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BRIEF REPORT

Simple and rapid detection of swine hepatitis E virus by reverse transcription loop-mediated isothermal amplification

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Abstract Hepatitis E virus (HEV) is an enteric pathogen of humans and animals, and pigs have been considered an important reservoir of this virus. Recent evidence has indicated the cross-species transmission of hepatitis E virus (HEV) from pigs to humans, causing zoonosis, mostly via consumption of uncooked or undercooked animal meat/viscera. In this study, we have developed a one-step
RT-LAMP assay for rapid detection of swine HEV. Specific primer sets targeting the ORF3 gene were designed. The sensitivity of the RT-LAMP assay was 10^1 copies/μl of RNA template, which was tenfold higher than that of RT-nPCR. The specificity of this assay was demonstrated by the lack of amplification of DNA/RNA from other swine viruses. Furthermore, a total of 41 bile samples were subjected to RT-LAMP and RT-nPCR. Eighteen positive samples were detected by RT-nPCR, while 36 positive samples were detected by RT-LAMP, indicating that the sensitivity of the RT-LAMP assay was higher than that of the conventional RT-nPCR assay. The RT-LAMP assay reported here may be used for diagnosis of swine HEV, not only in laboratories but also under field conditions.

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Introduction

Hepatitis E virus (HEV), the causative agent of acute human hepatitis E, is responsible for a disease that is important for public health in many developing countries of Asia and Africa [2, 19]. HEV is transmitted through oral consumption of contaminated food and water. Accumulating evidence indicates that hepatitis E is a zoonotic disease, and swine are the main reservoirs. Swine HEV was first isolated from pigs in the USA in 1997 [8]. Genetic analysis of strains isolated from pigs showed a high degree of similarity to strains of HEV isolated from humans [7]. This was the first evidence showing that HEV is a zoonotic agent. Further studies have shown that occupational groups (e.g., veterinarians, swine breeders) with close contact to pigs have an increased risk for HEV infection [1, 9], and direct transmission through the consumption of contaminated food products such as pork have been reported in Japan [21]. The ubiquitous nature of the virus in domestic pigs raises public-health concerns for zoonosis and food safety. Consequently, it is important to develop a simple, rapid and sensitive detection method for enhancing surveillance for swine HEV infections in swine.

A loop-mediated isothermal amplification (LAMP) method has been developed that can amplify nucleic acids with high specificity, sensitivity and rapidity under isothermal conditions [16]. It has the additional advantage that only simple equipment, such as a heating block or a water bath, is required. In addition, the results can be ascertained easily by the naked eye. The method is

Table 1 Primers for RT-LAMP for detection of swine HEV

Primer description	Primer name	Sequence (5'-3')	Genome position[a]
Forward outer	F3	GCGCTCTCGGGCTCTTC	5190-5206
Reverse outer	B3	GCGAAGGGGTTGGTTGGA	5358-5375
Forward inner	F1c	ACGGCCAGACGGCTGAC	5256-5272
	F2	TTTCTGCTCCTCGTGCTT	5209-5226
Reverse inner	B1c	GGTGCCGACGGTGGTTT	5293-5309
	B2	GATGAATATAGGGGAGGGCGAA	5338-5359

[a] The RT-LAMP primer positions are shown relative to swine hepatitis E virus isolate WH09 (GenBank accession number GU188851)

applicable to amplification of RNA templates by combination with a reverse transcription reaction (RT-LAMP) [17]. In this study, we developed a one-step RT-LAMP assay for simple, rapid, and sensitive detection of swine HEV.

Materials and methods

Design of RT-LAMP assay primers for swine HEV

The swine-HEV-specific primers used in the RT-LAMP assay were designed to be specific for the ORF3 gene. A set of four primers comprising two outer primers (forward primer F3 and reverse primer B3) and two inner primers (forward inner primer FIP and reverse inner primer BIP) that recognize six regions on the target sequence was designed by use of the LAMP primer design software Primer-Explorer (version 4; Ekien Chemical Co., Japan; http://primerexplorer.jp/elamp4.0.0/index.html). All of the primers used in this study were synthesized by Shanghai Invitrogen Co., Ltd. The details for each primer and the positions of the primers in the genomic sequences are shown in Table 1.

