Journal of Microbiological Methods 151 (2018) 62–65

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A real-time loop-mediated isothermal amplification method for rapid detection of *Lawsonia intracellularis* in porcine fecal samples

Yanan Li[a,1], Jianchang Wang[b,c,1], Jinfeng Wang[b,1], Libing Liu[b,c], Ruoxi Zhang[d], Ruihan Shi[b], Qingan Han[d], Jiguo Sun[a], Wanzhe Yuan[a,*]

[a] College of Veterinary Medicine, Agricultural University of Hebei, Baoding, Hebei 071001, China
[b] Center of Inspection and Quarantine Technology, Hebei Entry-Exit Inspection and Quarantine Bureau, Shi Jiazhuang, Hebei 050051,China
[c] Hebei Academy of Science and Technology for Inspection and Quarantine, Shijiazhuang 050051, China
[d] Hebei Animal Disease Control Center, Shijiazhuang 050050, China

ARTICLE INFO

Keywords:
Lawsonia intracellularis
16S rRNA
Real-time LAMP
Visual inspection
Molecular diagnosis

ABSTRACT

Porcine proliferative enteritis is a common diarrheal disease characterized by thickening of the intestinal mucosa in swine due to enterocyte proliferation, which is caused by *Lawsonia intracellularis*. In this study, a real-time loop-mediated isothermal amplification (LAMP) assay was developed to detect L. *intracellularis* based on the conserved region of the 16S ribosomal RNA gene. The optimal reaction conditions of the real-time LAMP was 65 °C for 60 min. The LAMP products could be detected by both real-time turbidity and direct visual inspection. The assay was specific for L. *intracellularis*, as no cross-reaction was observed with other pathogens. The detection limit of the real-time LAMP assay was 1.4×10^{-1}pg of L. *intracellularis* DNA, which was the same as that of real-time PCR and approximately 100 times more sensitive than that of conventional PCR. Of the 136 clinical samples, *L. intracellularis* DNA was identified in 60 samples by real-time LAMP, which was the same as real-time PCR and higher than conventional PCR (36.8%, 50/136). The specific, sensitive and rapid real-time LAMP assay developed in this study could be a useful alternative tool in point-of-care (POC) diagnosis of L. *intracellularis* infection.

1. Introduction

Porcine proliferative enteropathy (PPE) is a transmissible enteric disease of swine and characterized by thickening of the intestinal mucosa due to enterocyte proliferation (Lawson and Gebhart, 2000). PPE is widely distributed among pig herds in many pig-producing countries, and affected pigs may exhibit impaired growth, diarrhea, or subclinical infection (Lawson and Gebhart, 2000; Mcorist, 2005). PPE is considered among the most economically important infections in the swine industry worldwide (Lawson and Gebhart, 2000).The etiologic agent of the PPE is *Lawsonia intracellularis* which is an obligate intracellular, motile, curved, gram-negative bacterium that resides freely within the apical cytoplasm of infected intestinal enterocytes (Rowland and Lawson, 1992; Lawson and Gebhart, 2000). Until now, it has only been possible to cultivate L. *intracellularis in vitro* in cell cultures, which is not adaptable for the detection of L. *intracellularis* in clinical samples (Lindecrona et al., 2002; Yeh et al., 2006).

The above facts clearly indicate that the simple and rapid detection of L. *intracellularis* would be extremely important in the prevention and control of PPE. Currently, several conventional methods are available for the detection of L. *intracellularis*, including Warthin–Starry silver stain (Rowland and Lawson, 1992), immunofluorescence testing (Guedes et al., 2002), and *in situ* hybridization (Gebhart et al., 1994), which are useful for the identification of active disease. Nevertheless, these conventional methods are often time-consuming, requiring well-trained technicians, unsuitable for detection of subclinical infections, epidemiological and herd surveillance studies. A substantial number of polymerase chain reaction (PCR) assays have been reported and used widely for PPE diagnosis (Lindecrona et al., 2002; La et al., 2006; Richter et al., 2010). However, the PCR assays require high-precision, sophisticated and expensive instruments and good laboratory facilities. Therefore, they are unsuitable for being applied in smaller, under-equipped laboratories and point-of-care (POC) applications.

