

Validation of Single Real-Time TaqMan[®] PCR Assay for the Detection and Quantitation of Four Major Genotypes of Hepatitis E Virus in Clinical Specimens

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Since the characterization of the genome of the hepatitis E virus (HEV) in 1990, a large genetic diversity has been described. A single real-time reverse transcription (RT)-PCR assay with TaqMan[®] technology has been validated which uses only one set of primers and probe within the ORF2 HEV region (nt 5207–5292) for the detection and quantitation of the four major genotypes of HEV. This assay proved to be as efficient as the conventional RT-PCR methodology for the detection of HEV in clinical samples testing positive previously. The real-time RT-PCR and conventional RT-PCR were performed comparatively on 60 pairs of sera and stools collected during a recent outbreak of hepatitis E in Darfur. The real-time RT-PCR assay was 10- to 100-fold sensitive than for conventional RT-PCR assays used in this study with a range quantitation from 1.8×10^1 to 7.2×10^3 RNA copies/ μ l in clinical samples (serum and stools). **J. Med. Virol. 78:1076–1082, 2006.** © 2006 Wiley-Liss, Inc.

KEY WORDS: HEV; diagnosis; quantitation; outbreak

INTRODUCTION

The genetic heterogeneity of hepatitis E virus (HEV) has been well documented, and HEV isolates have been classified into from four major to at least nine genotypes according to partial or full-length sequences: genotype 1 (Asian and African strains), genotype 2 (the prototype Mexican strain and a few African strains), genotype 3 (strains from sporadic cases in industrialized countries), and genotype 4 (strains from sporadic cases in China, Japan, and Taiwan) [Schlauder and Mushahwar, 2001; Emerson and Purcell, 2003; Mansuy et al., 2004a]. The molecular detection of the various HEV genotypes by conventional reverse transcription (RT)-PCR currently involves the use of various specific sets of primers designed to amplify conserved regions within ORF1,

ORF2, and ORF3 [Tam et al., 1991; Hsieh et al., 1998; Schlauder and Mushahwar, 2001]. A universal RT-PCR assay able to detect divergent strains of HEV was developed recently and validated, but this technique is based on a conventional RT-PCR [Cooper et al., 2005].

Real-time RT-PCR assays using SYBR Green dye or TaqMan[®] probes have been developed successfully for the detection of HEV specimens, but these researchers did not report examining their assay with the various HEV genotypes in circulation [Orru et al., 2004]. In addition, SYBR Green dye binds to double-stranded nucleic acids generated during the amplification, allowing sensitive detection of products in real time. However, unlike PCR assays using internal fluorescent probes, this approach does not avoid the quantitation of non-specific amplification products [Ririe et al., 1997]. The development of a broadly reactive real-time assay using the TaqMan[®] chemistry is preferable to other fluorescent assay types based on lower relative cost, relative ease of design, and the higher specificity of the probe-based TaqMan[®] assay versus non-probe approaches like SYBR Green. Two real-time RT-PCR assays using TaqMan[®] chemistry have been described. Mansuy et al. [2004a] described a method sensitivity of 1,000 copies/ml of serum sample, but did not enhance the ability of the assay to detect multiple HEV genotypes. The second assay [Jothikumar et al., 2006] able to detect all HEV genotypes with high specificity was a TaqMan[®] assay validated from HEV isolates. But no quantitation of the viral load from a large number of human and

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animal clinical samples has been reported. Consequently, the aim of this study was to develop a single real-time LightCycler RT-PCR assay using TaqMan[®] probe technology to detect any of the four major genotypes of HEV and to quantify HEV in clinical samples, sera, and stools.

MATERIALS AND METHODS
HEV Strain Alignment and Design of Primers and Probe

Twenty-six full-length human and swine HEV sequences including strains representative of the four major genotypes were aligned with the multiple sequence editor CLUSTALX (1.82). The Genbank accession numbers of the complete genomes are listed in Figure 1. Local alignments were performed in several regions using all complete and partial HEV sequences available at the time of the study. The sequences were manipulated using DNA strider 1.3.6 (CEA). Homologous sequences were extracted from Genbank using BLAST 2.2.9 (NCBI). Mfold 3.2 (Washington University) was used to predict the stability of secondary RNA structures. The most conserved regions were then subjected to sequence analysis using the Primer 3 and Oligo 6 (Molecular Biology Insights) softwares to design primers and a probe [Rozen and Skaletsky, 2000].