Viruses

The analytical specificity of the RT-LAMP assay was evaluated by testing classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), foot-and-mouth disease virus (FMDV), porcine circovirus type 2 (PCV2), H3N2 swine influenza virus (SIV), and pseudorabies virus (PRV). All of these virus isolates were obtained from MOA Key Laboratory for Animal Vaccine Development, Guangdong, China.

Clinical samples and RNA extraction

A total of 41 bile samples from symptomatic swine were collected from Gongdong Province of China. Each sample was added to PBS (PH 7.2) to yield a 10 % suspension. The suspension was centrifuged at 10,000 rpm for 10 min at 4 °C. Genomic viral RNA was extracted from 250 µl of supernatant using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA was stored at −70 °C until used.

RT-LAMP assay

RT-LAMP was carried out in a final reaction volume of 25 µl using a Loopamp RNA amplification kit (Eiken Chemical Co., Ltd. Tokyo, Japan) with 5 pmol (each) of the primers F3 and B3, and 40 pmol (each) of the primers FIP and BIP. Five microliters of the extracted RNA was used as template per reaction. RNase-free water was added to "no-template" controls. All of the RT-LAMP assay reactions were conducted at 63 °C for 60 min and were then inactivated at 80 °C for 5 min. The RT-LAMP reactions were conducted with either a LA-320C Loopamp real-time turbidimeter (Teramecs, Japan) or in a water bath. The RT-LAMP products were detected by several methods: monitoring the accumulation of magnesium pyrophosphate spectrophotometrically at 650 nm using the LA-320C software package [6], 2 % agarose gel electrophoresis with a UV light transillumination, observation of turbidity, and naked-eye determination of color change after addition of 2 µl SYBR green I dye (Jetway Biotech Co., Ltd., Guangzhou, China) or 1 µl Loopamp fluorescent detection reagent (FD; Eiken Chemical Co., Ltd., Tokyo, Japan) under normal light conditions. The amplification products were digested with the restriction enzyme Hinf I (Fermentas, Lithuania), which was expected to yield 138-bp fragments that could be observed by electrophoresis on 2 % agarose gel.

Reverse transcription nested polymerase chain reaction (RT-nPCR)

For RT-nPCR, the outer primers were FES (GAR GCY TCT AAT TAT GCY CAG TA) and FEA (AAA GCC ARA GCA CAT CAT TAG C), and the inner primers were NIS (TTG GCG CTC RGT TGA GAC CTC) and NIA (TRG CTA TAC CCT TRT CCT GCT G). The first-round PCR was performed with the outer primers. The nested PCR using the first-round PCR products as templates was performed with the inner primers. RNase-free water was

added to "no-template" controls. Both PCR reactions were performed in a model 9700 Gene Amp PCR system (Applied Biosystems, Inc., CA) at 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and a final extension for 10 min at 72 °C. The final amplified products were then analyzed using 1 % agarose gel electrophoresis.

In vitro transcription and quantification

To obtain a quantitative RNA standard, the ORF3 gene, which contained the target region for the RT-LAMP, was cloned into a plasmid vector and transcribed *in vitro*. The PCR produced the ORF3 gene with the forward primer F1 (5'-CCC**AAGCTT**ATGGAGATGCCACCATGCG-3') and the reverse primer R1 (5'-CCG**GATATC**TACGGCGAAGC CCCAGC-3'). The HindIII and EcoRI restriction sites are indicated in bold type. The PCR product was cloned into pcDNA3.1(+)/myc-His A (Invitrogen, USA) after being digested by HindIII and EcoRI (Takara, Japan). Plasmids with an insert in the correct orientation were confirmed by sequencing with primer T7 (TAATACGACTCACTATA-GGG). Plasmids that were confirmed to be correct were linearized by digestion with EcoRI and purified using a Wizard SV Gel and PCR Clean-Up System (Promega, USA). The linearized plasmids were then transcribed *in vitro* with a T7 RiboMAX^{TM} Express Large Scale RNA Production System (Promega, USA) according to the manufacturer's instructions, and purified target RNA was thus obtained. The concentration (ng/μl) of the target RNA was determined by measuring the optical density (OD) at 260 nm using a NanoDrop ND-1000 apparatus (Thermo Scientific). The target RNA copy number was calculated by using the following formula: concentration of RNA (g per μl)/ [(RNA length $\times$ 660) $\times$ (6.022 $\times$ 10^{23})]. Serial dilutions ranging from 10^3 to 10^0 RNA copies/μl were used to determine the concentration range.

