three faecal samples and three independent RNA extractions serially diluted until 10^{-7} (Table 4). For each dilution, the same RNA extract was tested in duplicate with the conventional nested RT-PCR and the TaqMan system D. In this experiment, the limit of detection (LOD) for a positive signal was set at a fractional recovery level of approximately 50% (3 positive results out of 6). The conventional nested RT-PCR showed LOD values of 10^{-2} for samples swSH73 and swSH79 and 10^{-3} for sample swSH26, while LOD values of 10^{-3}, 10^{-3} and 10^{-4} were observed for the same samples with the TaqMan system D. All negative controls were negative with both detection systems.

Evaluation of HEV/FCV multiplex TaqMan real-time assay

Concentration of primers and probe previously defined during the optimization phase of both systems (HEV detection system D and FCV) were used in the multiplex assay. Standard curves were established individually for each system and then as a duplex assay using the corresponding cloned amplicon serially diluted from 1×10^8 to 1×10^0 copies. All real-time RT-PCR reactions were performed in triplicate. Individual standard curves showed an efficiency of 101·9%, a regression coefficient of 0·999, a slope of −3·278 and an intercept of 37·60 for HEV system D and an efficiency of 106·4%, a regression coefficient of 0·999, a slope of −3·177 and an intercept of 34·49 for FCV TaqMan system. Under the multiplex assay, the standard curve parameters obtained were an efficiency of 106·0%, a regression coefficient of 0·995, a slope of −3·187 and an intercept of 36·16 for HEV system D and an efficiency of 109·7%, a regression coefficient of 0·995, a slope of −3·110 and an intercept of 34·17 for FCV. When using a faecal sample positive for HEV that was artificially inoculated with $3{\cdot}2 \times 10^3$ PFU of the FCV strain F9 as a control sample, Ct values of 26·93 and 27·66 were observed for individual and duplex HEV detection assays respectively and Ct values of 28·46 and 28·81 for individual and duplex FCV detection assays (data not shown).

The detection of $3{\cdot}2 \times 10^3$ PFU of FCV artificially inoculated before the RNA extraction in 10 faecal samples contaminated with HEV and 10 faecal samples negative for HEV was equivalent. Variance analysis using an unpaired t-test with Welch's correction indicates that presence or not of HEV in the sample had no impact on the detection of internal control FCV ($P = 0{\cdot}3948$). The Ct value means and standard deviations for FCV detection were 30·11 ± 0·76 and 30·43 ± 0·13 with or without HEV. Interestingly, HEV was not detected in one sample (swSH1) under the multiplex TaqMan assay, while the detection result was positive in the individual real-time assay (Table 5).

Table 3 Comparison of three TaqMan real-time molecular detection systems (System A, Mansuy; System C, Anh; System D, Jothikumar) for the detection of HEV on three independent RNA extractions from three swine faecal samples contaminated with HEV

	RNA extraction 1	RNA extraction 2	RNA extraction 3		
TaqMan detection system	Ct	Ct	Ct	Mean	Standard deviation
Sample swSH26					
System A	34·98/34·56	34·11/34·03	34·66/34·28	34·44[a]	0·36
System C	28·87/29·22	28·64/28·21	29·07/29·18	28·87[b]	0·39
System D	25·72/24·40	24·97/24·30	25·42/24·50	24·89[c]	0·59
Sample swSH73					
System A	No Ct/No Ct	39·67/38·97	40·93/No Ct	39·86[a]	0·99
System C	31·88/32·28	31·61/32·57	32·19/31·84	32·06[b]	0·35
System D	29·84/29·69	29·76/29·68	30·33/29·98	29·98[c]	0·25
Sample swSH79					
System A	39·61/41·60	No Ct/No Ct	39·82/39·90	40·23[a]	0·92
System C	34·27/34·62	33·03/34·23	33·12/33·86	33·86[b]	0·65
System D	29·31/29·94	29·48/29·97	30·01/30·09	29·80[c]	0·32

Means with a different superscript letter are significantly different ($P < 0·05$) for sample swSH26, swSH73 or swSH79.

