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Original article

Development and application of isothermal amplification methods for rapid detection of F4 fimbriae producing *Escherichia coli*

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the causative agent of a wide range of diseases, which are the important cause of illness and mortality in piglets. ETEC strains expressing F4 fimbriae are frequently associated with post-weaning diarrhea (PWD) and lead to great economic losses in swine production industry worldwide. The aim of this study was to establish a rapid and effective isothermal amplification method for detection of F4 fimbriae. Loop-mediated isothermal amplification (LAMP), Polymerase spiral reaction (PSR) and cross-priming amplification (CPA) were used to develop and optimize the detection method first time. Subsequently, the specificity and sensitivity of these methods were evaluated, and the clinical samples were detected with these methods. All the F4-positive samples could produce ladder-like amplifications products and lead the chromogenic substrate SYBR Green I produce green fluorescence, while in blank control and negative samples lack of this pattern or remained orange. The sensitivity of LAMP and CPA were 10 times higher than PSR method. Meanwhile, these three methods were validated with clinical samples, 7 were found positive, while 125 samples were negative, the testing results were consisted with the real-time PCR method. These findings suggested that the isothermal amplification based on the F4 fimbriae is a rapid, effective and sensitive method under resource constrains.

Key words: *Escherichia coli*, F4 fimbriae, isothermal amplification, rapid detection, clinical samples

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea and death in neonatal and post-weaning pigs, which brings severe mortality and great economic losses to the swine production industry, especially in developing countries (Liu et al. 2014, Luo et al. 2015). Among the factors leading to post-weaning diarrhea (PWD), F4 fimbriae adhesin and its variants play a critical role in the pathogenicity of ETEC, which allows ETEC to colonize the small intestine of piglets by mediating adhesion to the brush borders of villous enterocytes (Xia et al. 2015). The ETEC produces heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) when it combines with specific receptors. The produced LT and ST cause water and electrolyte disorder across the mucosa leading to watery diarrhea (Liu et al. 2019). At present, vaccination and antibiotic treatment are crucial methods in prevention and treatment of PWD, but unreasonable abuse of antibiotics increases the risk of drug resistance (Fairbrother et al. 2005). Therefore, a rapid, accurate and effective method for the detection of F4 fimbriae producing by *Escherichia coli* is necessary to prevent PWD infection.

The traditional diagnostic method for F4 fimbriae producing Escherichia coli normally includes bacterial culture, biochemical identification, ELISA, slide agglutination test and polymerase chain reaction (PCR) (Vazquez et al. 1996, Guo et al. 2008, Akase et al. 2009). However, most of isolations and culture procedures are limited by their premium costs, time consumption, and low sensitivity, even though enzyme-linked immunosorbent assay (ELISA), slide agglutination test and polymerase chain reaction (PCR) are simple, time-saving, sensitive and reproducible. The application of those methods is still limited by their shortcomings, like PCR-based methods which require a sophisticated thermal cycling instrument to denature template DNA, which significantly restrict its application in the field diagnostics (Gupta et al. 2009, Byun et al. 2012). Currently, novel methods based on isothermal amplification are commonly used for assaying the food, agriculture and medical samples, which have achieved good application prospect (Yano et al. 2007, Xu et al. 2012).

Isothermal amplification technique is a novel nucleic acid amplification method, which works under isothermal conditions and do not require advanced or expensive equipment, in addition, it is faster than the traditional PCR method. A wide variety of isothermal amplification techniques have been reported for amplification of DNA or RNA in recent years (Mao et al. 2012, Liu et al. 2019). Loop-mediated isothermal amplification (LAMP) technique has shown its superiority in detecting microorganism under the conditions that lacks the standard laboratory equipment. Because of its high specificity and sensitivity, LAMP has been successfully used to detect many pathogens (Notomi et al. 2000, Zhang et al. 2011). Cross-priming amplification (CPA) is a rapid detection technique developed by Ustar Biotechnologies Co., Ltd (Fang et al. 2009). Bst DNA polymerase is used for CPA, which has strand displacement activity and eliminates the denaturation step (Xu et al. 2012). Five or six primers recognizing unique regions of the target DNA were used in CPA, the test results can be obtained within 1 h under isothermal conditions and do not require specialized equipment or expensive reagents (Zhang et al. 2015). CPA overcomes the disadvantages of PCR-based detection assays, such as high cost, time consumption and special instrument, which has become the convenient diagnostic method for the grass-roots veterinary departments (Niczyporuk et al. 2015). Polymerase spiral reaction (PSR) is also a recently developed nucleotide amplification method with high specificity and sensitivity, which can be performed at a constant temperature and the results can be directly detected by monitoring turbidity or fluorescence (Liu et al. 2015). This method could amplify the target DNA rapidly and efficiently with 2-4 primers. It has been successfully used in the detection of genes of pathogens and antibiotic-resistance genes.

In the present study, we established three different isothermal amplification methods (LAMP, PSR, and CPA) for detection of F4 fimbriae producing by ETEC, which are good tools for rapid and specific detection of F4 fimbriae. These new developing techniques may improve the monitoring abilities for the surveillance of environmental quality and the public health in the world, especially in the developing countries.

Materials and Methods

Bacterial origin and DNA extraction

In this study, 23 bacterial strains (16 *Escherichia coli* strains and 7 other strains) were used to test the specificity (Table 1). ETEC standard isolate C83905 was used for standardization, conditions optimization, specificity and sensitivity assessment of CPA, LAMP and IMSA assays. From December 2016 to June 2018, a total of 186 clinical specimens were collected from different piggeries with diarrhea in the northeast China, which were streaked on Mac-Conkey agar plates (Solarbio, Beijing, CN) and incubated at 37°C for 16 h. Subsequently, standard biochemical procedures were used to confirm the strains of *Escherichia coli*, which included indole production, MR test, VP tset, citrate

Table 1. Bacterial strains used in this study.