Finally, the specificity of the primers and probe for HEV genomes were tested by searching for homologous sequences in Genbank using the Blast software.

Specimens

Two types of samples (RNA and clinical samples) were tested by the real-time RT-PCR assay.

The first type was composed of 52 HEV RNA preparations from serum and stools provided by the French National Reference Centre for hepatitis E. All 52 were positive previously by conventional RT-PCR performed at the French National Reference Centre for HEV at the present time of the study. The HEV RNA samples were stored at -80°C (Table I). HEV RNA of genotypes 1 and 2 had been amplified with oligonucleotides targeting HEV ORF2 (nt 6653-7100) as described elsewhere [Tam et al., 1991]. HEV RNA of genotype 3 was detected by conventional PCR using degenerate primers targeting ORF1 and ORF2 [Schlauder et al., 1999]. HEV genotype 4 RNA extracts, collected in Cambodia from humans with sporadic cases of acute hepatitis and from swine, were previously amplified with primers for ORF1 [Hsieh et al., 1998].

The second type of specimens was composed of 120 human clinical samples, including 60 pairs of serum and fecal samples collected during the hepatitis E outbreak

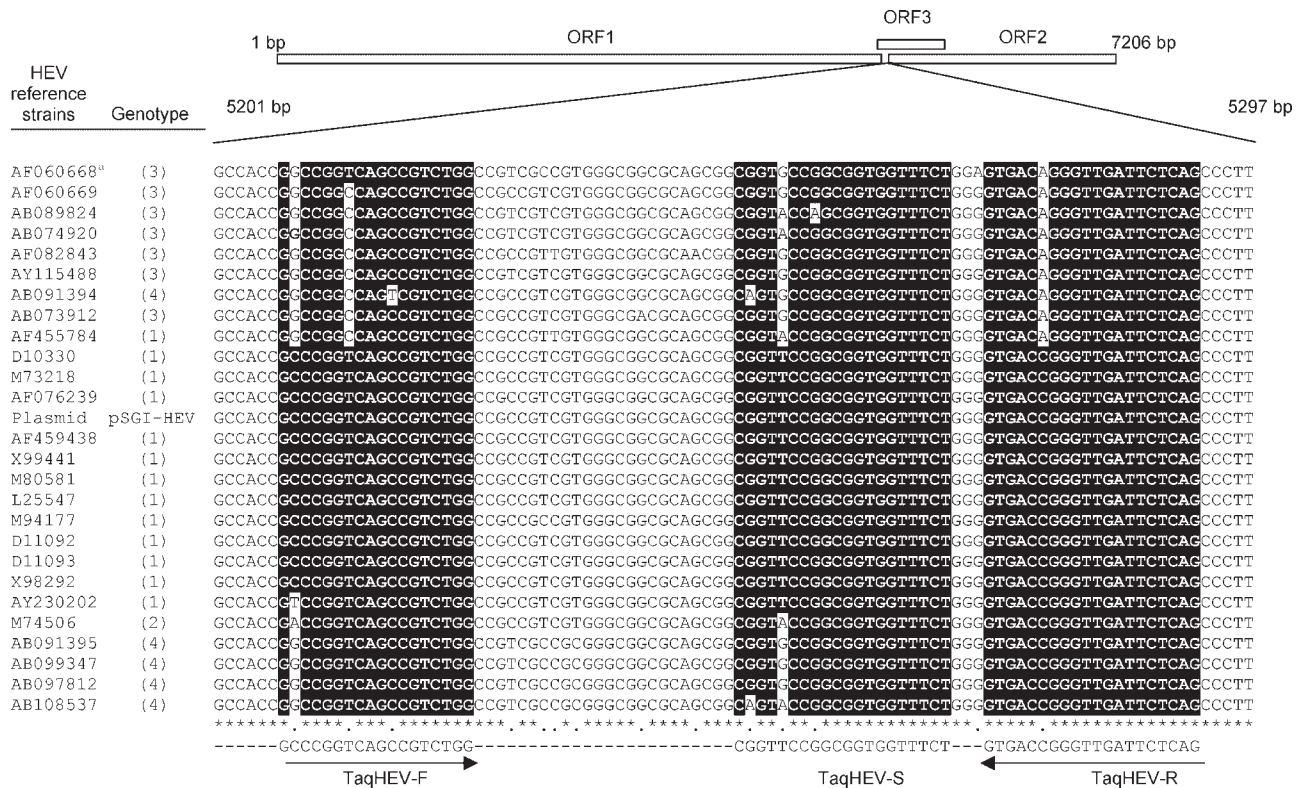


Fig. 1. Sequence alignment within HEV ORF2 region of 26 complete HEV genomes and the plasmid pSGI-HEV, and position of primers (TaqHEV-F and Taq HEV-R) and probe (TaqHEV-R). a: accession number; sequences in white boldface were used for the consensus sequence for primers and probe.

TABLE I. Fifty-Two HEV Clinical Isolates of the Four Major Genotypes Provided by The French National Reference Centre for Hepatitis E and Tested by Real-Time RT-PCR

Genotype	Origin	Specimens	Travel history	No. of strains	Genbank accession number	
Genotype 1	Human	Stools	Pakistan, India	6	AY568365	
		Serum	Taneftdur, Algeria	11	AY596463	
						AY568336
						AY568363
						AY568362