Table 4 Analytical sensitivity comparison of TaqMan RT-PCR system D (Jothikumar) with conventional nested RT-PCR from three faecal samples contaminated with HEV and three extracted RNA dilution sets

	Conventional nested RT-PCR			TaqMan RT-PCR (System D)		
RNA dilution	Extraction 1	Extraction 2	Extraction 3	Extraction 1	Extraction 2	Extraction 3
Sample swSH26						
ND	+/+	+/+	+/+	25·72/24·40	24·97/24·30	25·42/24·50
10^{-1}	+/+	+/+	+/+	36·47/27·08	27·27/27·25	27·81/27·60
10^{-2}	+/+	+/+	+/+	30·91/30·33	31·01/30·46	30·92/29·97
10^{-3}	+/+	+/+	+/+	33·73/35·41	33·79/33·85	33·81/33·71
10^{-4}	−/−	−/−	−/+	36·93/36·79	36·59/No Ct	37·35/36·78
10^{-5}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct
10^{-6}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct
10^{-7}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct
Sample swSH73						
ND	+/+	+/+	+/+	29·84/29·69	29·76/29·68	30·33/29·98
10^{-1}	+/+	+/+	+/+	32·46/32·16	31·68/31·84	32·15/32·79
10^{-2}	+/+ (w)	+/+	+/+	36·71/35·64	36·66/35·97	35·41/35·54
10^{-3}	−/−	−/−	−/+ (w)	No Ct/39·10	38·66/39·59	40·39/39·20
10^{-4}	−/−	−/−	−/−	No Ct/No Ct	No Ct/41·31	No Ct/36·64
10^{-5}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct
10^{-6}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct
10^{-7}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct
Sample swSH79						
ND	+/+	+/+	+/+	29·31/29·94	29·48/29·97	30·01/30·09
10^{-1}	+/+	+/+	+/+	32·26/31·77	32·19/32·73	31·87/33·36
10^{-2}	+/+	+/+	+/+	31·71/35·55	35·38/36·21	34·93/34·99
10^{-3}	+/−	−/−	−/−	40·58/No Ct	40·70/43·39	37·18/39·55
10^{-4}	−/−	−/−	−/−	No Ct/No Ct	40·13/No Ct	No Ct/No Ct
10^{-5}	−/−	−/−	−/−	37·60/No Ct	No Ct/No Ct	No Ct/No Ct
10^{-6}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct
10^{-7}	−/−	−/−	−/−	No Ct/No Ct	No Ct/No Ct	No Ct/No Ct

ND, not diluted; W, weak reaction.

Table 5 Detection using the HEV/FCV multiplex TaqMan RT-PCR assay of $3{\cdot}2 \times 10^3$ PFU of FCV artificially inoculated in swine faecal samples contaminated and not contaminated with HEV

	Detection of HEV	Detection of FCV
	Ct	Ct
Samples contaminated with HEV		
swSH1	No Ct/No Ct	31·42/30·92
swSH7	31·99/31·44	29·70/29·76
swSH9	33·38/35·30	29·22/29·22
swSH12	34·93/36·16	29·34/29·72
swSH13	35·07/34·66	30·06/29·59
swSH20	30·18/30·82	29·79/30·82
swSH31	34·04/32·09	30·22/30·00
swSH42	28·56/28·87	30·24/30·59
swSH46	32·86/31·96	29·96/30·00
swSH47	32·10/31·89	29·85/29·65
Mean (standard deviation)	32·57 (2·18)	30·00 (0·57)*
Samples not contaminated with HEV		
swSH15	No Ct/No Ct	30·31/29·94
swSH16	No Ct/No Ct	29·22/30·06
swSH23	No Ct/No Ct	30·02/30·03
swSH24	No Ct/No Ct	29·52/29·89
swSH25	No Ct/No Ct	30·48/30·57
swSH27	No Ct/No Ct	31·99/32·33
swSH28	No Ct/No Ct	29·27/30·09
swSH29	No Ct/No Ct	29·05/28·61
swSH30	No Ct/No Ct	31·30/31·43
swSH33	No Ct/No Ct	30·28/29·96
Mean (standard deviation)	No Ct	30·22 (0·95)*

*Presence or not of HEV in the sample had no impact on the detection of FCV ($P = 0{\cdot}3948$ unpaired *t*-test with Welch's correction).