Bacterial strain	Source	LAMP	PSR	CPA
Enterotoxigenic *E. coli*	CIVDC 83901 (F4ab)	+	+	+
Enterotoxigenic *E. coli*	CIVDC 83902 (F4ac)	+	+	+
Enterotoxigenic *E. coli*	CIVDC 83903 (F4ad)	+	+	+
Enterotoxigenic *E. coli*	CIVDC 83905 (F4ac)	+	+	+
Enterotoxigenic *E. coli*	PD-12-03 (Clinical-isolate)	+	+	+
Enterotoxigenic *E. coli*	PD-25-01 (Clinical-isolate)	+	+	+
Enterotoxigenic *E. coli*	CIVDC 83915	-	-	-
Enterotoxigenic *E. coli*	CIVDC 83917	-	-	-
Enterotoxigenic *E. coli*	CIVDC 83920	-	-	-
Enterotoxigenic *E. coli*	CMCC 44102	-	-	-
Enterotoxigenic *E. coli*	ATCC 25922	-	-	-
Enterotoxigenic *E. coli*	ATCC 35401	-	-	-
Escherichia coli	ATCC 700728 (O157: H7)	-	-	-
Escherichia coli	PD-02-01 (Clinical-isolate)	-	-	-
Escherichia coli	PD-11-01 (Clinical-isolate)	-	-	-
Escherichia coli	PD-15-02 (Clinical-isolate)	-	-	-
Salmonella Enteritidis,	ATCC 13076	-	-	-
Proteus mirabilis	ATCC 12453	-	-	-
Aeromonas hydrophila	ATCC 7966	-	-	-
Staphylococcus aureus	ATCC 25923	-	-	-
Staphylococcus aureus	ATCC 29213	-	-	-
Listeria monocytogenes	ATCC 19115	-	-	-
Yersinia enterocolitica	ATCC 23715	-	-	-

ATCC: American Type Culture Collection. CIVDC: China Institute of Veterinary Drug Control. CMCC: National Center for Medical Culture Collections. Clinical-isolates were preserved in our lab. +: positive result. -: negative result.

utilization, glucose and lactose fermentation, hydrogen sulfide production, etc. (Liu et al. 2014). DNA template was extracted from the enrichment culture broth of all bacterial strains by boiling method following the instructions as described and stored at -20°C (CapitalBio. Ltd., Beijing, CN).

Primer design

F4 fimbriae-specific primers were designed based on the oligonucleotide sequence of faeG conserved region published in the GenBank database (GenBank Number: M29374). The LAMP design principle was set following Notomi and colleagues (Notomi et al. 2000). The primers were designed using the Primer Explorer Version 5 (http://primerexplorer.jp/lampv5e). A set of four primers comprising two outer primers (forward outer primer, F3 and backward outer primer, B3) and the two inner primers (forward inner primer, FIP and backward inner primer, BIP) was designed and synthesized. For the PSR method, the design principle has been described by Liu's research group (Liu et al. 2015). The spiral primers (Ft and Bt) were designed based on the faeG gene sequence, which consist of forward primer (F) and reverse primer (B). Nr and N sequences were abstracted from a heterologous gene. Additionally, two auxiliary accelerated primers IF and IB were designed using the Primer Premier 5 (PREMIER Biosoft International Co., CA, USA) to enhance the reaction. For the CPA assay, a set of five primers were designed based on the mechanism of CPA (Fang et al. 2009) using the Primer Premier 5.0 software, which consisted of two displacement primers, one cross-primer, and two detector primers.

A pair of fluorescence real-time PCR primers was introduced, which was used as a standard reference for the sensitivity assays (Wang et al. 2017). All the sequences of the oligonucleotide primers are shown in Table 2 and the complementarity was assessed with the Primer-BLAST tool from the NCBI.

Reaction system

The LAMP, PSR and CPA assays were conducted in a final volume of 25 µL containing: 2.5 µL 10 × ThermolPol reaction buffer, 2.0 mM dNTPs (Takara Bio, Ohtsu, Japan), 3 mM $MgCl_2$ (Sigma, St. Louis, MO, USA), 0.8 M betaine (Sigma), 1 µL (8 U) Bst DNA

Table 2. Primers used for LAMP, CPA, IMSA and real-time PCR assays.

	Primers Name	Primer Sequences (5'-3')
LAMP primers	F3	GTACAGGTCTTAATGGATTTGG
	B3	TTTCACCATCAGGGTTTCT
	FIP	GGCCTAACAAAATTGGCTTATTACC-CCTGACCAATGGTGGAAC
	BIP	CATTTGCTACGCCAGTAACTGG-GTACTACAGAAGCTCCTTCA
CPA primers	1s	TCCACCATTGGTCAGGTCATTC-GTCAGAAATGGGAATGGAAAG
	2a	TCCACCATTGGTCAGGTCATTC
	3a	CCAAATCCATTAAGACCTGTACCA
	4s	GGTAGTATCACTGCAGATGA
	5a	GTCAGTAAATGCAATATGAGGAATT
PSR primers	Ft	gtcaaagcgatcccgccttac-TGGGAATGGAAAGTTGGT
	Bt	cattccgccctagcgaaactg-CAGAAGCTCCTTCATAGTCAG
	F1	ATGACTGGTGATTTCAATGGTT
	B1	TTTTATTAGTTTCACCATCAGG
Real-time PCR primers	Forward	AATGCATCTTATGCCGGTG
	Reverse	CGTCCGCAGAAGTAACCCCACCT

