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Development of isothermal amplification methods for rapid and sensitive detection of heat-labile enterotoxin producing *Escherichia coli*

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ABSTRACT

The objective of this study was to establish a novel isothermal amplification method for detection of heat-labile enterotoxin (LT-I)-producing *Escherichia coli*. Loop-mediated isothermal amplification (LAMP), cross-priming amplification (CPA), and isothermal multiple-self-matching-initiated amplification (IMSA) were developed and evaluated. Optimal conditions, specificity, and sensitivity tests were performed and compared to qPCR findings. All three methods could produce ladder-like products with LT-I positive samples, while no products were generated with the negative controls. The amplified products could be directly visualized as negative or positive in the isothermal amplification (IAM) tube, which saved time and prevented the possibility of cross-contamination. The detection limits of each assay were similar, and all three assays could directly detect the DNA of *Escherichia coli* in clinical samples successfully. This is the first report on the application of CPA and IMSA methods for the detection of LT-I. The findings suggest that the three assays may be important tools for the rapid detection of enterotoxigenic *Escherichia coli* (ETEC) in the clinic.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the primary cause of bacteria-induced diarrheal disease in young children and infants located in developing and low-income countries (Albert et al., 1995; Paniagua et al., 1997). ETEC strains have caused waterborne outbreaks of diarrheal disease on cruise ships, food-borne epidemics at schools and restaurants, and major outbreaks among military personnel located in developing countries (Reischl et al., 2004). The typical symptoms associated with ETEC in humans include mild-to-severe watery diarrhea of considerable volume, along with abdominal pain, nausea, malaise, and vomiting. There are two strains of ETEC based on the type of enterotoxin it produces, including heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) (Moseley et al., 1983; Yano et al., 2007). The enterotoxin is present in all ETEC isolates from environmental and clinical sources. Although the disease is self-limiting in healthy adults, outbreaks of ETEC-LT infections remain a substantial public health concern in several countries, including Japan, China, and the United States (Norton et al., 2012).

The traditional methodologies for the identification and differentiation between ETEC-LT and ETEC-ST involve tissue-culture assays and suckling-mouse assays, respectively. However, most immunological procedures and strain isolation procedures are limited by their premium costs, substantial time commitment, and low sensitivity. In the past decade, enzyme-linked immunosorbent assays (ELISA), membrane-based DNA hybridization assays, and polymerase chain reaction (PCR) detection methods garnished increasing popularity in the scientific community due to their simplicity, small time commitment, cost-effectiveness, high sensitivity, and improved replicability (Olive, 1989; Tsen and Jian, 1998; Yavzori et al., 1998). Currently, quantitative real-time PCR (qPCR) and other novel techniques based on isothermal amplification are being employed increasingly for the detection of many pathogenic microorganisms (Reischl et al., 2004; Yano et al., 2007). These methods provide highly specific and sensitive results with rapid amplification. These methodologies are excellent alternatives for rapidly diagnosing the disease in the clinic, especially for isothermal amplification, as it requires no advanced or expensive equipment.

Loop-mediated isothermal amplification (LAMP), which is a more established technique used for isothermal amplification, is commonly used for the detection of poultry pathogens and ETEC around the globe (Jiang et al., 2012; Mori and Notomi, 2009; Yano et al., 2007). Cross-priming amplification (CPA), an isothermal DNA amplification

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technique developed by Ustar Biotechnologies (Hangzhou, China), eliminates the first denaturation step and requirement of a nicking enzyme (Chow et al., 2008). Using five or six primers, the rapid amplification process (< 1 h) shows high sensitivity and specificity under a constant temperature ranging from 55 to 65 °C (Xu et al., 2012; Niczyporuk et al. 2015). During the CPA denaturation step, primer annealing and extension of the products occur dynamically, and the products are generated as interval tandem repeats (Cui et al., 2012). Recently, Ding and colleagues developed a novel isothermal amplification technology called isothermal multiple-self-matching-initiated amplification (IMSA), which uses a single pair of forward and reverse stem primers, SteF and SteR, and two pairs of hybrid nested primers. The method resembles the LAMP, achieving detection by use of a strand displacement DNA polymerase (Ding et al., 2014).

In the current study, we developed and assessed three isothermal amplification assays (LAMP, CPA, and IMSA) for the identification of LT-I-producing ETEC. The methods described in this study should facilitate the rapid and accurate identification of LT-I, which may limit future outbreaks caused by LT-I-producing ETEC.

2. Materials and methods

2.1. Bacterial strain

A total of 16 bacterial strains were used in this study, which included eight strains of *Escherichia coli* and eight strains of non-*Escherichia coli*, as shown in Table 1. *Escherichia coli* C83903 was maintained at our laboratory and used for the determination of CPA, LAMP, and IMSA sensitivity.

2.2. Primers

The LT-I-specific primers for LAMP, CPA and IMSA were designed based on the complete sequence of ETEC_118–5 *elt-I*, which was obtained from the GenBank database (No: JX504011.1), as shown in Fig. 1. Four primers that targeted the A subunit segment of LT-I were designed using the online LAMP software, known as Primer Explorer Version 5 (http://primerexplorer.jp/lampv5e), and included F3 (forward outer primer), B3 (backward outer primer), FIP (forward inner primer) and BIP (backward inner primer). The set of primers could recognize six distinct sequences of the *LT-I* gene, and the design principle was described in a previous report (Mori and Notomi et al., 2009). The CPA mechanism was described by Fang and colleagues (Fang et al., 2009), and included a set of five primers (two displacement primers, one cross primer, and two detector primers) that could recognize five distinct regions of the B subunit segment of LT-I. The primers were designed with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA). For IMSA, the primers were designed based on the principles of LAMP, and specifically recognized seven distinct regions of the A subunit of LT-I (Ding et al., 2014). The six primers consisted of two stem primers (SteF/SteR) and two pairs of hybrid nested primers (two outer primers of DsF/DsR and two inner primers of FIT/RIT). The basic principles of the three isothermal detection methods are similar, all of which are dumbbell structures formed by primer amplification, and ultimately form high molecular weight reaction products with different molecular weights. A pair of fluorescent qPCR primers for the LT-I toxin A subunit were designed (Postollec et al., 2011), which was used as a standard reference for the sensitivity assays and sample detection.