Discussion

Previous studies revealed that HEV detection results could be influenced by the age of the animal and the sample type (Huang *et al.* 2002; Takahashi *et al.* 2005). Because the concentration of HEV particles tends to be higher in faeces than in blood (Choi *et al.* 2003), RNA extraction and molecular detection could be more easily achieved with faecal samples. Pooled swine faeces collected from the pen floor can provide a good indication of the presence of HEV in the farm environment thus avoiding direct sampling from animals.

The most commonly used procedures in the diagnosis of HEV infections are enzyme immunoassays for the detection of IgG and IgM in serum samples. However, immunoassays often have limited sensitivity (Wang *et al.* 2001; Innis *et al.* 2002), cannot be used for genotype determination and therefore provide limited information for epidemiological studies. The detection of RNA by conventional RT-PCR enables both early diagnosis and genotype determination. It is well established now that real-time RT-PCR assays normally achieve a higher sensitivity, save time, can be automated and could provide viral load quantification compared with conventional individual or nested RT-PCR assays (Gyarmati *et al.* 2007). Several real-time RT-PCR have been proposed recently for the detection of HEV. These proposed assays are based on various chemistry of detection such as SYBR Green (Orru *et al.* 2004), TaqMan probe (Mansuy *et al.* 2004; Ahn *et al.* 2006; Enouf *et al.* 2006; Jothikumar *et al.* 2006) and Primer-Probe Energy Transfer (PriProET) (Gyarmati *et al.* 2007). Each of these technologies possesses its benefits and weaknesses. The SYBR Green chemistry is the easiest to apply, but its specificity is lower than that under molecular probe-based real-time methods and cannot be easily multiplexed for inclusion of an internal control. These disadvantages could be overcome with the TaqMan probe technology. However, the TaqMan detection assays may be less effective than other molecular probe technologies for dealing with mutations because the probe annealing and hydrolysis phase could be affected by some mismatches between the probe and the target sequence. The PriProET is a very robust technology that is less influenced by mismatches located in the target nucleotide sequence. However, the performance of this hybridization technology seems to be lower compared with the TaqMan probe technology (Gyarmati *et al.* 2007).

In this study, four different proposed TaqMan real-time RT-PCR primers and probe sets were evaluated for their ability to detect swine HEV genotype 3 strains currently circulating within the province of Quebec (Canada). The systems A (Mansuy *et al.* 2004) and C (Ahn *et al.* 2006) are targeting nucleotide sequences within the ORF2 (capsid protein), whereas the system B (Enouf *et al.* 2006) and the system D (Jothikumar *et al.* 2006) are directed towards the ORF2/ORF3 overlapping region. The ORF3 encodes a small cytoskeleton-associated phosphoprotein of about 123 amino acids. The ORF2/ORF3 overlapping region was found to be the best target region for PCR amplification of various HEV strains (Inoue *et al.* 2006). Systems A and C reported to be able to detect HEV RNA belonging to genotype 3, while systems B and D were reported to be able to detect all 4 mammalian genotypes (Mansuy *et al.* 2004; Ahn *et al.* 2006; Enouf *et al.* 2006; Jothikumar *et al.* 2006). All these TaqMan systems were optimized in this study for being used and compared on the same platform (Stratagene Mx3005P) in a one-step RT-PCR assay under a two-steps amplification programme (denaturation at 95°C and annealing/polymerization at 60°C). Under these conditions, the TaqMan detection system D showed the best performance parameters for the detection of swine HEV RNA in 87 faecal and 103 plasma samples followed

by the system C. The TaqMan system B, mainly used for the detection of human HEV strains in France, was unable to detect any Canadian genotype 3 swine HEV RNA (0/32) tested in this study.