						AY568361
						AY568360
						AY596462
						AY568359
						AY568358
						AY568357
Genotype 2	Human	Stools	Chad	4	DQ151637	
		Serum	Nigeria	5	AY903950	
						AF 172999
						AF 173000
Genotype 3	Human	Stools	No travel history (France)	6		
		Serum	No travel history (France)	2		
Genotype 4	Human	Stools	Cambodia	1		
		Serum	Cambodia	1		
	Swine	Stools	Cambodia	15		
		Serum	Cambodia	1		

in Mornay Darfur, Sudan. The samples were collected from members of the internally displaced population presenting acute hepatitis in 2004 at the hospital camp, then shipped to the French National Reference Centre for Hepatitis E (cold chain transport + 4°C) and tested. Diagnosis of hepatitis E was confirmed by detection of anti-HEV IgG (HEV Elisa, Genlabs Diagnostic, Singapore), anti-HEV IgM (HEV IgM Elisa, Genlabs Diagnostic) and molecular detection of HEV by conventional HEV RT-PCR as described previously. A strong anti-HEV IgG reactivity was found in 60/60 and anti-HEV IgM in 55/60 serum, reflecting recent hepatitis E infection. HEV RNA was amplified from 14 sera and 20 stools by conventional PCR [Tam et al., 1991]. The sequencing of several PCR products generated by conventional PCR indicated that genotype 1 was the virus circulating in West Darfur (GenBank accession number [AY903948]). Real-time TaqMan[®] PCR was used to quantify HEV RNA in these clinical samples. Two serum samples collected from two anti-HEV-negative, healthy subjects were used as negative samples.

RNA Extraction and Reverse Transcription

RNA was extracted from clinical serum and stool samples. One-tenth volume of 10× phosphate buffer saline (PBS) was added to the stool samples and the suspensions were mixed thoroughly and clarified by centrifugation at 12,000g at room temperature for 2 min. Total RNA was then extracted from 200 µl samples of sera and stool suspensions with the RNA Isolation kit I (Roche Diagnostics Corporation, Indianapolis, IN) using

