Reverse Transcription–Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Hepatitis E Virus

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The one-step single-tube betaine-free reverse transcription (RT)—loop-mediated isothermal amplification assay was developed for rapid diagnosis of hepatitis E virus. This assay amplified the target gene in less than 45 min (even as short as 20 min) under isothermal conditions at 63°C, and the sensitivity of this assay was 100-fold greater than that of RT-PCR. This assay demonstrated a detection limit of 0.045 fg (nine copies/reaction).

Hepatitis E is a very important public health disease in many developing countries and is also sporadically present in some industrialized countries (1, 3, 11, 13). The disease mainly affects young human adults and generally has a low mortality rate (1%), but this rate can rise to 25% in pregnant women (5, 6). Transmission of the hepatitis E virus (HEV), the causative agent of hepatitis E, is mainly through the fecal-oral route, via contaminated food and water (7, 10). HEV infection occurs not only in humans but also in animals, such as swine, wild boar, wild deer, and wild mongoose, and the disease can be considered an anthropozoonosis (2, 4, 8, 12). Current diagnostic methods depend on enzyme-linked immunosorbent assay and reverse transcription-PCR (RT-PCR). In recent years, a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) was developed by Notomi et al. (9), and due to its high specificity, sensitivity, time-saving merit, and isothermal amplification conditions, it is easier to apply than PCR is. In this study, we developed a betaine-free one-step single-tube accelerated RT-LAMP assay for rapid detection of HEV. The sensitivity, specificity, and applicability of the method are reported.

A total of 68 specimens comprising 59 fecal samples, 3 liver samples, and 3 bile samples from swine and 3 serum samples from humans were collected from the Gansu province of China. Of the 68 specimens, 18 were confirmed cases of HEV infection. The statistical analysis indicated that the hepatitis E virus infection rate was 1%; however, this rate can rise to 25% in pregnant women (5, 6). Transmission of the hepatitis E virus (HEV), the causative agent of hepatitis E, is mainly through the fecal-oral route, via contaminated food and water (7, 10). HEV infection occurs not only in humans but also in animals, such as swine, wild boar, wild deer, and wild mongoose, and the disease can be considered an anthropozoonosis (2, 4, 8, 12). Current diagnostic methods depend on enzyme-linked immunosorbent assay and reverse transcription-PCR (RT-PCR). In recent years, a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) was developed by Notomi et al. (9), and due to its high specificity, sensitivity, time-saving merit, and isothermal amplification conditions, it is easier to apply than PCR is. In this study, we developed a betaine-free one-step single-tube accelerated RT-LAMP assay for rapid detection of HEV. The sensitivity, specificity, and applicability of the method are reported.

A total of 68 specimens comprising 59 fecal samples, 3 liver samples, and 3 bile samples from swine and 3 serum samples from humans were collected from the Gansu province of China. Of the 68 specimens, 18 were confirmed cases of HEV infection (3 for genotype I and 15 for genotype IV) and used as positive controls, while 9 samples were collected from HEV-free pigs (negative controls). Viral genomic RNA was extracted from the diluted specimen supernatants by using a Qiagen RNeasy minikit (Qiagen, Hilden, Germany) and was used as a template for RT-LAMP and RT-PCR.

Primers used for RT-LAMP and nested PCR were designed from the capsid protein gene. A set of six RT-LAMP primers comprising two outer primers, two inner primers, and two loop primers that recognize eight distinct regions on the target sequence were used. These primers were F3 (T5172GAGATGCCACCATGCCTCTCG5198), B3 (A5445CGCCAAGGGCCGCCAGATGGAC5423), FIP (A5320CGCCGGCTGTCGCCGGC5350), NIA (T6352TRTCCTGAGTGGTGGAG6430), and the inner primers were NIS (C5974CGACGAG6430), and LOOP-B (C5376C5326TGCTCTTCGTGCTTCTGCCTATGCTGC5240). BIP (G5320GTGGTTTCTGGGGTGACCGGGTT5342C5376C5326GAAAGGTGTITGGATGTAATAGG5342), LOOP-F (C5273GACCGGCAGCAGCGCTGAC5324), and LOOP-B (G5320ATTCTCACGCCCCTGCCCCCT5342). The betaine-free RT-LAMP reaction was carried out in a 25-μl reaction mixture containing 5 pmol (each) of the outer primers F3 and B3, 40 pmol (each) of the primers FIP and BIP, 20 pmol (each) of the loop primers LOOP-F and LOOP-B, 2.8 mM deoxynucleoside triphosphates, 10 mM (NH4)2SO4, 12 mM MgSO4, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 8 U of Bst DNA polymerase (New England Biolabs), 50 U of PrimeScript reverse transcriptase (TaKaRa, Japan), and 2 μl of RNA. The mixture was incubated at 63°C for 45 min in a heat block, followed by heating at 80°C for 10 min to terminate the reaction. To confirm that the RT-LAMP products had the corresponding sequences of the selected target, 1-μl portions of the amplified products were digested in a 20-μl total reaction volume with the restriction enzyme BsiEI (for 2 h at 60°C), with 208-, 150-, and 136-bp fragments, the expected products. Because betaine was found to markedly elevate amplification efficiencies in LAMP (9), RT-LAMP containing 1 M betaine (Sigma) in the reaction mixture was also performed as a comparative test.