The performance was significantly different between TaqMan systems A, C and D ($P < 0{\cdot}0001$). System D showed Ct values that were consistently lower than in all other detection systems for every sample (Table 3). These results suggest that the TaqMan system D was more sensitive and reliable than the three other systems evaluated in this study for the detection of HEV in swine faecal sample. The RNA conformation, the availability of the targeted region for the reverse transcription step or the nucleotide composition of the targeted region can explain the differences observed in efficiency and sensitivity between the real-time TaqMan detection systems tested.

The analysis of HEV RNA dilution series revealed that the LOD observed with the TaqMan system D assay was consistently 10-fold more sensitive than the conventional nested RT-PCR considered as the gold standard for the detection of HEV. As both molecular detection assays have been performed on the same RNA extracts, viral RNA extraction recovery could be excluded. This increased sensitivity could explain that the TaqMan system D assay was able to detect one more HEV RNA in faecal samples than the conventional nested RT-PCR. As all nested amplifications of the 5′ end of the ORF2 capsid gene were previously confirmed by sequencing, no false positive results were obtained in this study. This genomic region was also used in other studies for HEV phylogenetic analysis (Lu *et al.* 2006; Ward *et al.* 2008). These two RT-PCR systems could complement each other well: the real-time system, being of greater sensitivity, could be used for screening and the nested RT-PCR system could be used for further molecular characterization of HEV strains in clinical, food and environmental samples.

Faecal samples contain substances such as polysaccharides, phenolic and metabolic compounds that could inhibit or interfere with RT-PCR reactions. The presence and concentration of these possible inhibitors could be very heterogeneous from sample to sample and this may explain the variability in PCR performances (Rutjes *et al.* 2007). To monitor the presence of possible PCR inhibitors, an internal control should be included within the RT-PCR reactions. The internal control is generally a modified PCR product cloned into a plasmid containing a T7 RNA-polymerase promoter. The T7 RNA transcript can be amplified simultaneously with the target RNA using the same primer set (Escobar-Herrera *et al.* 2006; Rutjes *et al.* 2007; Scipioni *et al.* 2008). By integrating an internal control in the assay, inhibitors of RT-PCR, which could lead to false negative results, can be easily identified in analytical samples. When inhibition of amplification reactions is detected, 10 and 100-fold diluted RNA templates or addition of Bovine Serum Albumin (BSA) in the RT-PCR mix could be used to overcome the inhibitory effect (Rutjes *et al.* 2007; Scipioni *et al.* 2008). Template RNA dilution is often effective to dilute the inhibitory compounds; however, the target RNA must be in sufficient quantities for being amplified after dilution. In this study, the FCV was integrated as internal quality control. An amount of $3{\cdot}2 \times 10^3$ PFU of FCV was artificially inoculated in the samples before RNA extraction and used as control to monitor the recovery of viral particles, the RNA extraction process and the amplification procedure in a TaqMan multiplex assay. Extraction and detection of FCV were reproducible from extraction to extraction and detection results were consistent in the multiplex TaqMan assay without interfering with the detection of HEV.

The results obtained in this study indicate that the TaqMan real-time RT-PCR system developed by Jothikumar *et al.* (2006) (system D) was more suitable, reproducible and reliable for the detection of current circulating Canadian swine HEV strains than the three other real-time systems tested. This real-time system also showed a 1 log better analytical sensitivity than a commonly used conventional nested RT-PCR. However, these two molecular systems complement well each other for the detection, quantification and molecular characterization of HEV strains in clinical, food and environmental samples. FCV was integrated successfully as an internal control to monitor the RNA extraction process and amplification procedure in a TaqMan multiplex assay. An amount of $3{\cdot}2 \times 10^3$ PFU of FCV was artificially inoculated in faecal samples before the viral RNA extraction step and its detection by multiplex TaqMan RT-PCR was reproducible from extraction to extraction in presence or not of HEV RNA. This new multiplex TaqMan FCV/HEV detection system will be a valuable assay to identify complications arising during RNA extraction or the presence of RT-PCR inhibitors that may result in false negative results.