The primers were assessed in the Primer-BLAST tool from the National Center for Biotechnology Information (NCBI) to confirm their specificity. The details of the primers used in this study are shown in Table 2.

2.3. DNA extraction

For DNA extraction, an overnight culture of *Escherichia coli* was centrifuged at 10,000 rpm for 5 min. The resulting pellet was suspended in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and heated at 100 °C for 10 min. Next, the samples were centrifuged at 10,000 rpm for 3 min, and the supernatant was collected and stored at −20 °C for future experiments. Two µL of the supernatant was used as the DNA template.

2.4. Reactions

The LAMP, CPA, and IMSA assays were conducted in a total volume of 25 µL containing 2.5 µL of 10× Bst buffer, 8 U Bst-DNA large fragment polymerase (New England Biolabs, Ipswich, MA, USA), 0.5 µM dNTPs (Takara Bio, Dalian, China), 4 µM $MgSO_4$ (Sigma-Aldrich, St. Louis, MO, USA), 0.8 M betaine (Sigma-Aldrich), and 2 µL of an appropriate concentration of target DNA. The primer-mix for the CPA assay contained 2.0 µM of the cross primer 1 s, 0.8 µM each of primers 2a and 3a, 0.2 µM each of displacement primers 4 s and 5a. For the LAMP assay, the primers consisted of 0.5 µL of 10 µM F3 and B3 primers, 5 µL of 10 µM FIP and BIP primers. For IMSA, the concentration of the outer primers DsF/DsR was settled at 0.2 µM, while FIT/RIT and SteF/SteR were set to 0.8 µM. The three reactions mixture were incubated in a water bath for 60 min at 60 °C, and then incubated for 5 min at 80 °C (Fig. 2).

The qPCR assay was performed using the Bio-Rad iQ5 system (Bio-Rad, Hercules, CA). The amplifications were conducted in a final volume of 50 µL, according to the manufacturers' instructions for the specific reaction systems. The following conditions were used: one cycle

Table 1
Bacterial strains used in this study.

Species	Strain	Source
Enterotoxigenic *E. coli*	C83903 (LT-I$^+$, Stb$^+$)	*China Institute of Veterinary Drug Control*
Enterotoxigenic *E. coli*	ATCC 43886 (LT-I$^+$)	*American Type Culture Collection*
Enterotoxigenic *E. coli*	WP-12-C	Self-isolate
Enterotoxigenic *E. coli*	WP-25-B	Self-isolate
Enterotoxigenic *E. coli*	C83920 (Sta$^+$)	*China Institute of Veterinary Drug Control*
Enterotoxigenic *E. coli*	C44498 (Stx2e$^+$)	*China Institute of Veterinary Drug Control*
Enterotoxigenic *E. coli*	O157:H7 (Stx1 +, Stx2$^+$)	*China Institute of Veterinary Drug Control*
Enterotoxigenic *E. coli*	OS-1 (LT-II$^+$)	Self-isolate
Aeromonas hydrophila	ATCC 7966	*American Type Culture Collection*
Clostridium perfringens	ATCC13124	*American Type Culture Collection*
Salmonella enteritidis	ATCC 13076	*American Type Culture Collection*
Salmonella typhimurium	ATCC 13311	*American Type Culture Collection*
Staphylococcus aureus	ATCC 25923	*American Type Culture Collection*
Staphylococcus aureus	ATCC 29213	*American Type Culture Collection*
Yersinia enterocolitica	ATCC 23715	*American Type Culture Collection*
Vibrio parahaemolyticus	ATCC 27519	*American Type Culture Collection*