Phylogenic analysis

All of the RT-nPCR products from swine-HEV-positive specimens were sequenced. Phylogenetic analysis was carried out by analyzing the data obtained here together with those of other sequences of HEV from the GenBank database. A neighbor-joining nucleic acid tree was constructed in MEGA 4.0 by bootstrap analysis with 1,000 replications.

Results

Detection of RT-LAMP products by several methods

The results of real-time monitoring of RT-LAMP products are shown (Fig. 1A). The amplification curve indicated a positive reaction, while no amplification curve appeared in the negative reaction. Also, the positive results of the RT-LAMP assay showed a typical ladder pattern in 2.0 % agarose gel electrophoresis (Fig. 1B). Furthermore, the high amplification efficiency of the RT-LAMP reaction yielded large amounts of pyrophosphate by-product, which led to the formation of a white precipitate of magnesium pyrophosphate on the bottom of the tube after centrifugation (Fig. 1C). Alternatively, SYBR green I dye and Loopamp fluorescent detection reagent were added to the terminated and initial reaction, respectively. Both color changes in the reaction solution could be seen with the naked eye; the solution changed from orange to green for a positive reaction and remained orange for the negative reaction (Fig. 1D). The results determined by these methods consistently matched.

Specificity of the RT-LAMP assay

To evaluate the specificity of the primer set used for the RT-LAMP assay, DNA and RNA extracted from CSFV, PRRSV, FMDV, PCV2, SIV, and PRV were detected by RT-LAMP assay. As expected, the amplification was only detected in the assay using HEV genomic RNA samples as templates. In addition, no amplification of other genomic viral DNA/RNA as or NTC (no-template control) could be detected. The specificity of the RT-LAMP product was also confirmed by digestion with Hinf I. The predicted product of 138 bp was resolved on the 2 % agarose gel as expected.

Sensitivity of the RT-LAMP assay

The sensitivity of the RT-LAMP was determined by testing serial tenfold dilutions of *in vitro*-transcribed target RNA (from 10^0 to 10^3 copies/μl) in duplicate, and samples of the same concentration could also be detected by RT-nPCR. The detection limit of RT-LAMP assay was 10^1 copies/μl of target RNA, which was tenfold higher than that of RT-nPCR.

Detection of swine HEV from clinical samples

Detection of swine HEV by RT-LAMP was done with 41 bile samples. Conventional RT-nPCR was also done with the same samples for comparison. The results obtained using the RT-LAMP and RT-nPCR methods are summarized in Table 2. Eighteen positive samples were detected by RT-nPCR, while 36 positive samples were detected by RT-LAMP, indicating that the sensitivity of RT-LAMP assay was higher than that of the conventional RT-nPCR assay.