References

Aggarwal, R. and Krawczynski, K. (2000) Hepatitis E: an overview and recent advances in clinical and laboratory research. *J Gastroenterol Hepatol* **15**, 9–20.

Ahn, J.M., Rayamajhi, N., Gyun Kang, S. and Sang Yoo, H. (2006) Comparison of real-time reverse transcriptase-polymerase chain reaction and nested or commercial reverse transcriptase-polymerase chain reaction for the detection of hepatitis E virus particle in human serum. *Diagn Microbiol Infect Dis* **56**, 269–274.

Balayan, M.S., Andjaparidze, A.G., Savinskaya, S.S., Ketiladze, E.S., Braginsky, D.M., Savinov, A.P. and Poleschuk, V.F. (1983) Evidence for a virus in non-A, non-B hepatitis

transmitted via the fecal-oral route. *Intervirology* **20**, 23–31.

Bidawid, S., Malik, N., Adegbunrin, O., Sattar, S.A. and Farber, J.M. (2003) A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. *J Virol Methods* **107**, 163–167.

Caron, M., Enouf, V., Than, S.C., Dellamonica, L., Buisson, Y. and Nicand, E. (2006) Identification of genotype 1 hepatitis E virus in samples from swine in Cambodia. *J Clin Microbiol* **44**, 3440–3442.

Chobe, L.P., Lole, K.S. and Arankalle, V.A. (2006) Full genome sequence and analysis of Indian swine hepatitis E virus isolate of genotype 4. *Vet Microbiol* **114**, 240–251.

Choi, I.S., Kwon, H.J., Shin, N.R. and Yoo, H.S. (2003) Identification of swine hepatitis E virus (HEV) and prevalence of anti-HEV antibodies in swine and human populations in Korea. *J Clin Microbiol* **41**, 3602–3608.

Cooper, K., Huang, F.F., Batista, L., Rayo, C.D., Bezanilla, J.C., Toth, T.E. and Meng, X.J. (2005) Identification of genotype 3 hepatitis E virus (HEV) in serum and fecal samples from pigs in Thailand and Mexico, where genotype 1 and 2 HEV strains are prevalent in the respective human populations. *J Clin Microbiol* **43**, 1684–1688.

Emerson, S.U. and Purcell, R.H. (2003) Hepatitis E virus. *Rev Med Virol* **13**, 145–154.

Emerson, S.U., Anderson, D., Arankalle, A., Meng, X.J., Purdy, M., Schauder, G.G. and Tsarev, S.A. (2005) Hepevirus. In *Virus taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses* ed. Fauquet, C.M.., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. pp. 853–857. London: Elsevier/Academic Press.

Enouf, V., Dos Reis, G., Guthmann, J.P., Guerin, P.J., Caron, M., Marechal, V. and Nicand, E. (2006) Validation of single real-time TaqMan PCR assay for the detection and quantitation of four major genotypes of hepatitis E virus in clinical specimens. *J Med Virol* **78**, 1076–1082.

Escobar-Herrera, J., Cancio, C., Guzman, G.I., Villegas-Sepulveda, N., Estrada-Garcia, T., Garcia-Lozano, H., Gomez-Santiago, F. and Gutierrez-Escolano, A.L. (2006) Construction of an internal RT-PCR standard control for the detection of human caliciviruses in stool. *J Virol Methods* **137**, 334–338.

Feagins, A.R., Opriessnig, T., Guenette, D.K., Halbur, P.G. and Meng, X.J. (2007) Detection and characterization of infectious Hepatitis E virus from commercial pig livers sold in local grocery stores in the USA. *J Gen Virol* **88**, 912–917.