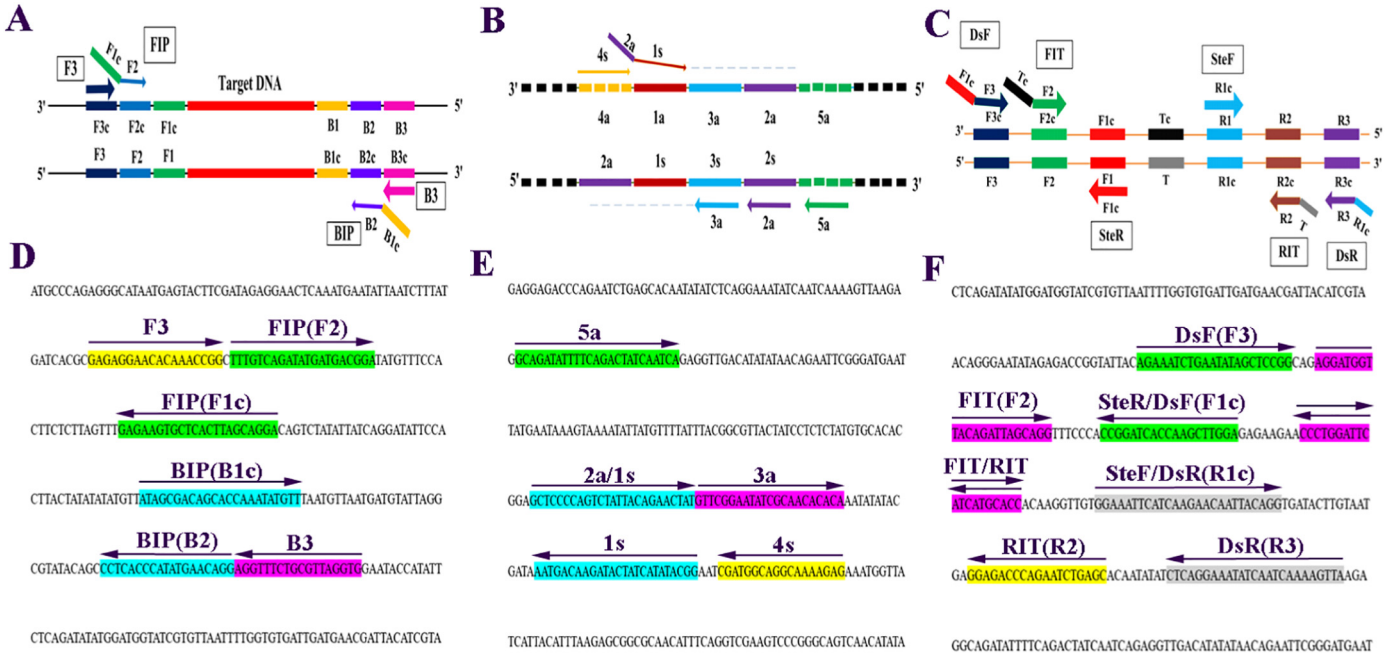

Fig. 1. Ideographic, location, and primer sequences for LAMP, CPA and IMSA within the conservative region of heat-labile enterotoxin (GenBank Accession No: JX504011.1). (A-C): Ideographic of the LAMP, CPA, and IMSA assays, respectively. (D-E): Location of primers for LAMP, CPA, and IMSA assays, respectively. Arrows and colored characters indicate the direction of primer extension in the target sequences.

Table 2
Primers of the LAMP, CPA, IMSA, and qPCR assays.

	Primer	Primer Sequence (5′-3′)
LAMP Primers	F3	GAGAGGAACACAAACCGG
	B3	CACCTAACGCAGAAACCT
	FIP	TCCTGCTAAGTGAGCACTTCTC-TTTGTCAGATATGATGACGGA
	BIP	ATAGCGACAGCACCAAATATGTT-CCTGTTCATATGGGTGAGG
CPA primers	1 s	GCTCCCCAGTCTATTACAGAACTAT-CCGTATATGATAGTATCTTGTCATT
	2a	GCTCCCCAGTCTATTACAGAACTAT
	3a	GTTCGGAATATCGCAACACACA
	4 s	CTCTTTTGCCTGCCATCG
	5a	GCAGATATTTTCAGACTATCAATCA
IMSA primers	DsF	TCCAAGCTTGGTGATCCGG-AGAAATCTGAATATAGCTCCGG
	DsR	GGAAATTCATCAAGAACAATTACAGG-TAACTTTTGATTGATATTTCCTGAG
	FIT	GGTGCATGATGAATCCAGGG-AGGATGGTTACAGATTAGCAGG
	RIT	CCCTGGATTCATCATGCACC-GCTCAGATTCTGGGTCTCC
	SteF	GGAAATTCATCAAGAACAATTACAGG
	SteR	TCCAAGCTTGGTGATCCGG
Q-PCR primers	Forward	AGGAGGTTTCTGCGTTAGGTG
	Reverse	GATGAATCCAGGGTTCTTCTCTC

of denaturation at 95 °C for 5 min; amplification in 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; followed by the final extension at 72 °C for 5 min. An amplification vwcurve analysis was performed with temperatures from 60 to 95 °C in a ramp speed of 0.5 °C/s for 10 s, followed by continuous fluorescence measurements.

For analysis of the CPA, LAMP, and IMSA products, a special color under normal light or ultraviolet (UV) light was observed in the isothermal amplification tubes (IAM) (Hua-feng Corporation, Guangzhou, P. R. China) following the addition of 1 μL of 2000× SYBR green I (Solarbio, Beijing, China). Green was positive and orange was negative. All products were also analyzed by electrophoresis using 2.0% agarose gels that were stained with ethidium bromide and visualized using an UV transilluminator.

2.5. Optimization of the LAMP, CPA, and IMSA assays

The reaction temperature was optimized by incubating the reaction mixture under isothermal conditions between 60 and 70 °C for 60 min. The concentration of *Bst* DNA polymerase was set at 6 U, 8 U, 10 U, or 12 U, while the concentration of Mg^{2+} was set at 1.0 mM, 2.0 mM, or 3.0 mM respectively. The incubation time was optimized from 30 to 120 min at the optimal temperature. *Escherichia coli* C83903 (1 ng/reaction) was used as the positive controls. The amplified DNA products from the CPA, LAMP, and IMSA assays were visualized by agarose gel electrophoresis.

2.6. Specificity of the LAMP, CPA, and IMSA assays

The specificity of the CPA, LAMP and IMSA assays were confirmed with the C83903, ATCC 43886, isolated strain OS-1 (LT-II$^+$), C83920, C44498, O157:H7 strains, and non-*Escherichia coli* strains. All assays were conducted as described above and the reactions were performed for 60 min. Before the reaction, 1 μL of 2000× SYBR Green I was added into the IAM tubes. All tests were repeated in triplicate.

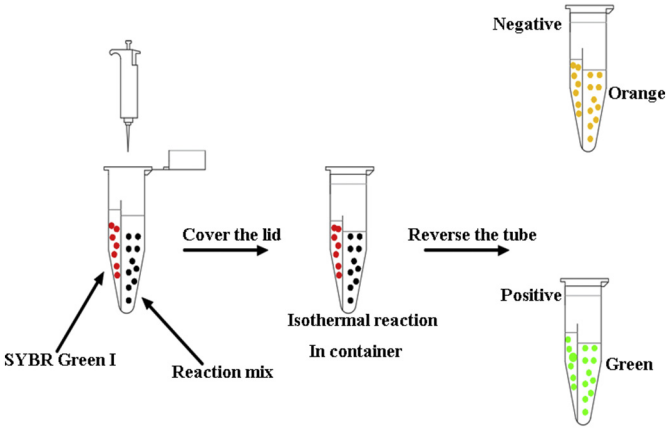

Fig. 2. Simulated diagram of the isothermal reaction process. The reaction was performed in the Hua-feng tubes without opening the cover during operation, effectively limiting the potential for cross-contamination.