polymerase (New England Biolabs, Ipswich, MA, USA), 2 µL of appropriate concentration of target DNA. For the LAMP assay, reaction primers consisted of FIP and BIP (40 pmoL) and F3 and B3 (10 pmoL). The primes for the PSR assay contained 40 pmoL for Ft and Bt, 20 pmoL for IF and IB. The CPA assay contained 40 pmoL of the cross-primer, 20 pmoL each of primers 2a and 3a, 10 pmoL each of displacement primers 4s and 5a. The three reactions were carried out in a water bath at 63°C for 60 min, and then incubated at 80°C for reaction termination. For analysis of the products, 1 µl of SYBR green I (2 000 ×) (Solarbio, Beijing, China) were mixed with the reacted product and observed under normal light or UV light, the bright green fluorescence represented positive and orange represented negative. Subsequently, the products were also analyzed by electrophoresis using 2.0% agarose gel.

Optimization of the LAMP, PSR and CPA assays

In this study, the reaction temperature, concentration of Bst DNA polymerase and reaction time of the three isothermal amplification techniques were optimized, respectively. Briefly, the reaction mixtures with different constituents were incubated at a temperature gradient of 61°C - 71°C for 60 min. The concentration of Bst-DNA polymerase was set at 6 U/tube, 8 U/tube, 10 U/tube and 12 U/tube, respectively. The incubation time was optimized at 30, 45, 60, 75, 90 and 120 min at the optimal temperature. After incubation, the amplified DNA products were analysis with 2.0 % agarose gels and stained with ethidium bromide (0.5 µg/mL).

Specificity of the Assays

The specificity of LAMP, PSR and CPA assays were evaluated by using 23 different DNAs extracted (16 *E. coli* strains and 7 other bacteria strains listed in Table 1). *E. coli* C83905 was used as the positive controls and the sterilized deionized water as the blank control. The reaction tubes were performed as described above at corresponding conditions and all tests were repeated ≥3 times. 5 µL of the products were subjected to electrophoresis with 2.0 % agarose gel, stained with SYBR Green I (2 000 ×) and visualized under ultraviolet light.

Sensitivity of the assays

To evaluate the detection limits of these three isothermal amplification assays, the extracted DNA of *Escherichia coli* C83905 was diluted from 10^8 CFU/mL to 5 CFU/mL and subjected to LAMP, PSR, CPA and real-time PCR assays in triplicate. The reactions were performed at the corresponding amplification conditions. Next, 1 µL of SYBR Green I (2 000 ×) was added to each reaction tube, after mixing the tube was visualized under natural light and ultraviolet light. At the same time, the amplification products were assayed by electrophoresis with 2.0 % agarose gel, respectively.

Clinical sample detection

To assess the efficiency and reliability of these three isothermal amplification assays, the clinical samples were tested. A total of 132 diarrhea specimens from suckling pigs were gathered from 2016 to 2018. DNA was extracted from all the samples and 2 µL of extracted DNA was used as template in the LAMP,