Gyarmati, P., Mohammed, N., Norder, H., Blomberg, J., Belak, S. and Widen, F. (2007) Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and Primer-Probe Energy Transfer. *J Virol Methods* **146**, 226–235.

Huang, F.F., Haqshenas, G., Guenette, D.K., Halbur, P.G., Schommer, S.K., Pierson, F.W., Toth, T.E. and Meng, X.J. (2002) Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J Clin Microbiol* **40**, 1326–1332.

Innis, B.L., Seriwatana, J., Robinson, R.A., Shrestha, M.P., Yarbough, P.O., Longer, C.F., Scott, R.M., Vaughn, D.W. *et al.* (2002) Quantitation of immunoglobulin to hepatitis E virus by enzyme immunoassay. *Clin Diagn Lab Immunol* **9**, 639–648.

Inoue, J., Takahashi, M., Yazaki, Y., Tsuda, F. and Okamoto, H. (2006) Development and validation of an improved RT-PCR assay with nested universal primers for detection of hepatitis E virus strains with significant sequence divergence. *J Virol Methods* **137**, 325–333.

Jothikumar, N., Paulmurugan, R., Padmanabhan, P., Sundari, R.B., Kamatchiammal, S. and Rao, K.S. (2000) Duplex RT-PCR for simultaneous detection of hepatitis A and hepatitis E virus isolated from drinking water samples. *J Environ Monit* **2**, 587–590.

Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J. and Hill, V.R. (2006) A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J Virol Methods* **131**, 65–71.

Leblanc, D., Ward, P., Gagne, M.J., Poitras, E., Muller, P., Trottier, Y.L., Simard, C. and Houde, A. (2007) Presence of hepatitis E virus in a naturally infected swine herd from nursery to slaughter. *Int J Food Microbiol* **117**, 160–166.

Li, T.C., Chijiwa, K., Sera, N., Ishibashi, T., Etoh, Y., Shinohara, Y., Kurata, Y., Ishida, M. *et al.* (2005) Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis* **11**, 1958–1960.

Lu, L., Li, C. and Hagedorn, C.H. (2006) Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Rev Med Virol* **16**, 5–36.

Mansuy, J.M., Peron, J.M., Abravanel, F., Poirson, H., Dubois, M., Miedouge, M., Vischi, F., Alric, L. *et al.* (2004) Hepatitis E in the south west of France in individuals who have never visited an endemic area. *J Med Virol* **74**, 419–424.

Mattison, K., Brassard, J., Houde, A., Simard, C., Pagotto, F., Jones, T. and Trottier, Y.-L. Octobre (2007) *The Feline Calicivirus (FCV) As an Internal Control for the Detection of RNA Viruses from Foods*. Federal Food Safety and Nutrition Research Meeting. Winnipeg, Manitoba, Canada.

Meng, X.J., Purcell, R.H., Halbur, P.G., Lehman, J.R., Webb, D.M., Tsareva, T.S., Haynes, J.S., Thacker, B.J. *et al.* (1997) A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci USA* **94**, 9860–9865.

Meng, X.J., Halbur, P.G., Shapiro, M.S., Govindarajan, S., Bruna, J.D., Mushahwar, I.K., Purcell, R.H. and Emerson, S.U. (1998) Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J Virol* **72**, 9714–9721.

Meng, X.J., Dea, S., Engle, R.E., Friendship, R., Lyoo, Y.S., Sirinarumitr, T., Urairong, K., Wang, D. *et al.* (1999) Prevalence of antibodies to the hepatitis E virus in pigs

from countries where hepatitis E is common or is rare in the human population. *J Med Virol* **59**, 297–302.

Meng, X.J., Wiseman, B., Elvinger, F., Guenette, D.K., Toth, T.E., Engle, R.E., Emerson, S.U. and Purcell, R.H. (2002) Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. *J Clin Microbiol* **40**, 117–122.