2.7. Sensitivity analysis of the LAMP, CPA, and IMSA assays

To determine the sensitivity limits of the three assays, template DNA from C83903 (1.0×10^6 CFU/mL) was prepared and diluted in a ten-fold serial dilution. The concentration was adjusted to 25 CFU/mL, 15 CFU/mL, and 5 CFU/mL. All reactions were performed at the corresponding temperature for 60 min. Next, 1 μL of SYBR Green I was added to the IAM tubes and the reaction was visualized by sight. At the same time, the templates were assessed by qPCR.

2.8. Sample culture, isolation, and detection

A total of 213 *Escherichia coli* isolates, consisting of 127 fecal samples from suckling pigs with diarrheal disease and 86 fecal samples from cattle with diarrheal disease, were collected from 2016 to 2017 from different farms in northeast China. All samples were collected directly from the rectum using sterile swabs, placed into Eppendorf tubes, transported to the laboratory within 10 h, and directly inoculated on MacConkey agar plates at 37 °C for 18 h. Standard biochemical procedures and visual appearance were used to identify the strains of *Escherichia coli* for every single colony. The properties included colony

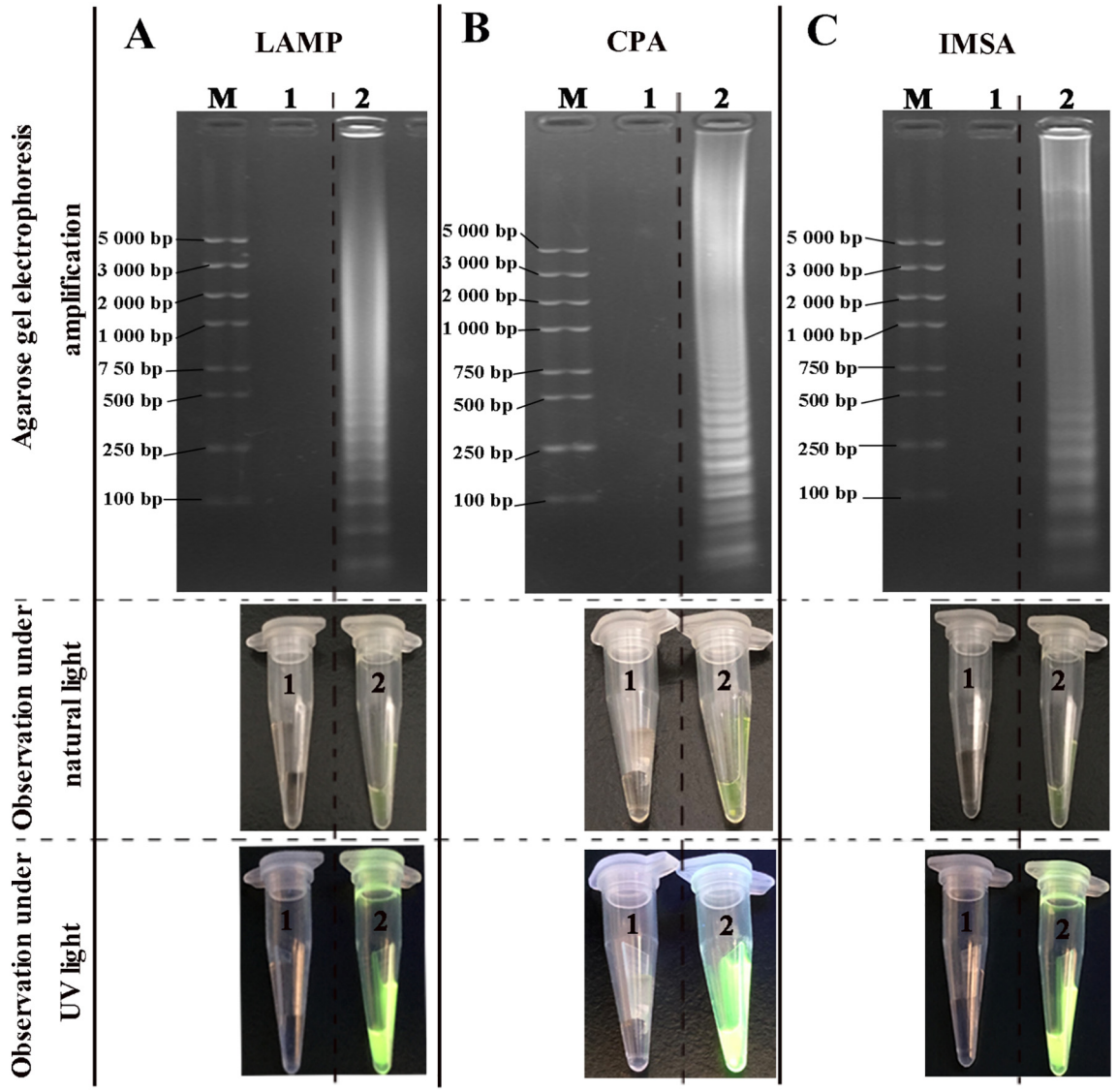

Fig. 3. LAMP, CPA and IMSA amplification results for the LT-I gene. (A-C): Observation of agarose gel electrophoresis, natural light, and UV light (with SYBR green I) for LAMP, CPA, and IMSA methods, respectively. M: Trans 2 K plus II DNA marker; 1. negative control; 2. detection for LT-II gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