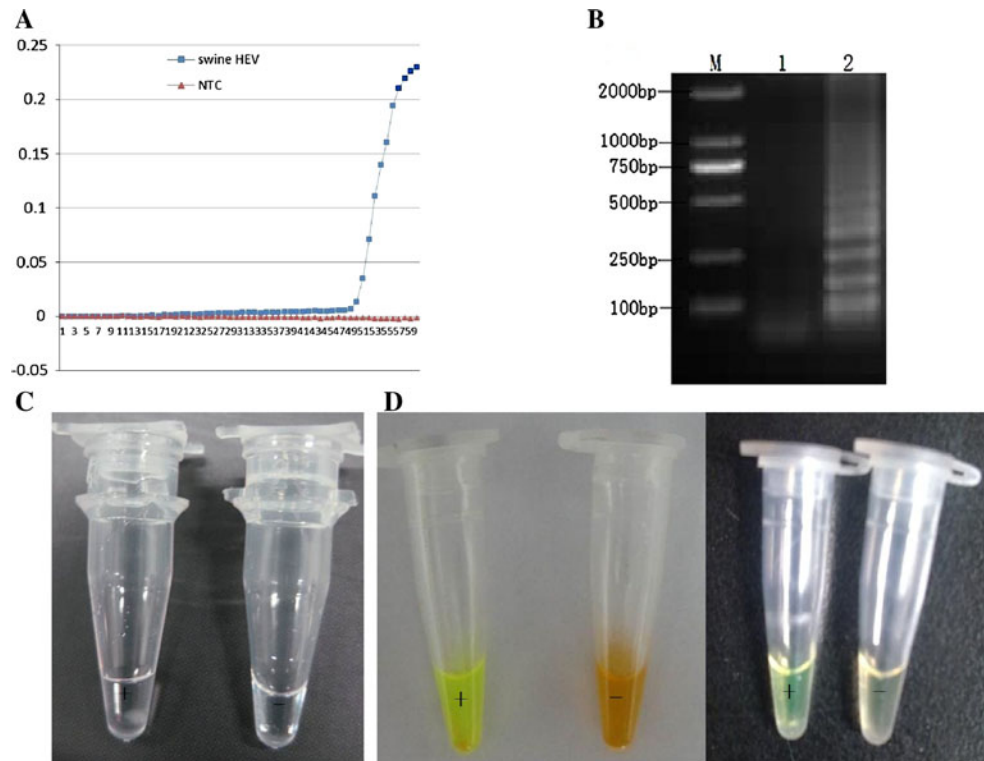

Fig. 1 Detection of swine HEV RT-LAMP products. (**A**) Real-time monitoring of RT-LAMP products. (**B**) Detection of RT-LAMP products by 2% agarose gel electrophoresis. Lane M, DNA marker DL-2000 (Takara, Japan); lane 1, no-template control; lane 2, swine HEV. (**C**) Detection of RT-LAMP products by turbidity. +, positive sample; −, negative control. (**D**) Detection of RT-LAMP products by color change. Left, color change after addition of 2 μl SYBR green I dye; right, color change after addition of 1 μl Loopamp fluorescent detection reagent. +, positive sample; −, negative control

Table 2 Comparison of results of the RT-nPCR and RT-LAMP assays

No. detected	Result for indicated method	
	RT-nPCR	RT-LAMP
17	+	+
19	−	+
1	+	−
4	−	−
Total (positive)	18	36

+, positive; −, negative

Phylogenic analysis

Phylogenetic trees for the partial gene of swine HEV from the 18 swine-HEV-positive specimens are shown in Fig. 2. All of them belong to genotype 4. Also, the swine HEVs were closely related to human HEV, indicating that swine HEV has a high homology with human HEV.

Discussion

Hepatitis E accounts for a significant proportion of the enterically transmitted form of viral hepatitis in humans. Although the overall mortality of hepatitis E is less than 1 % in the general population, it can reach up to 28 % in infected pregnant women [14, 18]. Hepatitis E is now considered as a zoonotic disease, and domestic pigs are reservoirs for HEV [11, 13]. Swine HEV infection is widespread in swine farms worldwide in both developing and industrialized countries, regardless of whether HEV is endemic in the corresponding human populations [10, 12, 20]. Because of the potential risk of HEV transmission to humans, it is important to develop a simple, rapid and sensitive detection method for swine HEV.

The one-step RT-LAMP assay relies on autocycling strand-displacement DNA synthesis performed with avian myeloblastosis virus reverse transcriptase and Bst DNA polymerase, which has a high degree of strand displacement activity [5]. Since the initial RT reaction and the amplification reaction can be carried out at the same time, RT-LAMP is easier to operate and saves time compared with RT-PCR. Because of the high sensitivity and specificity coupled with the requirements for only a water bath and endpoint detection using a color-change reaction visible with the naked eye [3, 4, 15], it has been applied for the analysis of various viruses. In this study, a one-step RT-LAMP assay was developed for the diagnosis of swine HEV.

The primer set used in this study was designed specifically for the ORF3 gene, which is highly conserved among swine HEVs. In addition, the four primers recognize six distinct regions on the target RNA sequence, which makes the RT-LAMP assay more specific than conventional PCR

