Full Length Research Paper

Development of a loop-mediated isothermal amplification assay for rapid detection of *Streptococcus suis* serotype 2

Aschalew Z. Bekele¹, Yogesh Chander^{1,2}, Jonathan Erber¹, John Tomaszkeski¹ and Sagar M. Goyal^{1*}

¹Veterinary Diagnostic Laboratory, Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN, USA.

²Lucigen Corporation, Middleton, WI, USA.

Received 16 August, 2013; Accepted 28 April, 2014

The development of a loop mediated isothermal amplification (LAMP) assay for the rapid and sensitive detection of *Streptococcus suis*, an economically important swine pathogen is reported. Primers were designed targeting conserved region of the capsular polysaccharide (*cps2J*) gene of *S. suis* serotype 2 and 1/2. The LAMP assay produced reliable amplification in 60 min at isothermal conditions of 60°C. Genomic DNA extracted from *S. suis* serotype 2 and other related bacterial species causing illness in swine were used to optimize and validate the sensitivity and specificity of the assay. Based on a serially diluted genomic DNA of *S. suis*, the LAMP assay was found to be 100-fold more sensitive than the currently used conventional polymerase chain reaction for *S. suis*. These results indicate that LAMP can be an alternate method for the rapid and sensitive detection of *S. suis*, especially in resource-poor countries.

Key words: *cps2J* gene, loop-mediated isothermal amplification, *Streptococcus suis*.

INTRODUCTION

Streptococcus suis is an important bacterial pathogen that causes a wide range of diseases primarily in swine but also in humans and other animal species including cats, dogs, horses, birds, deer and ruminants (Devriese et al., 1990; Hommez et al., 1988; Wertheim et al., 2009). Infection in swine is associated with sudden death, dysentery, septicemia, endocarditis, arthritis, pneumonia, meningitis and abortion causing annual losses of more

than 300 million dollars in the US alone (Staats et al., 1997). The organism is known to inhabit the upper respiratory tract of apparently healthy pigs, which can serve as a source of infection to other pigs (Marois et al., 2007). In humans, *S. suis* causes severe meningitis followed by hearing loss in 50-70% of infected patients (Dupas et al., 1992; Lun et al., 2007). Human cases of *S. suis* infections in North America are limited to persons working

*Corresponding author. E-mail: goyal001@umn.edu. Tel: 612-625-2714.

Table 1. Primers used to amplify the *cps2J* gene of *Streptococcus suis*.

Assay	Primer name*	Sequence (5' - 3')	Reference
LAMP	F3	ATGAATTTTAAACAGCCGATTC	This study
	B3	CGGAAATTATACATTTAGTAAGTGG	This study
	FIP	GTCATAACAAACCATTCACCAATTCACCACGGAACTTTTGAAC	This study
	BIP	AATCGTTTATACCACGTAGAATGCAACCATCAGAATGATGCCA	This study
	BLP	ATGGAGAACAATATCGAAACAGCTC	This study
Conventional PCR	F	GCAGCGTATTCTGTCAAACG	[20]
	R	CCATGGACAGATAAAGATGG	[20]

* F3 = Forward outer; B3 = reverse outer; FIP = forward inner; BIP = reverse inner; BLP = reverse loop; F = forward; R = reverse.

with animals while it is the most frequently diag-nosed cause of streptococcal meningitis in Southeast Asia (Huy et al., 2012; Mai et al., 2008; Suankratay et al., 2004).

Based on the polysaccharide capsular antigens, thirty-five serotypes of *S. suis* (types 1 to 34 and type 1/2) have been described (Staats et al., 1997). On the basis of sequence analysis of the 16S rRNA and *cpn60* genes, serotypes 32 and 34 have now been reclassified as *Streptococcus orisratii* (Hill et al., 2005). A wide variation in virulence has been reported among the *S. suis* serotypes but serotype 2 is considered to be the most virulent worldwide and is most frequently isolated from outbreaks in swine as well as in humans (Staats et al., 1997).

