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<Short Communication>

Loop-mediated isothermal amplification assay for the detection of *Salmonella* spp. in pig feces

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Abstract : *Salmonella* are causative agents of gastroenteritis and systemic disease in animals. The *invA* gene was selected as a target sequence of loop-mediated isothermal amplification (LAMP) assay for diagnosis of *Salmonella* infection. The detection limits for broth dilution, spiked feces and enrichment were 10^4, 10^5 and 10^2 CFUs/mL, respectively. The LAMP assay developed in the present study may be a reliable method for detection of *Salmonella* spp. in pig feces.

Keywords : detection limit, *invA*, loop-mediated isothermal amplification assay, *Salmonella*

Salmonella (*S.*) are gram-negative bacilli belong to the family of *Enterobacteriaceae* and comprise a number of closely related serotypes, many of which are responsible for various illness and death in animals [5, 8]. Although most of the Salmonella cause gastrointestinal disorder, some Serovar such as Cholerasuis, Abortusequi, Gallinarum produce systemic diseases [3]. It is important that the identification of causative agent is simple and rapid. A variety of methods such as isolation and PCR have been applied for salmonellosis in animal and human beings. Although isolation of agents is golden method for diagnosis of the disease, it is laborious and time consuming. Thus, molecular diagnostics such as PCR have been applied to detect *Salmonella* spp. in feces and pork [1]. However, there are shortcomings such as carryover contaminations and non-specific reactions. Recently, loop-mediated isothermal amplification (LAMP) has been developed and used as an alternative for molecular diagnosis [13]. LAMP is a nucleic acid amplification technique that relies on an auto-cycling strand displacement DNA synthesis under isothermal condition [10, 11, 12, 13]. Although there are some reports for inspections such as eggs and pork [14, 19], there are few reports for developing LAMP for diagnostic purpose. Therefore, LAMP assay was developed for amplifying *invA* gene to detect the *Salmonella spp*. and evaluated for the detection limit and specificity.

Nineteen strains containing 6 *Salmonella* spp. (Cholerasuis, Derby, Enteritidis, Montevideo, Schwarzenground, Typhimurium) and 13 non-*Salmonella* spp. (*Acinetobacter haemolyticus* ATCC17906, *Acinetobacter johnsonii* ATCC17909, *Acinetobacter junii* ATCC17098, *Alcaligenes xylosoxydans* ATCC15173, *Burkholderia cepacia* ATCC25416, *Comamonas testosteroni* ATCC11996, *Enterobacter cloacae* ATCC13047, *Escherichia coli* ATCC25922, *Kocuria rosea* ATCC186, *Micrococcus luteus* ATCC4698, *Ochrobactrum anthropi* ATCC49188, *Serratia marcescens* ATCC13880, *Stenotrophomonas maltophilia* ATCC13637) were used. All strains of *Salmonella* were from field isolates and serogrouped by O and H antibody (Becton, Dickson and Company, USA) except *S.* Choleraesuis (ATCC 10708). The primer sequence is shown in Table 1. *Salmonella* invasion protein gene (*invA*) was targeted for the LAMP assay. Each primer was designed using PrimerExplorer V3 software (Eiken Chemical, Japan). The primers consisted of forward outer (F3), backward outer (B3), forward inner (FIP), backward inner (BIP), loop forward (LF), and loop backward (LB). The reaction was carried out in a 25 μL volume containing 15 μL of Isothermal Master Mix (OptiGene, UK), 50 μM of the primers FIP and BIP, 25 μM of the primers LF and LB, 5 μM of the primers F3 and B3 and 2 μL of DNA template. The amplified signal was detected by Genie (OptiGene) under the 63°C for 30 min. To compare the efficacy of LAMP, conventional PCR was carried out following the previously described method [1]. The PCR reaction was carried out in 20 μL volume containing 10 μL of Takara EmeraldAmp PCR Master Mix (Takara Bio, Japan), 0.5 μM of each primers and 2 μL of DNA template. Amplification was performed as follows: 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 30 sec at 54°C, and 30 sec at 72°C; and a final elongation step of 10 min at 72°C. Bacteria were suspended in 1 mL of distilled water and heated at 100°C for 10 min. After centrifugation at

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Table 1. Oligonucleotide sequences

	Primer	Sequence (5' to 3')	Position[a]
invA	FIP	CCAGTACGCTTCGCCGTTCAGATGAGTATTGATGCCGAT	538-558 (3'-F) 596-613 (5'-R)
	BIP	AGGGAAAGCCAGCTTTACGGGGCAATAGCGTCACCTTT	616-635 (5'-F) 664-681 (3'-R)
	F3	CCCGATTTTCTCTGGATGG	506-524
	B3	ACCGCCAATAAAGTTCACA	699-717
	LF	GCATCCGCATCAATAATACCG	
	LB	TTGACGGTGCGATGAAGT	

[a]Genome position relative to the *invA* gene of *Salmonella* Typhimurium (Accession No. M90846). FIP: forward inner, BIP: backward inner, F3: forward outer, B3: backward outer, LF: loop forward, LB: loop backward.