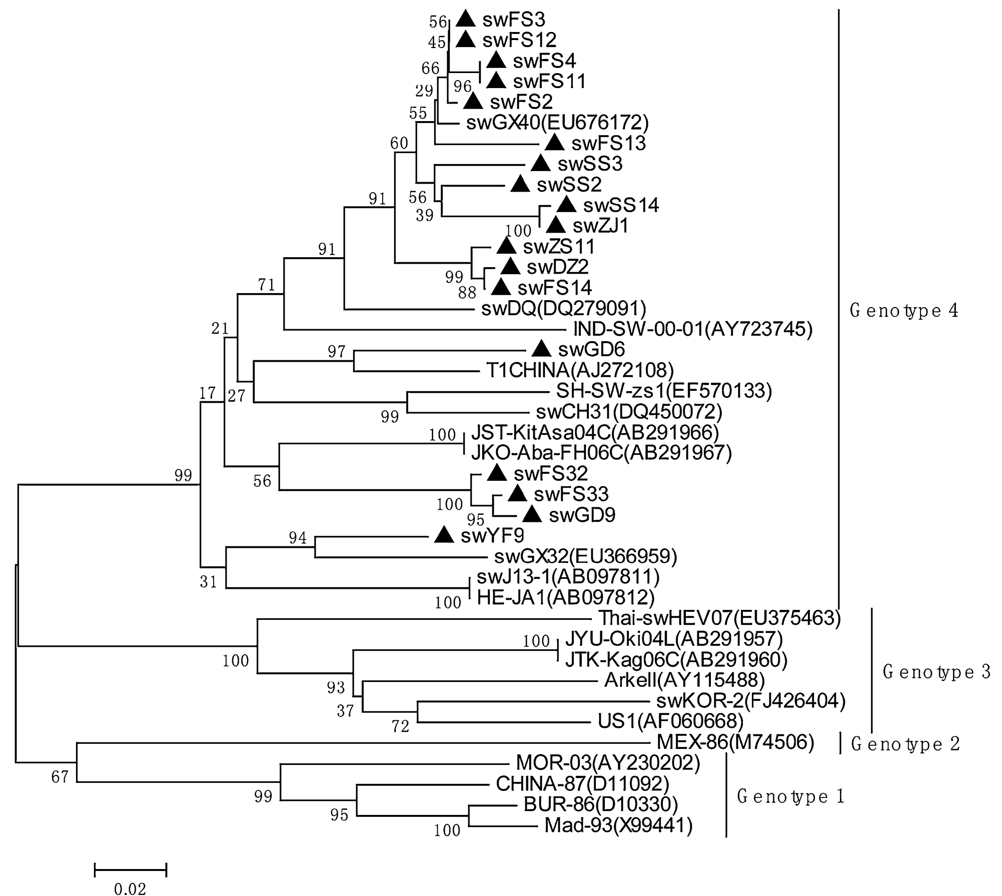

Fig. 2 Phylogenetic trees for the partial ORF3 gene of swine HEV from the 18 swine-HEV-positive specimens. ▲, 18 isolates from this study

(including RT-nPCR). In this study, restriction analysis confirmed the specificity of the amplified RNA fragments. The evaluation of the specificity was completed by showing the lack of amplification of DNA/RNA from other swine viruses.

The sensitivity of the RT-LAMP was 10^1 copies/μl of RNA, indicating that a very small amount of viral RNA was detectable by this assay. By contrast, the sensitivity of RT-nPCR was tenfold lower. Furthermore, detection of swine HEV from clinical samples demonstrated that the sensitivity of the RT-LAMP assay was higher than that of the conventional RT-nPCR assay.

Four different methods for detection of RT-LAMP products were shown to be feasible in this study. However, the real-time monitoring and agarose gel electrophoresis are not intended for routine use because they require specialized equipment. Thus, the methods of turbidity and change in color were selected for their simplicity and ease of interpretation. It is worth noticing that the method of adding SYBR green I dye requires opening the tube after the reaction is terminated. This creates a high chance of contamination of reagents, the environment and instruments, and it may result in a false positive result. This does not occur when using the method of adding FD, which can be added to the RT-LAMP reaction mixture without affecting the amplification reaction. Therefore, the detection methods based on turbidity and adding FD are preferred.

Sequence analysis of swine HEV isolates identified thus far revealed that there exist at least two genotypes of swine HEV worldwide, genotypes 3 and 4, both of which are known to cause sporadic cases of hepatitis E in humans, and swine HEV has high homology to human HEV. In this study, the four primers recognizing six distinct regions on the target cDNA were absolutely conserved in genotypes 3 and 4, and thus the RT-LAMP assay developed in this study could detect swine HEV not only from genotype 3 but also from genotype 4. In theory HEV genotypes 3 and 4 could be detected not only from swine but also from humans. All the RT-nPCR products from swine-HEV-positive specimens were sequenced. Phylogenetic analysis showed that all of them belong to genotype 4, and therefore the detection of genotype 3 by this method could not be verified due to the lack of samples with this genotype.

In conclusion, we describe a one-step RT-LAMP assay for detection of swine HEV, which is simple, rapid, sensitive, and specific. The RT-LAMP assay may be used for

diagnosis of swine HEV, not only in laboratories but also under field conditions.

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