While it is not difficult to cultivate and identify *S. suis* under laboratory conditions, it takes about 2-3 days to correctly identify the organism. Also, bacterial culture has low sensitivity and is often complicated by the presence of multiple microbes and prior use of antimicrobials. Polymerase chain reaction (PCR)-based assays have been developed and used for the detection of *S. suis* (Maroisse et al., 2004). While conventional PCR is a sensitive molecular diagnostic tool, it is time consuming and labor intensive as it requires extensive prior sample preparation to remove PCR inhibitors and post PCR analysis such as gel electrophoresis. Conventional PCR assays also need expensive thermocyclers that may not be accessible to resource poor countries (Fredricks and Relman, 1998).

To overcome the limitations of the conventional PCR assays and to make the technology accessible to resource limited laboratories, loop mediated isothermal amplification (LAMP) assays have been developed and used for molecular detection of pathogens (Hara et al., 2007; Hong et al., 2004; Notomi et al., 2000). LAMP is a simple, rapid, and cost-effective method for pathogen detection as compared to conventional PCR. The LAMP method uses four primers targeting six regions on the target DNA. Additionally, two more primers (loop primers) can be used to accelerate the reaction. The incorporation of colorimetric indicators such as calcein or hydroxynaphthol blue makes it possible to visually detect amplification results (Goto et al., 2007, 2008; Tomita et al.,

2008). Since amplification in LAMP is done at a constant temperature, no specialized thermocycler instrumentation is needed, making it easier to use in resource limited laboratories.

In the current study, the development of a serotype specific LAMP assay for the rapid and sensitive detection of *S. suis* type 2 is reported.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were: *S. suis* serotype 1 DSM 9683, serotype 2 ATCC 43765, *Mycoplasma hyopneumoniae* 232, *Haemophilus parasuis* strain 29775. *S. suis* serotype 7 and 9, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella* spp., *Escherichia coli* were controlled reference strains obtained from the archives of the Minnesota Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN.

DNA extraction

Total genomic DNA was extracted from overnight grown colonies of various bacteria using a commercial kit (PrepMan® Ultra, Applied Biosystems). In brief, colonies were picked with a moist sterile swab and dislodged into 200 µl of phosphate buffered saline (PBS) in a micro-centrifuge tube. The bacterial cells were pelleted by centrifugation, re-suspended in 200 µl of buffer and placed in a boiling water bath for 15 min. The DNA was separated from cell debris by centrifugation at 8,000 g for 2 min and the resulting supernatant was collected and used in subsequent analysis.

Development of LAMP assay

Primers targeting the conserved region of capsular (*cps2J*) gene (GenBank accession number. JN980172) of *S. suis* serotype 2, were designed using a PrimerExplorer V4 software (<http://primerexplorer.jp/e/index.html>). The Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to ensure primer specificity. The sequences of the primers are shown in Table 1. The LAMP reaction was carried out in a heating block and the reaction consisted of 12.5 µl of 2× reaction mix containing 40 mM Tris-HCl, 20 mM KCl, 16 mM MgSO₄, 20 mM [NH₄]₂SO₄, 0.2% Tween 20, 1.6 M betaine, 50 µM of calcein, 2.8 mM each deoxynucleotide triphosphates, 0.8 µM of each internal

Figure 1. Visual detection of loop-mediated isothermal amplification products from 10-fold serial dilutions of *S. suis* DNA template corresponding to 10^0 - 10^{-10} dilutions. a). Observation for color change with naked eye. Positive reactions are indicated by bright yellow color in tubes 1 to 7 (10^0 - 10^{-6}). b). Upon UV irradiation, tubes 1 to 7 (DNA dilutions of 10^0 - 10^{-6}) showed strong fluorescent green color, while tubes 8 and 9 had a weak fluorescent green color. No change of color was observed in tubes 10 and 11 (10^{-9} and 10^{-10}) including lane 12 that served as a no template negative control.

primers (BIP and FIP), 0.4 μ M of each outer primers (B3 and F3) 0.4 μ M of loop (LB), 1 μ l of the Bst DNA polymerase (8 U), 2 μ l of target DNA and appropriate amount of PCR grade water to make a final volume of 25 μ l. Optimum time and temperature for LAMP reaction was determined by performing the reaction at different temperatures ranging from 56 to 64°C (in increments of 2°C) for time periods ranging from 15 to 60 min (in increments of 15 min). The reaction was stopped by increasing the temperature to 80°C for 2 min.