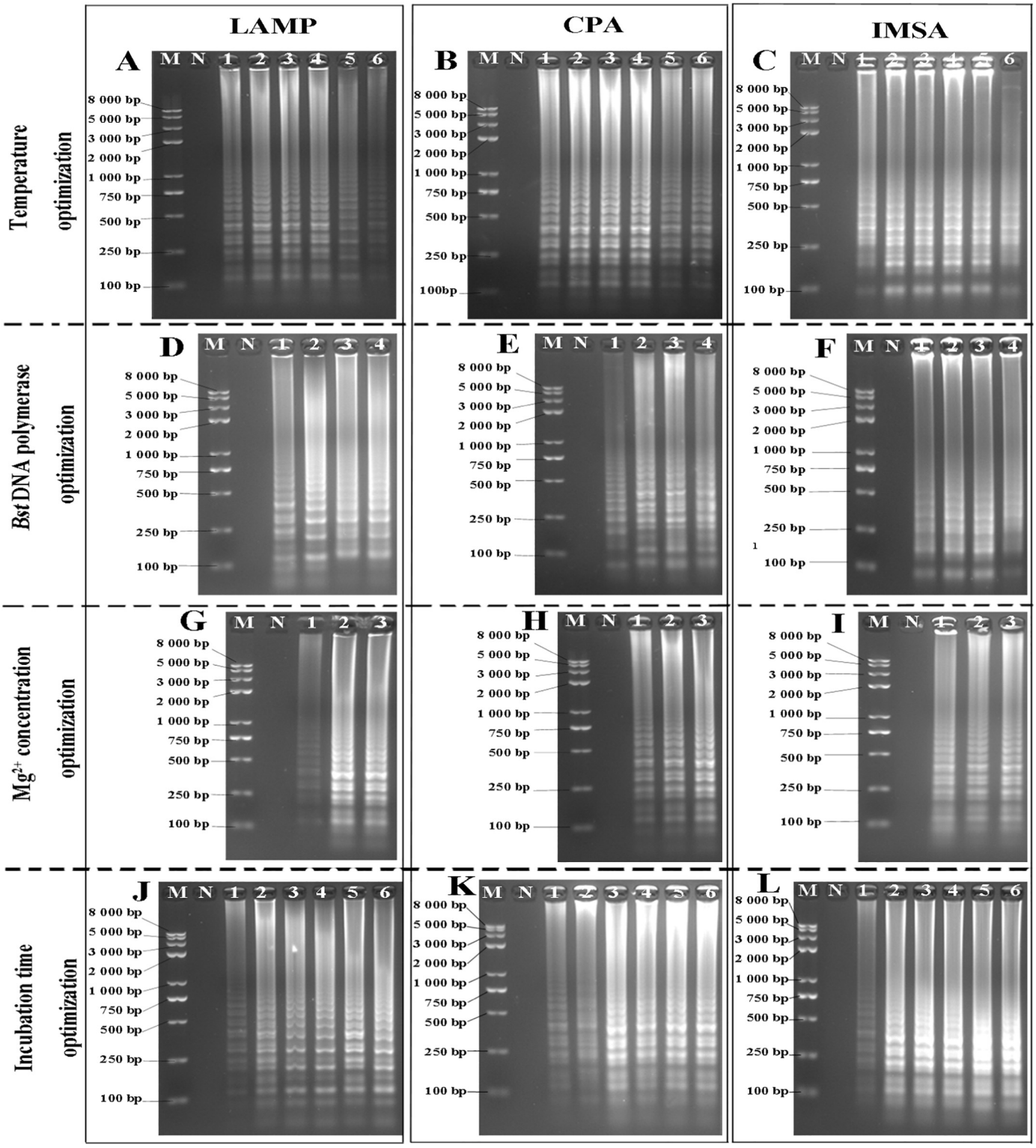

Fig. 4. Optimization of the reaction conditions for LAMP, CPA, and IMSA. (A-C): Optimization of the incubation temperatures for the LAMP, CPA, and IMSA assays, respectively; 1–6. Incubation temperature at 60, 62, 64, 66, 68 and 70 °C respectively; D-F: Optimization of *Bst* DNA polymerase concentration for LAMP, CPA and IMSA, respectively; 1–4. *Bst*-DNA polymerase concentration at 6 U, 8 U, 10 U, and 12 U, respectively; (G-I): Optimization of the Mg^{2+} concentration for the LAMP, CPA, and IMSA assays, respectively. 1–3. 1.0, 2.0, and 3.0 mM, respectively; (J-L): Optimization of incubation time for LAMP, CPA, and IMSA. 1–6. Product of amplification at 30, 45, 60, 75, 90 and 120 min, respectively; M. Trans 2 K plus II DNA marker; N. negative control.

appearance and color, indole production, citrate utilization, glucose and lactose fermentation, hydrogen sulfate production, and others (Liu et al., 2014). DNA samples of *Escherichia coli* were extracted and maintained at −20 °C. The LT-I toxin gene of the clinical samples of *Escherichia coli* was detected by CPA, LAMP, and IMSA, and the results were compared and analyzed with the qPCR findings. Next, 1 μL of SYBR Green I dye was added to the other side of the Hua-feng tube, and the results were observed directly under UV light after the reaction.