Since loop-mediated isothermal amplification (LAMP) was first reported in 2000 (Notomi et al., 2000), a number of pathogen-specific assays based on LAMP have been described, which showed high sensitivity and specificity, rapidness and cost-effectiveness (Ge et al., 2013; Marthaler et al., 2014; Yuan et al., 2014). LAMP uses a strand-

* Corresponding author at: College of Veterinary Medicine, Agricultural University of Hebei, No.38 Lingyusi Street, Baoding, Hebei 071001, China.
E-mail address: yuanwanzhe@163.com (W. Yuan).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.mimet.2018.06.002
Received 25 April 2018; Received in revised form 25 May 2018; Accepted 12 June 2018
Available online 13 June 2018
0167-7012/

displacing DNA polymerase along with two internal primers (FIP and BIP) and two outer primers (F3 and B3), the primers recognize 6 different regions on the target gene. LAMP reactions could be performed at constant temperature in the user-friendly real-time turbidimeter or water bath, and large amounts of nucleic acid product could be generated in approximately 60 min, which could be detected by the real-time turbidimeter or by naked eye inspection. The above characteristics make LAMP an easy and rapid method suitable for the POC detection of pathogens. In this study, a rapid and reliable real-time LAMP method was developed for the detection of L. *intracellularis* DNA using primers targeting the conserved region of the 16S ribosomal RNA (rRNA) gene.

2. Material and methods

2.1. Virus, bacteria strains and clinical samples

L. intracellularis (strain LX5), transmissible gastroenteritis virus (TGEV, strain HB-YX), porcine rotavirus (PRoV, strain HB-BD/2016), porcine epidemic diarrhea virus (PEDV, strain HB-LX), porcine parvovirus (PPV, strain BJ-2), *Escherichia coli* O157 (ATCC 43889), *Salmonella enterica* subsp. *enterica serovar* Typhisuis (ATCC 8321), *Clostridium perfringens* (ATCC 13124), *Shigella sonnei* (ATCC 25931)and *Campylobacter jejuni* (ATCC 33291) are all kept in our laboratory. The viral RNA of porcine deltacoronavirus (PDCoV) was extracted from the small intestinal sample tested to be PDCoV positive by real-time RT-PCR (Marthaler et al., 2014). The genomic DNA of Brachyspira hyodysenteriae and Brachyspira pilosicoli was kindly provided by Dr. Mi Lin from the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute.

One hundred and thirty-six clinical samples (including feces and ileum samples) were collected from pigs with diarrhea from different pig farms in Hebei province from January 2015 to September 2017. The ileum samples were homogenized with phosphate-buffered saline (PBS, pH 7.4) as a 10% (*w/v*) suspension, and the homogenates were centrifuged for approximately 1 min at 12000*g* at 4 °C. The precipitate was collected for DNA extraction.

2.2. DNA/RNA extraction and RNA reverse transcription

All the bacterial genomic DNA was extracted using the TIANamp Bacteria DNA kit (Tiangen, Beijing, China), viral DNA was extracted using the TIANamp Virus DNA kit (Tiangen, Beijing, China) and viral RNA was extracted using the Trizol Reagent (Invitrogen, Waltham, USA), which were all performed according to the manufacturer's instructions, respectively. One hundred nanograms of viral RNA was reverse transcribed to cDNA using the First strand cDNA Synthesis kit (Takara, Dalian, China) according to the manufacturer's instructions. Ten micrograms of ileum sample was used for DNA extraction using the TIANamp Genomic DNA kit (Tiangen, Beijing, China), and 200 micrograms of feces sample was used for DNA extraction using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany). Total DNA extracted from clinical samples was finally eluted in 50 μL of TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 7.4). All DNA and cDNA were quantified using a ND-2000c spectrophotometer (NanoDrop, Wilmington, USA) and stored at −80 °C until use.