Validation of the LAMP assay

The sensitivity of the LAMP assay was determined using 10-fold serial dilutions of the genomic DNA from *S. suis* serotype 2 (corresponding to 428 to 0.0428 fg/ μ l of DNA). Triplicates of each dilution were tested in three times in three different days. Upon completion of the amplification reaction, the tubes were inspected for color change with unaided eye and for fluorescence upon ultraviolet irradiation. In addition, the LAMP products were separated on a 2% agarose gel and visualized under UV light upon ethidium bromide staining. The specificity of the LAMP assay was tested by using DNA extracts from several related bacterial species causing illnesses in swine as mentioned under the section on bacterial strains.

Conventional PCR

For comparative purposes, the newly developed LAMP assay and the *S. suis* serotype-specific conventional PCR were run in parallel. PCR was performed using commercial PCR Master Mix kit (Qiagen). The reaction mixture contained 10 μ M of each primer, 12.5 μ l of master mix (Qiagen) and 2 μ l of template DNA in a total volume of 25 μ l. The cycling program consisted of an initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, annealing at 65°C for 1 min and extension at 72°C for 3 min with a final extension at 72°C for 10 min. PCR products were separated on

a 1.2 % agarose gel. From positive reactions, an amplicon of ca. 688 bp was obtained (Okuwumabua et al., 2003).

RESULTS

The LAMP assay successfully amplified serotype specific capsular polysaccharide (*cps2J*) gene from *S. suis* after 60 min at isothermal conditions of 60°C (Figure 1a). Initially, the LAMP amplification products were inspected for color change with unaided eye and were later visualized under ultraviolet light for fluorescence. Negative controls without added template retained light yellow color of the pre-incubation reaction mix while positive reactions showed green fluorescence (Figure 1a and 1b). When LAMP products were subjected to 2% agarose gel electrophoresis, typical ladder-like amplification products were detected in positive reactions (Figure 2a).

To determine the sensitivity of the LAMP assay, serial 10-fold dilutions of a *S. suis* DNA extract (original DNA concentration 428 ng/ μ l) were tested. Clear amplification that was detectable by fluorescence and by typical LAMP products on agarose gel was observed in dilutions as high as 10^{-6} with estimated DNA concentration of 0.428 pg/ μ l (Figures 1b and 2a). Although as low as 4.28 fg/ μ l of template DNA showed amplification products on agarose gel, they were not as clear as those with higher template concentrations. Therefore, a reliable detection limit of the LAMP assay was determined to be 0.428 pg/ μ l of template DNA. The conventional PCR that was run in parallel with LAMP had a detection limit of 42.8 pg/ μ l of template DNA (Figure 2b), which were 100 fold less than the newly developed LAMP assay. To ascertain the specificity of the

Figure 2. Agarose gel electrophoresis of amplification products. a). Typical ladder-like pattern, a characteristic feature of loop-mediated isothermal amplification (LAMP), can be seen. Lanes 1 - 9 depict LAMP products from 10-fold serial dilutions of *S. suis* DNA template corresponding to 10^0 - 10^{-8} dilutions. No product was detected in lane 10 and 11 (10^{-9} and 10^{-10}). Lane 12 was a no template negative control. b). Conventional polymerase chain reaction products showing single bands in lanes 1-5 (dilutions 10^0 - 10^{-4}).

assay, genomic DNA from pure cultures of other bacterial species known to colonize swine respiratory tract were tested and found negative. The newly developed LAMP did not cross react with *S. suis* serotypes 1, 7 and 9.

DISCUSSION

S. suis is one of the most economically important swine pathogen causing heavy losses to the swine industry (King et al., 2001). Recent reports indicate that *S. suis* is an emerging zoonotic pathogen that poses serious occupational health hazard to persons working with pigs (Tara et al., 2008). Serotype 2 is known to be the most virulent and most frequently isolated serotype from *S. suis* outbreaks (Staats et al., 1997). Multiplex PCR assays have been developed for the detection of several serotypes of *S. suis* (Okuwumabua et al., 2003).