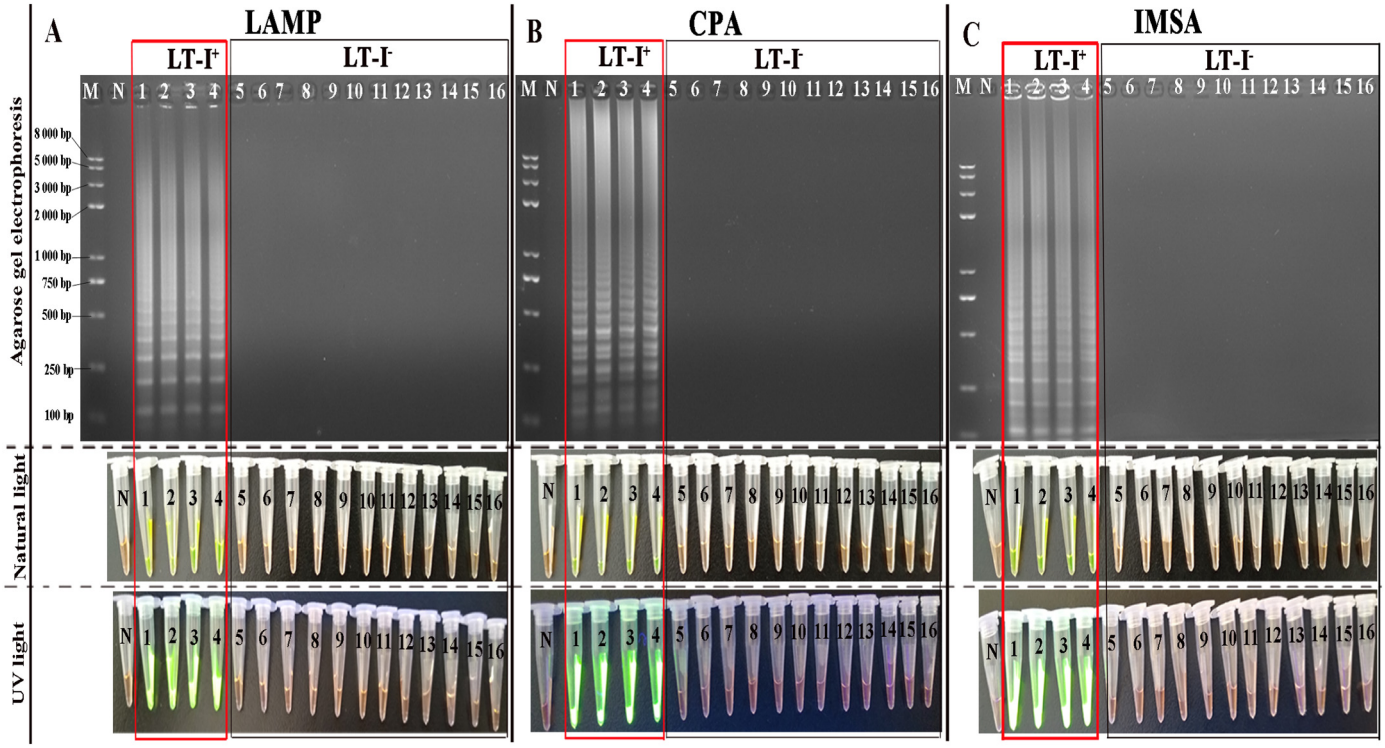

Fig. 5. The specificity of LAMP, CPA, and IMSA amplification. (A-C): Observation of the products from LAMP, CPA, and IMSA assays under agarose gel electrophoresis, natural light, and UV light (with SYBR green I), respectively. 1–16: C83903, ATCC 43886, WP-12-C, WP-25-B, C83920, C44498, O157:H7, OS-1, ATCC7966, ATCC13124, ATCC13076, ATCC13311, ATCC25923, ATCC29213, ATCC23715 and ATCC27519, respectively. M: Trans 2 K plus II DNA marker; N: negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.9. Ethics statement

The samples were collected with the permission of the owner of the farms. The present study did not involve endangered or protected species. The animal study complied with the Animal Welfare Act and followed the National Institute of Health (NIH) guidelines (NIH Pub. No. 85–23, revised 1996). The protocols were approved and supervised by the Animal Care and Use Committee of Northeastern University (Shenyang, Liaoning, P. R. China).

3. Results

3.1. Amplification of the LT-I gene by CPA, LAMP, and IMSA

The primers for the CPA, LAMP, and IMSA assays were designed for the LT-I gene. Positive products from the CPA, LAMP, and IMSA assays showed ladder-like patterns on the gel electrophoresis (Fig. 3A, D, G). After mixing of SYBR Green I, the products could be visualized directly by the naked eye (Fig. 3B, E, H), or under UV light (Fig. 3C, F, I). In contrast, the negative control sample did not show any characteristic changes.

3.2. Optimization of LAMP, CPA and IMSA assays for the LT-I gene

The initial standardization of these assays was carried out using the primers mentioned above. For LAMP, the reaction mixture was incubated at temperatures ranging from 60 to 70 °C, yet the optimal temperature was found to be 60 °C. The reaction times for the LAMP assays were 30, 45, 60, 75, 90 and 120 min at 60 °C. The subsequent results indicated that the DNA products showed the highest intensity with a reaction time of 45 min. The optimal reaction condition (LAMP for the LT-I gene) was 62 °C and 45 min (Fig. 4A, J). The optimal temperature and incubation time for the CPA reaction were also found to be 60 to 70 °C and 30 to 120 min. However, the optimal reaction condition was 60 °C for 60 min (Fig. 4B, K). Optimization of IMSA was also conducted using different temperatures between 60 and 70 °C for 60 min. The reaction time ranged from 30 to 120 min at 62 °C. The optimal condition was found to be 45 min at 62 °C (Fig. 4C, L). The optimal concentrations of *Bst* DNA polymerase for the three assays were 8 U/tube, 8 U/tube, and 6 U/tube (Fig. 4D-F). The optimal concentrations of Mg^{2+} for the three assays were 2.0 mM, 1.0 mM, and 1.0 mM, respectively (Fig. 4G-I). All the results were observed using agarose gel electrophoresis for the positive samples.

3.3. Specificity of the assays

The specificity results of the CPA, LAMP and IMSA assays are shown in Fig. 5. Production formation in the three tests was monitored by observing the color following the addition of SYBR Green I in the reaction mixture. The green color indicated positive findings. The amplification products were also confirmed using gel electrophoresis. Four of the 16 strains yielded positive results and showed no signs of cross-amplification with the other 14 non-LT-I^+ strains examined by the three techniques.

3.4. Sensitivity of the assays

The detection limit was determined for the CPA, LAMP and IMSA assays using the DNA prepared from C83903 as the template. Template DNA was tested at 1×10^6 CFU/reaction to 5 CFU/reaction. The LAMP and CPA reactions yielded positive results at ≥ 50 CFU, and the detection limit of IMSA was 25 CFU. The three methods were sufficient to give a positive result with the LT-I under these conditions. The detection limits of each assay were similar to that of qPCR, which yielded a positive signal at ≥ 50 CFU (Fig. 6).