2.3. Primers design

The 16S rRNA gene is highly conserved in the genome of L. *intracellularis*. According to the reference sequences of L. *intracellularis* (Acession number: AM180252, EU348664, L15739, NC_020127, NR_102487, U65995, U30147), the conserved region of the 16S rRNA gene was identified, and a set of LAMP primers were designed using Explored V4 Software (http://primerexplorer.jp/e/). All primers were synthesized by a commercial company (Sangon, Shanghai, China). Sequences for the primers are provided in Table 1.

Table 1
Sequences of primers and probes for L. *intracellularis* real-time LAMP, PCR, and real-time PCR.

Assays	Primers and probe	Sequence 5′-3′
Real-time LAMP	16S-F3	GCATCTCAGTCCGGATTGG
	16S-B3	CTTGTTACGACTTCACCCCA
	16S-FIP (F1c-F2)	GCATTCACCCGAGCATGCTGA-AGTCTGCAAC TCGACTCCAT
	16S-BIP (B1c-B2)	GTACACACCGCCCGTCACAC-GTAGACGACTGCCTCGATTG
PCR	Lint-146F	GATAATCTACCTTCGAGACGG
	Lint-745R	TGACCTCAGTGTCAGTTATCGT
Real-time PCR	16S-F	GCGCGCGTAGGTGGTTATAT
	16S-R	GCCACCCTCTCCGATACTCA
	16S-P	FAM-CACCGCTTAACGGTGGAACAGCCTT-BHQ1

2.4. Optimization of the real-time LAMP assay

The real-time LAMP reaction was performed in a final reaction volume of 25 μL by using a Loopamp DNA amplification kit (Eiken Chemical, Tokyo, Japan). The reaction system included 12.5 μL of 2 × Reaction Mix, 40 pmol each of inner primers 16S-FIP and 16S-BIP, 5 pmol each of outer primers 16S-F3 and 16S-B3, 8 U of Bst DNA polymerase, and 1 μL of DNA template. Amplification reactions were performed at different temperatures (60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, 66 °C, and 67 °C) for 60 min using a LA-320c Loopamp real-time turbidimeter (Eiken Chemical, Tokyo, Japan) to optimize the real-time LAMP reaction. The turbidity of the reaction was measured in real time, and the results were indicated by the graph on the monitor of the real-time turbidimeter.

Amplification reactions were also performed at the optimal temperature for 60 min in a water bath. In this case, 1 μL of fluorescent detection reagent (Eiken Chemical, Tokyo, Japan) was added into the 25 μL reaction system. The mixtures were heated at 80 °C for 10 min to terminate the reactions. LAMP products were also evaluated by direct naked eye inspection.

2.5. Analytical specificity and sensitivity of the real-time LAMP assay

Ten nanograms of bacterial DNA, viral DNA or cDNA were used for the analytical specificity analysis. The real-time LAMP assay was carried out to amplify the nucleic acids of a panel of pathogens including L. *intracellularis*, *E. coli O157*, *Salmonella enterica* subsp. *enterica serovar* Typhisuis, *C. perfringens, S. sonnei, C. jejuni, B. hyodysenteriae, B. pilosicoli,* PEDV, TGEV, PDCoV, PRoV, and PPV, which are all the important pathogens causing swine diarrhea. Five independent reactions were performed.

The L. *intracellularis* genomic DNA, ranging from 1.4×10^{4} to 1.4×10^{-3} pg/μL in TE buffer (pH 7.4), was used for the real-time LAMP analytical sensitivity assay. LAMP products were then evaluated with a fluorescent detection reagent. One micoliter of each dilution was amplified by real-time LAMP to determine the detection limit of the assay, and five independent reactions were performed.

2.6. Conventional PCR and real-time PCR

The conventional PCR and real-time PCR assays specific for L. *intracellularis* were performed as described previously (Lindecrona et al., 2002; La et al., 2006). Sequences for the primers and probe are provided in Table 1. The Premix Ex Taq™ (Takara, Dalian, China) was applied in the real-time PCR assay and the reaction was performed as follows: 95 °C for 10 s; then 40 cycles of 95 °C for 5 s and 60 °C for 35 s. The conventional PCR assay was performed as follows: 94 °C for 3 min and 32 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 39 s and 72 °C for 8 min. Nine microliters of the PCR products were analyzed by 1.5%