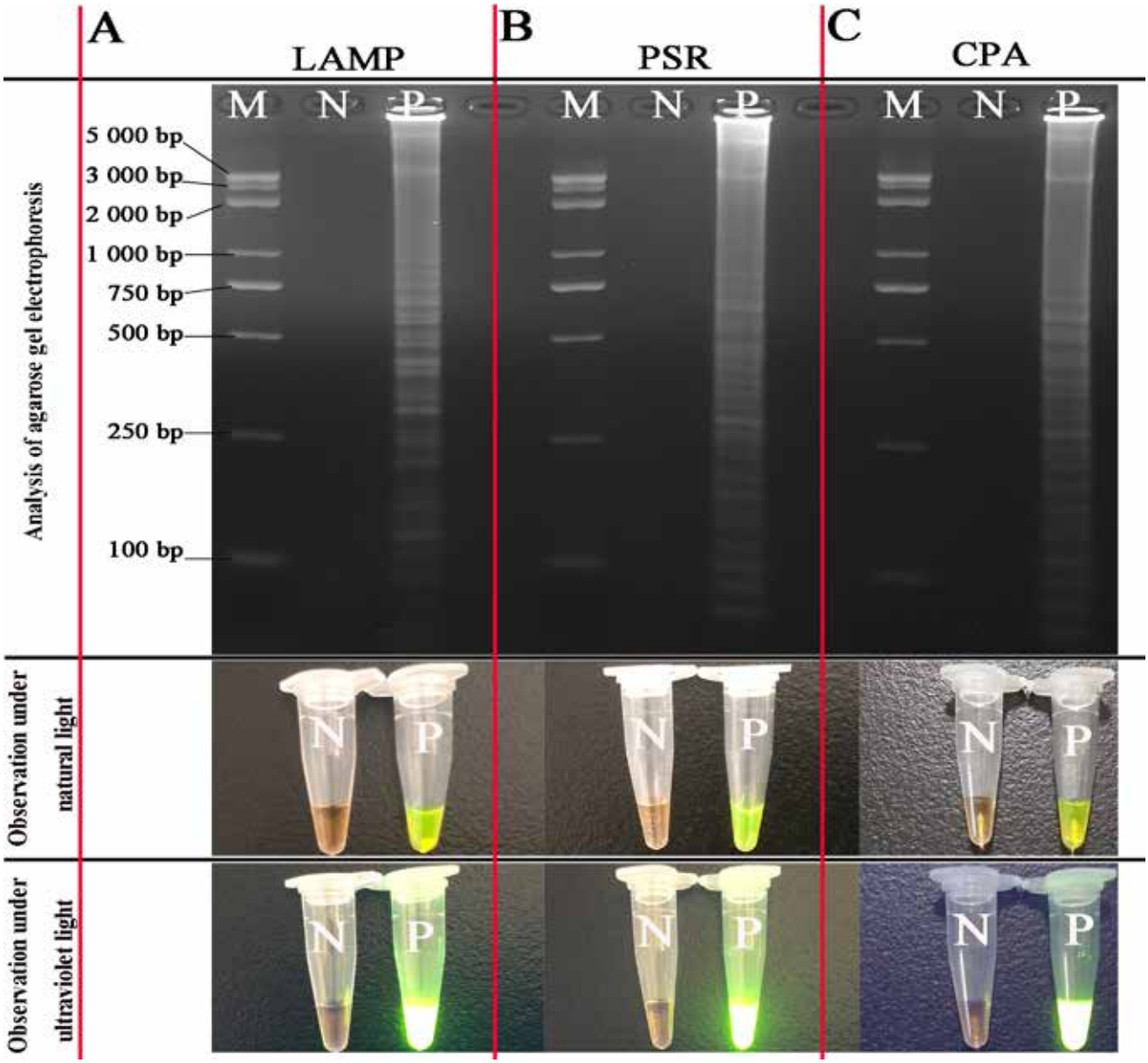

Fig. 1. Result from LAMP, PSR and CPA amplification for F4 fimbriae. A-C. Observation of agarose gel electrophoresis, natural light and UV light for LAMP, PSR and CPA methods, respectively; M. molecular weight marker (TransGen Biotech Co., Ltd, CN); N. negative control; P. C83905 amplified product.

PSR, CPA and real-time PCR assay. All the amplification products were assayed by electrophoresis with 2.0% agarose gel and visualized by ultraviolet light after mixing with SYBR Green I (2000 ×).

Ethics statement

The diarrheal samples were collected with the permission of the owner in the farms. 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 Northeast Agricultural University (Harbin, Heilongjiang, P. R. China).

Results

Amplification of the F4 fimbriae by LAMP, PSR and CPA

The specific primers for the three assays were designed based on the FaeG gene of F4 fimbriae. The products of positive amplification from LAMP, PSR and CPA assays showed typical ladder-like patterns after gel electrophoresis. After adding SYBR Green I, the positive products gave green fluorescence under natural light and UV light. In contrast, the negative control group presented orange (Fig. 1).