Mizuo, H., Yazaki, Y., Sugawara, K., Tsuda, F., Takahashi, M., Nishizawa, T. and Okamoto, H. (2005) Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan. *J Med Virol* **76**, 341–349.

Mushahwar, I.K. (2008) Hepatitis E virus: molecular virology, clinical features, diagnosis, transmission, epidemiology, and prevention. *J Med Virol* **80**, 646–658.

Nishizawa, T., Takahashi, M., Mizuo, H., Miyajima, H., Gotanda, Y. and Okamoto, H. (2003) Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. *J Gen Virol* **84**, 1245–1251.

Okamoto, H. (2007) Genetic variability and evolution of hepatitis E virus. *Virus Res* **127**, 216–228.

Orru, G., Masia, G., Romano, L., Piras, V. and Coppola, R.C. (2004) Detection and quantitation of hepatitis E virus in human faeces by real-time quantitative PCR. *J Virol Methods* **118**, 77–82.

Renou, C., Cadranel, J.F., Bourliere, M., Halfon, P., Ouzan, D., Rifflet, H., Carenco, P., Harafa, A. *et al.* (2007) Possible zoonotic transmission of hepatitis E from pet pig to its owner. *Emerg Infect Dis* **13**, 1094–1096.

Rutjes, S.A., Lodder, W.J., Bouwknegt, M. and de Roda Husman, A.M. (2007) Increased hepatitis E virus prevalence on Dutch pig farms from 33 to 55% by using appropriate internal quality controls for RT-PCR. *J Virol Methods* **143**, 112–116.

Scipioni, A., Bourgot, I., Mauroy, A., Ziant, D., Saegerman, C., Daube, G. and Thiry, E. (2008) Detection and quantification of human and bovine noroviruses by a TaqMan RT-PCR assay with a control for inhibition. *Mol Cell Probes* **22**, 215–222.

Smith, J.L. (2001) A review of hepatitis E virus. *J Food Prot* **64**, 572–586.

Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. and Okamoto, H. (2003) Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. *J Gen Virol* **84**, 851–862.

Takahashi, M., Nishizawa, T., Tanaka, T., Tsatsralt-Od, B., Inoue, J. and Okamoto, H. (2005) Correlation between positivity for immunoglobulin A antibodies and viraemia of swine hepatitis E virus observed among farm pigs in Japan. *J Gen Virol* **86**, 1807–1813.

Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.C., Bradley, D.W., Fry, K.E. and Reyes, G.R. (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185**, 120–131.

Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. (2003) Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* **362**, 371–373.

Wang, Y., Zhang, H., Li, Z., Gu, W., Lan, H., Hao, W., Ling, R., Li, H. *et al.* (2001) Detection of sporadic cases of hepatitis E virus (HEV) infection in China using immunoassays based on recombinant open reading frame 2 and 3 polypeptides from HEV genotype 4. *J Clin Microbiol* **39**, 4370–4379.

Ward, P., Muller, P., Letellier, A., Quessy, S., Simard, C., Trottier, Y.L., Houde, A. and Brassard, J. (2008) Molecular characterization of hepatitis E virus detected in swine farms in the province of Quebec. *Can J Vet Res* **72**, 27–31.

Williams, T.P., Kasorndorkbua, C., Halbur, P.G., Haqshenas, G., Guenette, D.K., Toth, T.E. and Meng, X.J. (2001) Evidence of extrahepatic sites of replication of the hepatitis E virus in a swine model. *J Clin Microbiol* **39**, 3040–3046.

Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y. and Okamoto, H. (2003) Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be foodborne, as suggested by the presence of hepatitis E virus in pig liver as food. *J Gen Virol* **84**, 2351–2357.

Yoo, D., Willson, P., Pei, Y., Hayes, M.A., Deckert, A., Dewey, C.E., Friendship, R.M., Yoon, Y. *et al.* (2001) Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus. *Clin Diagn Lab Immunol* **8**, 1213–1219.