In the present study, we successfully developed and validated a LAMP assay for sensitive and specific detection of *S. suis* serotype 2. Primers were designed based on the sequences of *S. suis* *cps2J* gene known to be specific to *S. suis* serotype 2 and the LAMP assay successfully amplified this gene and yielded reliable amplification products in 60 min (Figure 2a).

One of the characteristic features of LAMP is its ability to amplify nucleic acids under isothermal conditions (Notomi et al., 2000) allowing the use of simple and inexpensive equipment. As compared to the currently employed conventional PCR used to detect *S. suis*, the newly developed LAMP assay was 100-fold more sensitive. The use of two to three primer pairs designed to target six to eight sequences makes LAMP a highly sensitive and specific assay as shown in the current study as well as in previous reports (Das et al., 2012; Venkatesan

et al., 2012).

The robustness of LAMP is also ascribed to the formation of large amounts of pyrophosphate ion that enables visual monitoring of amplification products under natural light or UV irradiation (Mori et al., 2001). In the newly developed *S. suis* LAMP assay, calcein was used as a fluorescence detection reagent; strong green fluorescence was visually detected from positive reactions within 60 min. The degree of fluorescence corresponded to typical ladder-like LAMP amplification products upon agarose gel electrophoresis (Figures 1b and 2a).

Although, not all *S. suis* serotypes were tested, the LAMP assay tested negative for *S. suis* serotypes 1, 7 and 9 suggesting that the new LAMP is specific for serotype 2 of *S. suis*. This is not surprising because specific primers showing 100% sequence identity with the *cps2J* gene sequence of *S. suis* serotype 2 were used. In contrast to the 33 *S. suis* serotypes, the *cps2J* gene in *S. suis* serotype 2 and serotype 1/2 has been reported to have high sequence homology (Zhang et al., 2013). Since we did not test serotype 1/2, we cannot tell if our LAMP assay could discriminate between these two strains. Also, no cross reaction was observed with other bacterial species known to colonize swine respiratory tract. These results indicate that LAMP can be used as a rapid and diagnostic tool for the detection of *S. suis*. This study is based on using well characterized bacterial isolates. Further studies are necessary to determine if this assay will be applicable to samples collected from the field.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