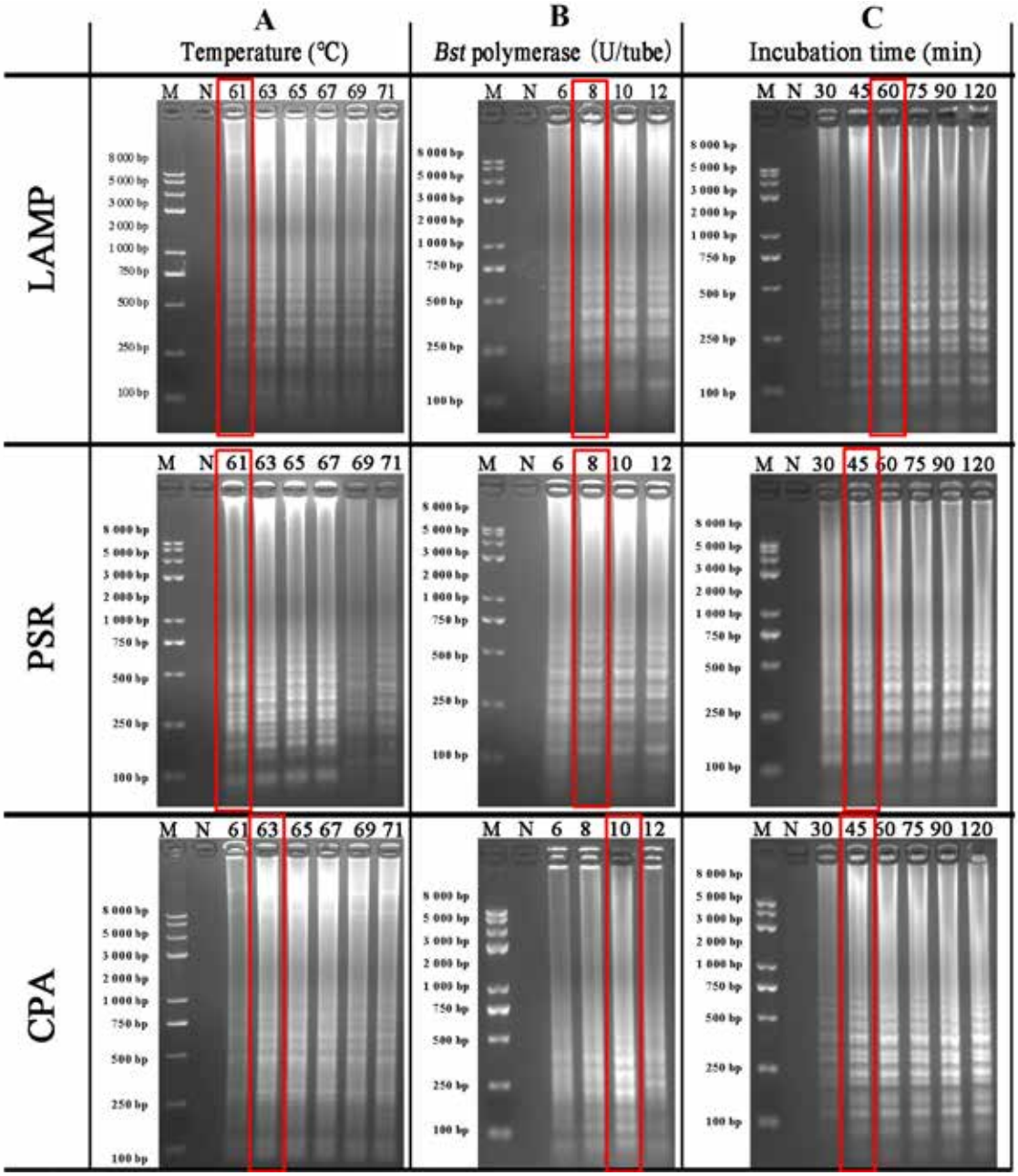

Fig. 2. Optimization of reaction Conditions for LAMP, PSR and CPA. A-C: Optimization of incubation temperature, bst DNA polymerase and incubation time of LAMP, PSR and CPA, respectively. Optimum conditions have been marked in the figures, respectively. M: molecular weight marker; N: negative control (double distilled water).

Optimization of the assays

To standardize these three assays, reactions were carried out using the specific primers and fixed reagents as mentioned above. Correspondingly, the reaction temperature, Bst DNA polymerase amount and incubation time were optimized, respectively. As shown in Fig. 2, LAMP assays were performed under isothermal conditions between 61°C to 71°C in 2°C increments. Based on the amounts of DNA amplicons and the optimal temperature for Bst DNA polymerase activity, we choose 61 °C and 8 U/tube as the final reaction temperature and Bst DNA polymerase amount, respectively. The incubation times for the LAMP assays ranged from 30 to 120 min at 61°C. The subsequent results indicated that the optimum reaction time is 60 min. For the PSR assays, the optimum temperatures ranging from 61 to 71°C at 2°C increments were used to optimize amplification, and the best results were obtained when the condition was standardized at 61°C for 45 min. According to the principle of cost saving, 8 U/tube of Bst DNA polymerase was set as the optimal concentration. The best choice for CPA reactions were 45 min at 63°C and 10 U/tube of Bst DNA large-fragment polymerase.