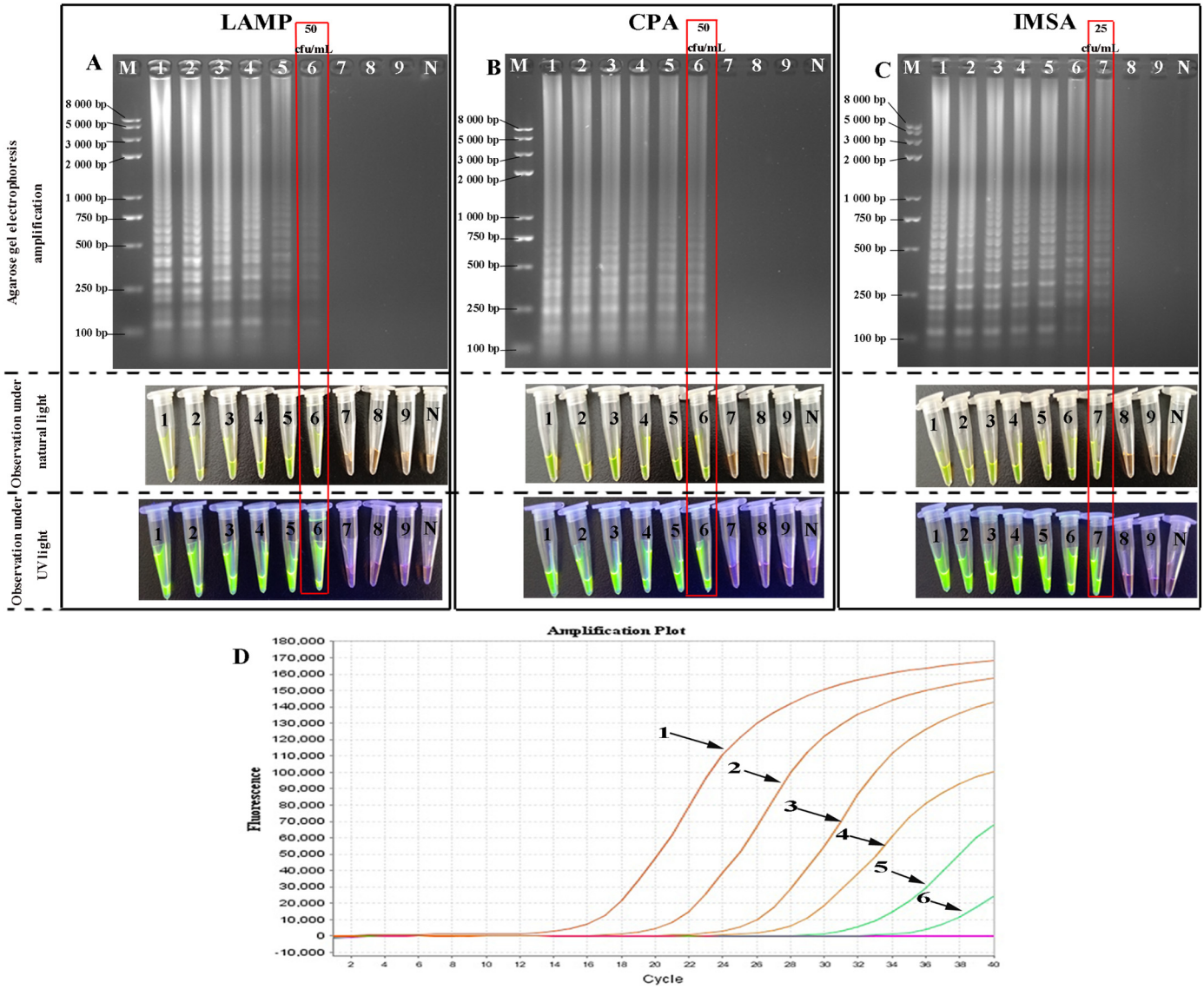

Fig. 6. The sensitivity of LAMP, CPA, and IMSA. (A-C): Observation of products obtained from LAMP, CPA, and IMSA by agarose gel electrophoresis, natural light, and UV light (with SYBR green I), respectively. (D): Amplification results of qPCR. M: Trans 2 K plus II DNA marker; N: negative control. 1–9: 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 50, 25, 15 and 5 CFU/mL, respectively. The minimum detection limits of the three methods are denoted with red lines, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Detection of clinical samples with LAMP, CPA, and IMSA

A total of 103 *Escherichia coli* strains (63 fecal samples from suckling pigs with diarrheal and 40 fecal samples from cattle with diarrheal) were isolated from 213 *Escherichia coli* strains by bacteriological cultivation and standard biochemical test and served as samples for clinical testing. The LT-I gene of *Escherichia coli* was tested for further evaluation and validation by used of LAMP, CPA and IMSA (Fig. 7). By adding the SYBR dye to the amplification product, UV light showed that 6, 17, 31, 59, 60, 71, 86 and 93 were LT-I positive (Table 3). The results of the three methods were the same and consistent with the qPCR findings (Fig. S1), which proved that the establishment of this method has clinical value and may be used for the rapid detection of the LT-I gene.

4. Discussion

Diarrheal disease caused by ETEC is often fatal in young children and infants from developing countries. In addition, the occasional food-borne outbreaks and mild-to-severe water-borne outbreaks have been shown to result from outbreaks of ETEC strains that produce heat-labile enterotoxin (LT-I) and heat-stable enterotoxin (ST). In cases of widespread diarrheal disease epidemics, reliable and rapid diagnostic methods are necessary to quickly identify the causative agents. Historically, qPCR and ELISAs have been the methods most commonly used for the detection of the LT-I pathogen. However, the equipment required for carrying out qPCR is expensive, which has rendered this method unsuitable for point-of-care testing in resource-limited countries or areas (Giljohann and Mirkin, 2009). In addition, the sensitivity of most ELISA kits is low, which has hindered the employment of ELISAs globally (Moore et al., 2000; de Bruin et al., 2006).