the automated MagNA Pure device according to the manufacturer's recommendations. RNA was eluted in 100 µl of kit I elution buffer, aliquoted, and stored at -80°C until testing. Five microliter samples of extracted RNA were used for RT. RNA was first incubated with 0.25 µg of oligo d(T)₁₅ (in a final volume of 8.5 µl) at 70°C for 5 min and immediately cooled on ice. Moloney murine leukemia virus reverse transcriptase (M-MLV Reverse Transcriptase) (Promega) was used for RT: a final volume of 12.5 µl of RT reaction containing annealed primer/template (8.5 µl), 2.5 µl of 5 × M-MLV buffer, 20 U of RNasin Plus Ribonuclease Inhibitor (Promega), 200 U of M-MLV reverse transcriptase, 5 mM each of dATP, dCTP, dGTP, and dTTP, was incubated in a Perkin-Elmer 9600 thermal cycler; the cycling parameters were 5 min at 25°C, 60 min at 42°C, 15 min at 70°C, followed by a 4°C hold.

Real-Time Quantitative PCR With LightCycler

LightCycler-FastStart DNA Master hybridization Probes (Roche Diagnostics) kit was used according to the manufacturer's instructions for real-time PCR. PCR was performed in a final volume of 15 µl containing 3 mM MgCl₂, 0.3 µM of each HEV-specific primer, 0.2 µM of internal probe, and 2 µl of the cDNA preparation. The thermocycling conditions were optimized to one cycle of denaturation for 10 min at 95°C, followed by 50 amplification cycles (temperature transition rate of 5°C/sec), each cycle comprising denaturation (95°C for 5 sec), and annealing/extension (60°C for 60 sec). Negative samples and distilled water were tested in duplicate in each run as negative controls.

Positive Control

The plasmid, pSGI-HEV containing a full-length genotype 1 HEV cDNA [Panda et al., 2000], was used as a DNA template in conventional PCR with sense and antisense primers designed from the HEV strain alignment: TaqHEV-F and TaqHEV-R. The resulting PCR product was inserted into pGEM-T (Promega) according to the manufacturer's instructions. The cloning region was sequenced and the recombinant plasmid, named pGEM-HEVtaqm, was purified by the MidiPrep Method (Qiagen) and was quantified with the Gen Quant II instrument according to the manufacturer's instructions (Pharmacia Biotech). Two microliters of stock DNA contained 2.5×10^9 genome equivalent (GE) copies of the plasmid. Serial 10-fold dilutions of pGEM-HEVtaqm were used as a standard to establish the reference amplification curve.

Controls and Interpretation

The linearity of the reference amplification curve was evaluated by amplification of serial 10-fold dilutions of the positive control pGEM-HEVtaqm in duplicate in two independent assays. Distilled water was used as a negative control. The crossing point (Cp) was the quantitative parameter and was determined by the second derivative maximum (SDM) (LightCycler Software 3.5.3). Samples with a Cp value below 43 and with no evidence of amplification in the negative control and negative samples (threshold not reached after 45 cycles) were considered as positive.

To check for the presence of contaminating DNA, negative samples and negative controls were included in each run of real time PCR. Experimental accuracy was improved by running samples in duplicate starting with the RT reaction [Stahlberg et al., 2004] and the resulting cDNA was subjected to two independent real-time PCR assays.

RESULTS

Primer and Probe Design

The aim of the study was to design a set of primers and probe that would allow the detection of the four main genotypes of HEV in a single real-time RT-PCR assay. After collection of a set of 26 full-length HEV sequences from Genbank, the sequences were compared and the most conserved regions of HEV genome were thereby identified. Three regions between nucleotides 1–150

(region I), 5000–5500 (region II), and 6300–6500 (region III) were selected and subjected to a more detailed analysis. For each region, the most conserved sub-regions were first determined by extracting and comparing all partial or complete HEV sequences available in the Genbank database at the time of the study. Then, the sequences that met all the criteria required to design a powerful consensus real-time RT-PCR based assay were identified. In particular, it was systematically excluded sequences predicted to form highly stable secondary RNA structures because such structures are likely to impair primer annealing. A 86-bp region within ORF 2 (nt 5207–5292) appeared to be highly conserved and suitable as the basis for a set of primers and probe for real-time PCR, despite its GC nucleotide composition of 72% (Fig. 1). The sequences of the primers TaqHEV-F, TaqHEV-R, and probe TaqHEV-S are shown in Table II. Extensive search for homologous sequences in sequence databases indicated that the primers and probe were highly specific for HEV sequences.