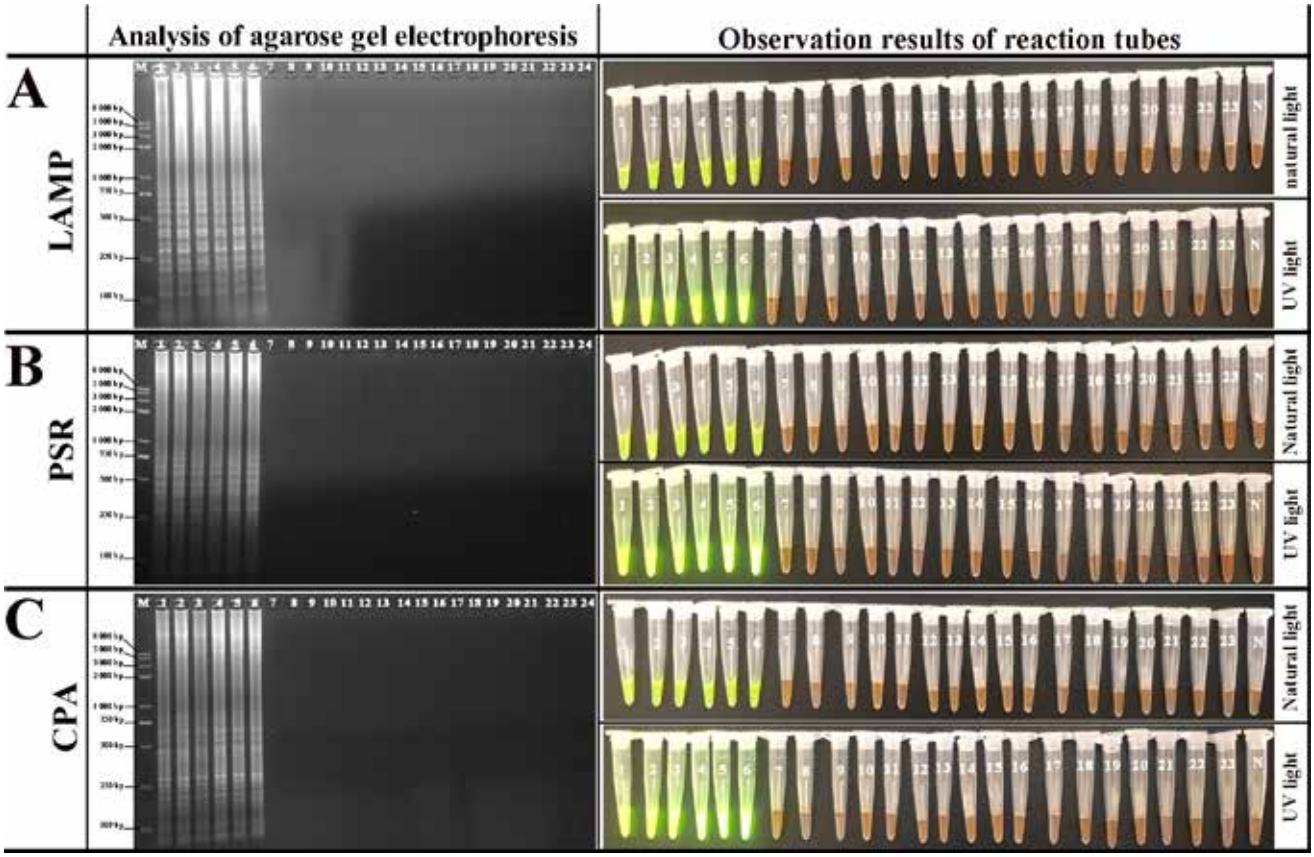

Fig. 3. The specificity of LAMP, PSR and CPA amplification. A-C: Observation of the products from LAMP, PSR and CPA under agarose gel electrophoresis, natural light and UV light (with SYBR green I), respectively; M: molecular weight marker; N: negative control; 1-23: CIVDC 83901, CIVDC 83902, CIVDC 83903, CIVDC 83905, PD-12-03, PD-25-01, CIVDC 83915, CIVDC 83917, CIVDC 83920, CMCC 44102, ATCC 25922, ATCC 35401, ATCC 700728 (O157: H7), PD-02-01, PD-11-01, PD-15-02, ATCC 13076, ATCC 12453, ATCC 7966, ATCC 25923, ATCC 29213, ATCC 19115, ATCC 23715.

Specificity of the assays

The results of specificity analysis are shown in Fig. 3. The LAMP, PSR and CPA assays were evaluated by amplifying the genomic DNA extracted from 16 *E. coli* strains and 7 other bacterial strains (Table 1). The amplification products of all the bacterial strains were tested using 2.0% agarose gel as described, the positive results with the typical ladder could be easily observed from F4 fimbriae amplification. In contrast, no positive DNA products were observed from other bacterial strains. According to the color of the reaction mixture after adding SYBR green I, the amplification results can be monitored (green indicated positive and orange indicated negative). Six of the 23 strains yielded positive results and the other 17 non-F4 fimbriae strains showed negative. This results clearly demonstrated that the CPA, LAMP and IMSA methods are specific for identification of ETEC having F4 fimbriae.

Sensitivity of the assays

To assess the sensitivity of LAMP, PSR and CPA assays, DNA was extracted from a 10-fold serial dilution of C83905 strain (1×10^8 CFU/mL to 5 CFU/mL) and was used as the DNA template to detected the sensitivity of these methods. The results showed that the detection limit of LAMP and CPA were down to 10^1 CFU/mL, which were ten times higher than the PSR amplification method. The sensitivity of the present three methods was same as the real-time PCR in detecting F4 fimbriae of ETEC (Fig. 4).

Detection of clinical diarrheal samples

A total of 132 ETEC strains were characterized from 186 clinical diarrheal samples by standard bacteriological culture and biochemical tests. Subsequently, the ETEC strains were further evaluated and validated using LAMP, PSR and CPA methods, and the real-time PCR was used as a reference. The results show that seven of the 132 clinical strains were positive by LAMP, PSR, CPA assays, and 125 strains were negative (Table 3). The testing results of the three methods were