In recent years, several isothermal amplification methods have been developed for the rapid diagnosis of infectious pathogens in the clinic. Some of these methods include LAMP, CPA, and IMSA. Using these techniques, the only instrument required for amplification is a heat block or water bath, which makes it accessible to low-income communities and developing countries. In this report, we described the development of LAMP, CPA, and IMSA methods for the detection of the *LT-I* gene. The three methods described in this study have similar

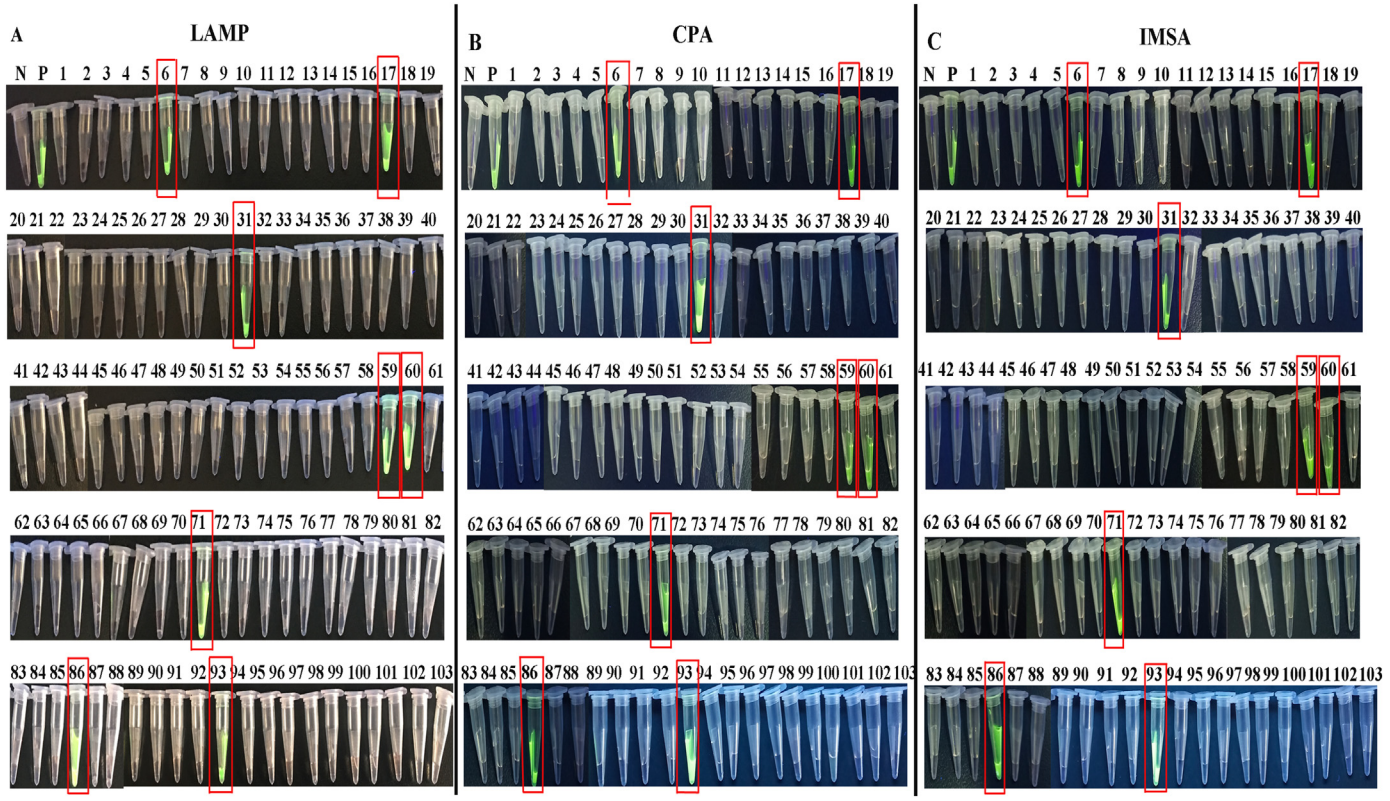

Fig. 7. Amplification results of clinical samples from the LAMP, CPA and IMSA assays. A-C: Amplification results of clinical samples detected by under UV light (with SYBR green I), respectively; The positive amplification of the three methods was labeled with red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Detection results of clinical samples amplified by LAMP, CPA, and IMSA.

Detection method	Porcine diarrheal specimens (n = 63)		Bovine diarrheal specimens (n = 40)		Total
	Positive	Negative	Positive	Negative	
LAMP	5	58	3	37	103
CPA	5	58	3	37	103
IMSA	5	58	3	37	103
qPCR	5	58	3	37	103

advantages, including rapid results, simple operation procedures, and easy readout of the results. For comparison, the regions of the target sequences used for designing the LAMP primers were also for the CPA and IMSA assay. In addition, the three assays used the same amount and type of templates. The IMSA method showed the highest sensitivity of the three methods, while the sensitivity values of the CPA and LAMP methods were two times lower than that of IMSA (with respect to the DNA concentration). In reference to the specificity, the IMSA assays showed a high degree of specificity to the target DNA, which is related to the six primers that specifically recognize seven distinct regions on the target sequence. This makes the IMSA assay a more attractive candidate assay for the detection of LT-I strains in clinical samples (Supplementary Fig. S1). Importantly, in this study, the Hua-feng tubes were used to ensure the amplifications were housed in a closed device, a sealed plastic separated device, which decreased the risk of sample or aerosol contamination, and prevented the likelihood of obtaining false-positive results.

In conclusion, all three amplification methods showed high specificity and sensitivity in the clinical samples, and the detection results were consistent with qPCR. These methods provide new tools for future epidemiological studies in the clinic.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.04.010.

Conflict of interest

No conflict of interest declared.

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