13,500 × g for 2 min, supernatant (2 μL) were used for the template of LAMP assay. The detection limit was determined by 10-fold dilution using *S.* Typhimurium as reference strain. Briefly, the bacteria were aerobically cultured in tryptic soy broth (Becton, Dickson and Company) at 37°C for 18 h. The broth was 10-fold diluted with saline and boiled for 15 min. After cooling in ice, the broth was centrifuged and supernatant being used for DNA templates. The cell numbers were calculated by standard plate counting method. The specificity was evaluated by performing the LAMP assay using DNA from 12 non-*Salmonella* strains. To see if the LAMP can be working in feces, One milliliter of the broth (2×10^9 colony forming units [CFU]/mL) was centrifuged at 13,200 × g for 5 min and serially diluted from 10^1 to 10^8. The 1 mL of diluents were mixed with 9 mL of feces mixture (feces : saline = 1 : 1). The each diluent was extracted by stool DNA extraction kit (Qiagen, Germany) according to manufacturer's instructions. To enrich the mixture, 1 mL of spiked feces was inoculated into 9 mL of tryptic soy broth and incubated for 18 h. The supernatant was extracted by boiling and subjected to LAMP assay.

To evaluate the specificity, LAMP was applied to the 13 non-*Salmonella* strains. No amplification was observed in the 13 non-*Salmonella* strains. The detection limit of broth dilution was 10^4 CFU/mL in LAMP and conventional PCR, respectively (Table 2). In spiked feces without enrichment, the detection limit was 10^5 CFU/g of feces. However, 10^5 CFU/g of feces was observed in the conventional PCR. Following enrichment, the LAMP detected 10^2 CFU/g of feces but that of PCR was 10^4 CFU/g of feces.

The present study describes the development of S*almonella*-specific LAMP assay targeting the *InvA* gene. The *InvA* gene has been used as a target in many reports [2, 14, 19], whereas *phoP* gene was used for detection of *Salmonella* in milk and minced pork [7]. However, the *phoP* gene is present in most *Enterobacteriaceae* and non-specific amplification might occur when testing samples. By contrast, the *invA* is only known as common gene encoded in *Salmonella* spp. and more specific than the *phoP* gene [6]. LAMP assay have been applied to eggs [2, 14] and pork [19] to detect the *Salmonella* spp. The detection limit of *Salmonella* targeting *rfbJ* and *fim* Y was 10^3 CFU/mL in previous reports [15, 18] which was lower than that of our LAMP assay. However, the reaction time was 60 min in a previous report [15], whereas the reaction time in this study was 30 min. As a result, the detection limit was 10^4 CFU/mL. In addition, LAMP assay in present study possessed 100% exclusivity among 13 non-*Salmonella* strains tested. These results clearly indicate that *InvA* specific LAMP was sensitive and specific assay for the detection of *Salmonella* spp. There are several DNA extraction methods which could influence the results of molecular assay. It is important that DNA extraction should be fast and simple in order to reduce the contamination [17]. Gram-negative bacteria can easily be extracted by boiling method, which was applied to *S.* Enteritidis and *S.* Typhimurium [9, 16]. Conventional PCR was widely used for detecting *Salmonella spp*. from different specimens such as stools and tissues. Until now, conventional PCR has been used for detection of *Salmonella*. However, there are shortcomings such as carry-over contaminations and skilled staffs. Besides, PCR inhibitors in samples reduce the sensitivity of PCR in case of detecting the target gene [4, 20]. The advantage of LAMP is to use simple equipments, such as heating block or water bath and the results of LAMP can be observed by turbidity of reagents. Also, LAMP reaction is carried out in a closed tube which prevents contamination and can be monitored in a real-time manner [16]. The LAMP assay developed in the present study is simple and reliable assay to detect the salmonellosis in pig samples.

Table 2. The detection limit of LAMP in broth dilution, spiked feces and enrichment

Method	Lowest $\log_{10}$CFU/g (mL)	
	LAMP	PCR
Broth dilution	4	4
Spiked feces without enrichment	5	6
Spiked feces with enrichment	2	4

LAMP: loop-mediated isothermal amplification, PCR: polymerase chain reaction.

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