For RT-PCR, the outer primers were FES (T5955GCGCGTTCDGTGGAGA5969) and FEA (G6449GBTBCGGAGAGGAGGA5430), and the inner primers were NIS (C5974GAGACGAAATCATTCGTC5991) and NIA (T5352TRTCCTCGTGWGCRCTTC6533). In the first round of PCR, a 497-bp fragment was amplified using the outer primers. A 379-bp fragment was amplified in the second round of PCR using 2 μl of undiluted first-round PCR product or 2 μl of the standard plasmid and inner primers. The thermal profile for RT-PCR was as follows: 30 min at 50°C; 2 min at 94°C; followed by 34 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C; and a final extension step of 10 min at 72°C.
As for RT-LAMP, ladder-like patterns of amplification products were observed on the gel 20 min after initiation of incubation, and the white precipitate of magnesium pyrophosphate in the positive reaction mixture was also observed by the naked eye after 45 min, whereas the negative reaction has no product. No significant difference was observed on the gel 20 min after initiation of incubation between the products amplified by the betaine-free or betaine-containing reaction mixture, although the products of the betaine-containing RT-LAMP were slightly brighter after 45 min (Fig. 1A). This may be because the melting temperature of all six primers was higher than 63°C, leading to strong binding of the primers to the template cDNA; this facilitated efficient amplification at 63 to 65°C, the optimal reaction temperature for Bst DNA polymerase. The fragments produced by BsiEI digestion were in good agreement with the predicted sizes of 208, 150, and 136 bp (Fig. 1B), and sequencing results were highly homologous to the expected nucleotide sequences (data not shown). The sequencing results in the study indicated that all HEV-positive specimens from swine belong to genotype IV and the samples from the patients belong to genotype I. The specificity for genotypes II and III could not be verified due to the scarcity of samples with these genotypes, but in this study, the 164-bp target sequence used in the RT-LAMP assay was the optimal conserved sequence of all the HEV genome genotypes that are deposited in GenBank and represented 93.2% to 99.3% nucleotide homology. The six primers recognizing eight distinct conserved regions on the target cDNA were absolutely conserved in the different genotypes of HEV, so the RT-LAMP assay demonstrated in this study could detect HEV not only from genotypes I and IV but also from genotypes II and III in theory.

Both methods demonstrated high specificity by amplifying sequences from the 18 HEV-positive samples, while no product was obtained from the nine negative controls. The betaine-free RT-LAMP reaction was significantly shorter than the 6-h nested PCR, taking less than 45 min to amplify the 164-bp target sequence of HEV at 63°C. The detection limit for the LAMP assay was found to be 0.045 fg or 9 copies/reaction, and the LAMP assay was 100-fold more sensitive than nested PCR (4.5 fg or 900 copies/reaction); this was determined using a 10-fold serial dilution of standard plasmid that contained the capsid protein gene of swCH189 (GenBank accession no. FJ610232) (Fig. 1C and D). In 68 specimens, the RT-LAMP assay detected 24 HEV-positive samples and 44 HEV-negative samples, while the RT-PCR (nested PCR) assay detected 20 HEV-positive samples and 48 HEV-negative samples. The sensitivities of the RT-LAMP assay and RT-PCR (nested PCR) assay were 100% and 92%, respectively.

In conclusion, a one-step single-tube RT-LAMP assay was developed in this study for the diagnosis of HEV. It is a rapid, sensitive, and convenient tool for clinical diagnosis and surveillance of HEV, as it requires only a regular laboratory water bath or heat block for incubation under isothermal conditions.

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REFERENCES