Reference Amplification Curve and Detection Limit

The detection limit of the real-time PCR was evaluated using serial 10-fold dilutions of pGEM-HEVtaqm, a plasmid that contained the target DNA sequence (Fig. 2). The assay had a linear response over at least a 9- \log_{10} concentration range, from 10 to 10^9 copies/reaction ($R^2 = 0.998$). The assay results were reproducible with a coefficient of variation (CV) in the Cp values range from 0.97% to 2.26% (mean of CV = 1.41%).

Efficiency and Reliability of Real-Time HEV PCR

A first set of experiments was designed to determine if the real-time RT-PCR assay was as sensitive as the various conventional RT-PCR assays currently used to detect the four major genotypes of HEV. The ability of the real-time PCR assay to detect HEV RNA in 52 fecal and serum samples of human and swine origin was evaluated (Table I). All these samples were found positive previously for HEV RNA on conventional RT-PCR assays performed at the French National Reference Centre for HEV. Independent of the genotype, all 52 samples gave amplification products in the single real-time RT-PCR assay (Cp values ranged from 28.02 to 35.4). No cross amplification was observed.

TABLE II. Primers and Probe Used for the Detection of HEV by Real-Time PCR Assay

Primer or probe	Sequence (5'-3')	Location
TaqHEV-F	GCCCGGTCAGCCGTCTGG	5207–5224 ^b
TaqHEV-R	CTGAGAATCAACCCGGTCAC	5273–5292 ^b
TaqHEV-S	FAM-CGGTTCGGCGGTGGTTTCT-TAMRA ^a	5250–5269 ^b

^aThe probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end and 6 carboxy-tetramethylrhodamine (TAMRA) at the 3' end. It was designed so that the predicted melting temperature was at least 5°C higher than that of the primers.

^bCorresponding nucleotide position of HEV virus (Burma) (accession no. M73218).