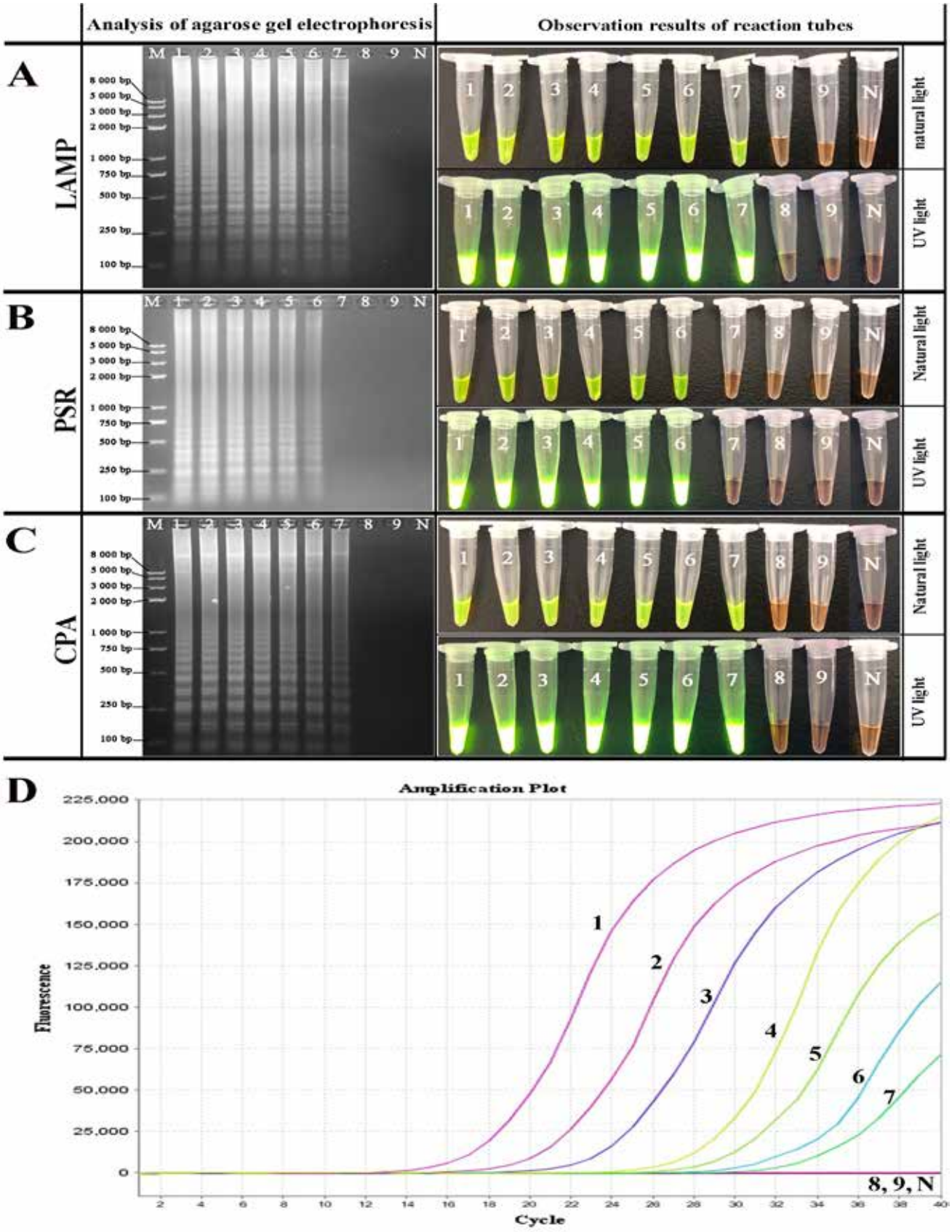

Fig. 4. The sensitivity of LAMP, PSR, CPA and real-time PCR assays. A-C: Observation of the amplification productions from LAMP, PSR and CPA by agarose gel electrophoresis, natural light and UV light, respectively. D: Amplification results of real-time PCR. M: molecular weight marker; N: negative control (double distilled water). 1-9: 1×10^{8}, 1×10^{7}, 1×10^{6}, 1×10^{5}, 1×10^{4}, 1×10^{3}, 1×10^{2}, 1×10^{1} and 5 CFU/mL, respectively.

Table 3. Detection results of clinical diarrhea samples.

Detection methods	Porcine diarrheal specimens (n=132)		total
	Positive	Negative	
culture-based method	7	125	132
LAMP	7	125	132
CPA	7	125	132
IMSA	7	125	132
real-time PCR	7	125	132

consistent with the results of real-time PCR. It indicated that these methods have valuable potential in clinical diagnosis and can be used for rapid detection of F4-fimbriae producing by ETEC.

Discussion

Loop-mediated isothermal amplification is the most widely used isothermal technique for detection of genomic DNA of the pathogen agents, which reveals significant advantages, such as simple operation, low cost, high stability and specificity (Mori et al. 2013). Subsequently, many isothermal amplification techniques have been established and applied for the rapid detection of the pathogen, including PSR, CPA and IMSA etc (Ding et al. 2014, Das et al. 2018). These methods rely on the auto-cycling strand displacement feature of Bst DNA polymerase with strand displacement activity in a constant temperature condition. In terms of primer usage, the PSR method has more advantages than LAMP and CPA method as the involvement of minority primers reduce the chances of cross-contamination and false positivity. In the present study, we established three isothermal methods targeting the conserved region of faeG gene. The faeG protein shows a significant effect on adhering to host epithelial cells and existed in all F4 fimbriae isolates. In particular, the serotypes of *E. coli* are numerous and complex compared with other bacterial species, and it is difficult to find the common and conserved sequences to detect all the virulence factors. Even for the enterotoxigenic *E. coli*, they also carry different virulence factors. Both adhesion factors and enterotoxins of ETEC strains are required to cause diarrhea. The ETEC strains that only carry the enterotoxin fails to cause diarrhea. Therefore, compared with the previously established method based on the detecting of enterotoxin, detecting F4 fimbriae is more suitable and practical for the detection of ETEC infection (Liu et al. 2019).

These three methods established in our study have the same advantages, such as rapid reaction, high sensitivity and ease to observe results. In the optimizing assays, we show the more important optimum conditions, while the amount of betaine, Mg^{2+} and dNTPs had little effect on the assays (data were not shown). Based on the previous studies, the betaine was added to improve the sensitivity and effectiveness of the isothermal amplification (Zhou et al. 2014). But in this study, the addition of betaine had no effect on the amount of amplification products (data not shown).

For the specificity analysis, 16 *Escherichia coli* strains of different serotypes and 7 other bacterial strains were tested. These three methods successfully amplified all of the F4 fimbriae strains. Moreover, three F4 antigenic variants (F4ab, F4ac and F4ad) were also used to validate the universality of the established method. In terms of sensitivity, the CPA method is consistent with that of LAMP and real-time PCR, which could achieve a high sensitivity level (10 CFU/mL), ten times higher than PSR method. Therefore, it could be a suitable alternative method for screening samples with small amount of F4 fimbriae. To evaluate the potential of these three methods in the diagnosis of PWD caused by F4 fimbriae, 132 clinical diarrhea samples were tested for further verification. All these three methods could correctly identify seven positive samples and the results were consisted with the real-time PCR method. Therefore, these results suggested that the novel methods can be used to detect PWD caused by F4 fimbriae of ETEC.

In conclusion, the diverse virulence factors and complex serotypes of ETEC increase the difficulty to identify the pathogen and control the PWD in swine industry. The developed FaeG gene-based isothermal techniques are rapid, reliable, sensitive and specific method for the detection of PWD.

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References

Akase S, Uchitani Y, Sohmura Y, Tatsuta K, Sadamasu K, Adachi Y **(2009)** Application of real time PCR for diagnosis of Swine Dysentery. J Vet Med Sci 71: 359-362.

Byun JW, Jung BY, Kim HY, Fairbrother JM, Lee WK **(2012)** Real-time PCR for differentiation of F4 (K88) variants (F4ab, F4ac, F4ad) of enterotoxigenic *Escherichia coli* from diarrhoeic piglets. Vet J 193: 593-594.

Das A, Kumar B, Chakravarti S, Prakash C, Singh RP, Gupta V, Singh KP, Agrawal RK, Chaturvedi VK, Abhishek, Shrinet G **(2018)** Rapid visual isothermal nucleic acid-based detection assay of *Brucella* species by polymerase spiral reaction. J Appl Microbiol 125: 646-654.