- Das A, Babiuk S, McIntosh MT (2012). Development of a Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Capripoxviruses. *J. Clin. Microbiol.* 50:1613-1620.
- Devriese LA, Sustronck B, Maenhout T, Haesebrouck F (1990). *Streptococcus suis* meningitis in a horse. *Vet. Rec.* 127: 68.
- Dupas D, Vignon M, Geraut C (1992). *Streptococcus suis* meningitis. A severe non compensated occupational disease. *J. Occup. Med.* 34:1102-1105.
- Fredricks DN, Relman, DA (1998). Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanetholesulfonate. *J. Clin. Microbiol.* 36:2810-2816.
- Goto M, Hayashidani H, Takatori K, Kudo YH (2007). Rapid detection of enterotoxigenic *Staphylococcus aureus* harboring genes for four classical enterotoxins, SEA, SEB, SEC and SED, by loop-mediated isothermal amplification assay. *Lett. Appl. Microbiol.* 45:100-107.
- Goto M, Honda E, Ogura A, Nomoto A, Hanaki K (2008). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques* 46:167-172.
- Hara KY, Nemoto J, Ohtsuka K, Segawa Y, Takatori K, Kojima T, Ikeda M (2007). Sensitive and rapid detection of vero toxin producing *Escherichia coli* using loop-mediated isothermal amplification. *J. Med. Microbiol.* 56:398-406.
- Hill JE, Gottschalk M, Brousseau R, Harel J, Hemmingsen SM Han GS (2005). Biochemical analysis, *cpn60* and 16S rRNA sequence data indicate that *St reptococcus suis* serotypes 32 and 34, isolated from pigs are *Streptococcus orisratti*. *Vet. Microbiol.* 107: 63-69.
- Hommez J, Wullepit J, Cassimon P, Castryck F, Ceyssens K, Devriese LA (1988). *Streptococcus suis* and other *streptococcal* species as a cause of extra mammary infection in ruminants. *Vet. Rec.* 123:626-627.
- Hong TC, Mai QL, Cuong DV, Parida M, Minekawa H, Notomi T, Hasebe F, Morita K (2004). Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. *J. Clin. Microbiol.* 42:1956-1961.
- Huy NT, Hang le TT, Boamah D, Lan NT, Van Thanh P, Watanabe K, Huong VT, Kikuchi M, Ariyoshi K, Morita K, Hirayama K (2012). Development of a single-tube loop-mediated isothermal amplification assay for detection of four pathogens of bacterial meningitis. *FEMS. Microbiol. Lett.* 337: 25-30.
- King SJ, Heath PJ, Luque I, Tarradas C, Dowson CG, Whatmore AM (2001). Distribution and genetic diversity of suilysin in *Streptococcus suis* isolated from different diseases of pigs and characterization of the genetic basis of suilysin absence. *Infect. Immun.* 69:7572-7582.
- Lun ZR, Wang QP, Chen XG, Li AX Zhu XQ (2007). *Streptococcus suis*: an emerging zoonotic pathogen. *Lancet Infect. Dis.* 7:201-9.
- Mai NT, Hoa NT, Nga TV, Linh LD, Chau TT, Sinh DX (2008). *Streptococcus suis* meningitis in adults in Vietnam. *Clin. Infect. Dis.* 46:659-67.
- Marois C, Bougeard S, Gottschalk M, Kobisch M (2004). Multiplex PCR assay for detection of *Streptococcus suis* species and sero-types 2 and 1/2 in tonsils of live and dead pigs. *J. Clin. Microbiol.* 42:3169-3175.
- Marois C, Le Devendec L, Gottschalk M, Kobisch M (2007). Detection and molecular typing of *Streptococcus suis* in tonsils from live pigs in France. *Can. J. Vet. Res.* 71:14-22.
- Mori Y, Nagamine K, Tomita N, Notomi T (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Bioph. Res. Co.* 289:150-154.
- 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.i-vii.
- Okwumabua O, O'Connor M, Shull E (2003). A polymerase chain reaction (PCR) assay specific for *Streptococcus suis* based on the gene encoding the glutamate dehydrogenase. *FEMS. Microbiol. Lett.* 218: 79-84.
- Staats JJ, Feder I, Okwumabua O, Chengappa MM (1997). *Streptococcus suis*: Past and present. *Vet. Res. Commun.* 21:381-407.
- Suankratay C, Intalapaporn P, Nunthapisud P, Arunyingmongkol K, Wilde H (2004). *Streptococcus suis* meningitis in Thailand. *Southeast Asian J. Trop. Med. Public Health.* 35: 868-876.
- Tara CS, Ana WC, Brenda B, Kendall PM, Gregory CG (2008). Occupational exposure to *Streptococcus suis* among US swine workers. *Emerg. Infect. Dis.* 14:1925-1927.
- Tomita N, Mori Y, Kanda H, Notomi T (2008). Loop mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat. Protoc.* 3: 877-882.
- Venkatesan G, Bhanuprakash V, Balamurugan V, Singh RK, Pandey AB (2012). Development of loop-mediated isothermal amplification assay for specific and rapid detection of camelpox virus in clinical samples. *J. Virol. Methods* 183: 34-9.
- Wertheim HF, Nghia HD, Taylor W, Schultz C (2009). *Streptococcus suis*: an emerging human pathogen. *Clin. Infect. Dis.* 48: 617-625.
- Zhang J, Zhu J, Ren H, Zhu S, Zhao P, Zhang F, Qi Z. (2013). Rapid Visual Detection of Highly Pathogenic *Streptococcus suis* Serotype 2 Isolates by Use of Loop-Mediated Isothermal Amplification. *J. Clin. Microbiol.* 51(10):3250-3256.