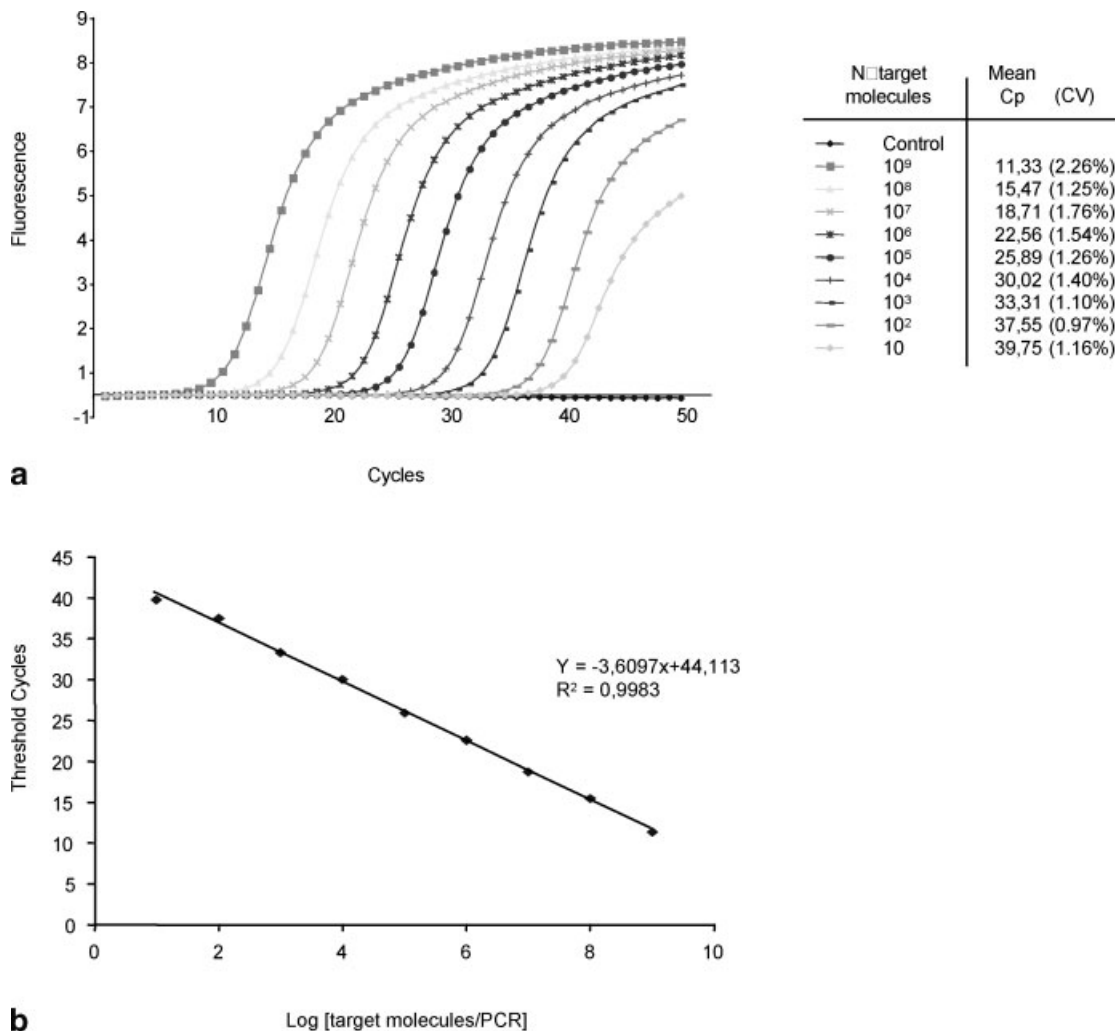


Fig. 2. Quantification of serially diluted suspension ($10-10^9$ molecules) of quantified HEV plasmid target obtained by real-time PCR with LightCycler instrument using Taqman technology. **a**: Fluorescence curve of F1/F2 signal formation during the amplification of a 86 bp fragment of HEV gene (each point represents the mean of the Cp from four independent assays. CV, coefficient of variation); **b**: standard curve constructed from the data in (a).

Performance of Real-Time HEV PCR From Clinical Samples

Preliminary experiments indicated that the real-time RT-PCR assay was as sensitive as conventional RT-PCR assays. As the primers and probe were designed in a highly conserved region, it was tested whether this real-time RT-PCR could detect HEV in samples from HEV-infected patients that have not been tested previously by conventional RT-PCR assays. The performances of these two PCR methods were compared using a total of 120 samples comprising 60 pairs of serum and fecal samples collected between 6 and 14 days after the onset of clinical signs of acute hepatitis. The serum samples were drawn 6–7 days after the onset of clinical signs for those with high viral loads and over 7 days after the onset of signs for those with lower viral loads. The fecal samples were collected between 6 and 14 days after the onset of clinical signs.

The 120 samples were analyzed by both conventional and real-time RT-PCR assays. Of the 120 samples, 34 samples (29%, 14 sera and 20 stools) were positive on both assays. And real-time RT-PCR detected HEV in additional samples (35 sera and 33 stools) with the overall amplification of 102 samples (85%) (49 sera and 53 stools) (Table III). The RT-PCR products of 33 stool samples scoring positive by real-time RT-PCR and negative by conventional RT-PCR were sequenced; the presence of HEV virus was confirmed. And the sequences of these isolates were identical to sequences of samples positive on both assays. HEV real-time RT-PCR-positive samples with Cp values below 31 (7.2×10^3 RNA copies/ μ l serum or μ g stools) scored positive by conventional RT-PCR.

The Cp values of sera only identified as positive by the real-time RT-PCR assay were between 36.01 and 40.79, corresponding to $6.15 \times 10^2-1.87 \times 10^1$ RNA copies/ μ l serum. The Cp values of stool samples only identified as

TABLE III. Analysis of 60 Pairs of Serum and Fecal Samples by Both Conventional and Real-Time RT-PCR

	Conventional RT-PCR ^a	Real-time RT-PCR assay result			
		Assay result	Positive	Negative	Total
Serum	Positive	14	0	14	<0.05
	Negative	35	11	46	
	Total	49	11	60	
Stool	Positive	20	0	20	<0.05
	Negative	33	7	40	
	Total	53	7	60	

^aConventional RT-PCR was performed with primers targeting HEV ORF2 as described elsewhere [Tam et al., 1991].