Ding X, Nie K, Shi L, Zhang Y, Guan L, Zhang D, Qi S, Ma X **(2014)** Improved detection limit in rapid detection of human enterovirus 71 and coxsackievirus A16 by a novel reverse transcription-isothermal multiple-self--matching-initiated amplification assay. J Clin Microbiol 52: 1862-1870.

Fairbrother JM, Nadeau E, Gyles CL **(2005)** *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim Health Res Rev 6: 17-39.

Fang R, Li X, Hu L, You Q, Li J, Wu J, Xu P, Zhong H, Luo Y, Mei J, Gao Q **(2009)** Cross-priming amplification for rapid detection of *Mycobacterium tuberculosis* in sputum specimens. J Clin Microbiol 47: 845-847.

Guo X, Xia X, Tang R, Zhou J, Zhao H, Wang K **(2008)** Development of a real-time PCR method for Firmicutes and *Bacteroidetes* in faeces and its application to quantify intestinal population of obese and lean pigs. Lett Appl Microbiol 47: 367-373.

Gupta R, Mirdha BR, Guleria R, Kumar L, Samantaray JC, Agarwal SK, Kabra SK, Luthra K **(2009)** Diagnostic significance of nested polymerase chain reaction for sensitive detection of *Pneumocystis jirovecii* in respiratory clinical specimens. Diagn Microbiol Infect Dis 64: 381-388.

Jiang X, Dong D, Bian L, Zou D, He X, Ao D, Yang Z, Huang S, Liu N, Liu W, Huang L **(2016)** Rapid Detection of Candida albicans by polymerase spiral reaction assay in clinical blood samples. Front Microbiol 7: 916.

Liu W, Dong D, Yang Z, Zou D, Chen Z, Yuan J, Huang L **(2015)** Polymerase Spiral Reaction (PSR): A novel isothermal nucleic acid amplification method. Sci Rep 5: 12723.

Liu W, Yuan C, Meng X, Du Y, Gao R, Tang J, Shi D **(2014)** Frequency of virulence factors in *Escherichia coli* isolated from suckling pigs with diarrhoea in China. Vet J 199: 286-289.

Liu W, Yuan C, Zhang L, Feng Y **(2019)** Development of isothermal amplification methods for rapid and sensitive detection of heat-labile enterotoxin producing *Escherichia coli*. J Microbiol Methods 161: 47-55

Liu Z, Zhang Q, Yang NN, Xu MG, Xu JF, Jing ML, Wu WX, Lu YD, Shi F, Chen CF **(2019)** Rapid and Sensitive Detection of *Salmonella* in Chickens Using Loop--Mediated Isothermal Amplification Combined with a Lateral Flow Dipstick. J Microbiol Biotechnol 29: 454-464.

Luo Y, Van Nguyen U, De La PY, Devriendt B, Cox E **(2015)** F4+ ETEC infection and oral immunization with F4 fimbriae elicits an IL-17-dominated immune response. Vet Res 46: 121-126.

Mao Z, Qiu Y, Zheng L, Chen J, Yang J **(2012)** Development of a visual loop-mediated isothermal amplification method for rapid detection of the bacterial pathogen *Pseudomonas* putida of the large yellow croaker (*Pseudosciaena crocea*). J Microbiol Methods 89: 179-184.

Mori Y, Kanda H, Notomi T **(2013)** Loop-mediated isothermal amplification (LAMP): recent progress in research and development. J Infect Chemother 19: 404-411.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T **(2000)** Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28: E63.

Vazquez F, Gonzalez EA, Garabal JI, Valderrama S, Blanco J, Baloda SB **(1996)** Development and evaluation of an ELISA to detect *Escherichia coli* K88 (F4) fimbrial antibody levels. J Med Microbiol 44: 453-463.

Wang W, Zijlstra RT, Ganzle MG **(2017)** Identification and quantification of virulence factors of enterotoxigenic *Escherichia coli* by high-resolution melting curve quantitative PCR. BMC Microbiol 17: 114.

Xia P, Song Y, Zou Y, Yang Y, Zhu G **(2015)** F4+ enterotoxigenic *Escherichia coli* (ETEC) adhesion mediated by the major fimbrial subunit FaeG. J Basic Microbiol 55: 1118-1124.

Xu G, Hu L, Zhong H, Wang H, Yusa S, Weiss TC, Romaniuk PJ, Pickerill S, You Q **(2012)** Cross priming amplification: mechanism and optimization for isothermal DNA amplification. Sci Rep 2: 246-249.

Yano A, Ishimaru R, Hujikata R **(2007)** Rapid and sensitive detection of heat-labile I and heat-stable I enterotoxin genes of enterotoxigenic *Escherichia coli* by Loop--Mediated Isothermal Amplification. J Microbiol Methods 68: 414-420.

Zhang X, Du XJ, Guan C, Li P, Zheng WJ, Wang S **(2015)** Detection of *Vibrio cholerae* by isothermal cross-priming amplification combined with nucleic acid detection strip analysis. Mol Cell Probes 29: 208-214.

Zhang XJ, Han QY, Sun Y, Belak S, Liu L, Qiu HJ **(2011)** Development of a loop-mediated isothermal amplification for visual detection of the HCLV vaccine against classical swine fever in China. J Virol Methods 171: 200-205.

Zhou D, Guo J, Xu L, Gao S, Lin Q, Wu Q, Wu L, Que Y **(2014)** Establishment and application of a loop-mediated isothermal amplification (LAMP) system for detection of cry1Ac transgenic sugarcane. Sci Rep 4: 4912.