^bChi Squared for paired samples.

positive by real-time RT-PCR assay were between 33 and 41 corresponding to 1.15×10^3 – 1.51×10^1 RNA copies/ μ g stool. These results based on a genotype 1 standard (pGEM-HEVtaqm) confirmed that conventional PCR-based assays used in this study were less sensitive than the single real-time RT-PCR.

The reproducibility of these data was assessed by testing each sample in duplicate.

DISCUSSION

Real-time nucleic acid amplification systems are reliable tools with high sensitivity and specificity, and fast turnaround times. The idea of performing both the RT and PCR in a single tube is attractive since it reduces handling and therefore minimizes the chance of pipetting errors and contamination. Unlike Jothikumar et al. [2006], a two-step RT-PCR method was used to compare results of conventional and real-time PCR with the same cDNA and moreover it was found that the one-step method is variable intrinsically and significantly less sensitive than the two-step method. Many researchers have reported these conclusions and it was shown that experimental variation in RT-PCR is attributable mainly to the RT step [Battaglia et al., 1998; Bustin, 2002; Stahlberg et al., 2004]. Jothikumar et al. [2006] described a TaqMan[®] RT-PCR assay evaluated with few HEV isolates tested previously from four major genotypes. The present development of a single consensus real-time TaqMan[®] RT-PCR has the advantage of being validated with RNA extracts from clinical samples (sera and stools) for all the four major genotypes of HEV. Moreover, quantitation of viral loads in serum and fecal specimens indicated that this real-time RT-PCR assay had a detection limit 10- to 100-fold lower than for conventional RT-PCR assays used in this study.

The main difficulty when developing a molecular assay for HEV virus is the design and construction of the primers and probe: they must be compatible with the genetic heterogeneity of this virus and the high G + C content of the viral genome. Because of the process, it was used to identify the most conserved regions of the viral genome. And two different set of primers were determined from nt 5260–5330 [Jothikumar et al., 2006] within ORF3 HEV region and from 5207 to 5292

within ORF2 HEV region in the present study. It is likely that this RT-PCR assay detects a broader set of genotypes. This, however, remains to be assessed.

An advantage of real-time RT-PCR is the improvement in the diagnosis of hepatitis E. The course of HEV infection is monitored currently by RT-PCR assays with samples collected during outbreaks of hepatitis E and from experimentally infected animals. With conventional RT-PCR, HEV RNA can be detected in serum and stools from most patients within 2 weeks [Clayson et al., 1995]. A study with sequential serum and stools collected from patients during an outbreak of hepatitis E reported viraemia until day 29 and fecal excretion until day 35; this is longer than both the clinical course and the duration of biochemical hepatitis [Aggarwal et al., 2000]. Suitable real-time RT-PCR may allow the duration of viraemia and fecal shedding to be determined more rigorously. Indeed, further analyses of sequential samples are required to determine the duration of infectiousness. HEV infections have been described in the absence of anti-HEV IgG and IgM [Nicand et al., 2001; Mansuy et al., 2004b]: the single HEV marker was HEV RNA either in serum or in stools. Using this powerful technique, such infections with a very low HEV loads (10^2 HEV molecules per reaction) could be detected.

The real-time TaqMan[®] PCR assay able to detect any genotype of HEV opens new opportunities. This sensitive method should allow HEV to be quantified in environmental samples, particularly water sources. Outbreaks, like the recent outbreak in the Western Darfur region of Sudan, were likely to have been associated with water supplies [Nicand et al., 2005].

It is concluded that the consensus real-time RT-PCR assay developed in this work is a highly sensitive and specific tool for accurate quantification of major genotypes of HEV virus. This assay will be useful as a routine diagnostic assay for clinical specimens.

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