DOI: 10.55415/deep-2022-0027.v1

Rapid and visual detection of pathogenic Streptococcus suis type 2 based on recombinase polymerase amplification assay coupled with lateral flow test

Yong Qi [1, #], Wei Li [2, #], Xiaoling Li [3], Wanpeng Shen [1], Ruichen Lv [1], Nianhong Lu [1], Jiameng Li [1], Susu Zhuang [2], Yingjia Xu [2], Qiyuan Gui [1], Hongbing Shen [2, *], Yuexi Li [1, 2, 3, *]

1 Huadong Research Institute for Medicine and Biotechniques, 210002, #293 Zhongshandonglu, Nanjing, Jiangsu, China

2 Nanjing Medical University, 211166, 101 Longmian Avenue, Jiangning District, Nanjing, Jiangsu, China

3 China Pharmaceutical University, 210009, #24 Tongjiaxiang, Nanjing, Jiangsu, China

# These authors contributed equally in this research

* Corresponding author addresses: liyxi2007@126.com for Y. L. and hbshen@njmu.edu.cn for H. S.

DOI: 10.55415/deep-2022-0027.v1

Abstract

Objective *Streptococcus suis* serotype 2 (SS2) is an important zoonotic pathogen causing serious diseases and even deaths in pigs and humans. Public health events as well as economic losses caused by SS2 have aroused widespread concerns. Due to unavailability of vaccines, development of rapid detection methods for timely diagnosis of SS2 infection or contaminated products, and normally monitoring its prevalence in susceptible animals and population, will be helpful for prevention and control of SS2 infections.

Methods Several sets of primers and one probe for recombinase polymerase amplification (RPA) assay targeting *cpsJ2* gene were designed and synthesized. Lateral flow (LF) test was introduced to produce visual results combined with RPA. Primers with high amplification efficiency were screened and the reaction system was optimized. Indicators of detection effectiveness were evaluated.

Results The established method had a detection limit of 100 copies/reaction in recognizing SS2 rather than other organisms. The sensitivity was 100% evaluated by infected animal samples. The detection can be completed in 20 min with requirement of a constant temperature equipment only.

Conclusion The established rapid, visual, sensitive, and specific RPA-LF assay showed superior detection performance and is expected to be widely applied to fight SS2 infection in resource-limited areas.

DOI: 10.55415/deep-2022-0027.v1

Keywords： *Streptococcus suis* serotype 2; recombinase polymerase amplification; lateral flow test; detection; *cpsJ2* gene.

Introduction

Streptococcus suis is an important zoonotic pathogen that can cause meningitis, pneumonia, endocarditis, arthritis and septicemia in pigs, as well as meningitis, endocarditis, septicemia, permanent deafness, toxic shock-like syndrome, and even death in humans [1, 2] . At least 29 serotypes have been identified, within which, *S. suis* serotype 2 (SS2) is the most pathogenic and widespread one [2]. SS2 has imposed serious threat on public health as well as breeding industry and has aroused widespread concerns [2]. In 1998 and 2005, SS2 caused large-scale public health events by infecting humans and causing deaths in Jiangsu province and Sichuan Province in China, respectively [3, 4]. Nowadays, due to unavailable of vaccines, development of rapid detection methods for timely diagnosis of disease or contaminated products, and normally monitoring the prevalence of SS2 in susceptible animals and population, will be helpful for prevention and control of SS2 caused public health events.

Now the detection of SS2 or diagnosis of its causing diseases relies on microbial culture, serologic tests and molecular methods [5]. The microbial culture is labor intensive and time consuming, and serological tests are frequently negative during the acute phase of the disease because antibodies develop 1-2 weeks after infection [5]. Molecular tests such as conventional polymerase chain reaction (PCR) and nested

DOI: 10.55415/deep-2022-0027.v1

PCR are often used to confirm the species coupling with sequencing, which is not convenient and time-consuming [6]. Real-time quantitative PCR (RT-qPCR) has been proved to be more useful in dealing with endemics such as the ongoing SARS-CoV-2 prevalence. However, it needs an expensive fluorescence quantitative machine, which is not suitable for resource-limited areas [7].

Nowadays, recombinase polymerase amplification (RPA) assay, an isothermal amplification method with a short amplification time of ≤20 min [8] provides a solution for detection of SS2 in places with limited conditions. Here in the present study, we aimed to develop a simple, fast, sensitive and specific method for detection of SS2 by combination of RPA with a lateral flow (LF) test.

Materials and Methods

Ethics statement and sample preparation

The genomic DNA of SS2 was extracted from strain 05ZYH33, using QIAamp Blood and Tissue Mini DNA kit (Qiagen, CA, USA) according to the manufacturer's instructions. The genomic DNA of control pathogens used in our previous study [9] were also prepared in the present study, with concentrations from 10^5 copies/μL to 10^8 copies/μL.

In addition, DNA from SS2-infected mouse spleens were also prepared. Briefly, 4 to 6-week-old female C3H/HeN mice were infected with sublethal dose of purified SS2 (strain 05ZYH33). Mice were sacrificed and spleens were separated on day 3

DOI: 10.55415/deep-2022-0027.v1

post infection. Spleen from 1 uninfected mouse was used as control. DNA in 10 mg of each spleen were purified using a QIAamp Blood and Tissue Mini DNA kit (Qiagen). The concentrations of the genomic DNA of SS2 (copies/μL) in these samples were determined by RT-qPCR targeting *cps2J* gene as described previously [5, 7].

Nasopharyngeal swab samples were collected from healthy volunteers and DNA was extracted as above-described.

The animal experiments were approved by the Ethics Committee of Huadong Research Institute for Medicine and Biotechniques and carried out in accordance with the approved guidelines. The use of human nasopharyngeal swab samples was approved by the committee and consent form was signed.

Plasmid, primers, and probe design and synthesis

The SS2 type-specific gene *cps2J* was used as the target in the present study. The target sequence was amplified from the genomic DNA using a conventional PCR using a Premix Ex Taq Version 2.0 kit (Takara, Beijing, China). The primer pairs were indicated in Table 1 with an annealing temperature of 55°C. The amplified product as well as plasmid pMD®18T vector (Takara) were linked as per the manufacturer's instructions. The recombinant plasmid *cps2J*-pMD18T was transformed into *Escherichia coli* DH5α competent cells, and further purified from cultured transformed *E. coli* cells using a QIAGEN Plasmid Mini Kit (Qiagen). The existence of target sequence in the recombinant plasmid was confirmed by PCR as

DOI: 10.55415/deep-2022-0027.v1

mentioned above. The concentration of the plasmid was determined using Nanodrop One, and the copies/ μL of plasmid was calculated.

Three forward and 2 reverse primers and 1 probe for RPA were designed as previously described [10] and synthesized by Genscript company (Nanjing, China). The reverse primers and probe were labeled as indicated in Table 1.

Table 1 Primer and probe sequences used in the study.

Methods	Primers and probe	Sequences（5'-3'）
PCR	S.s-F	ATGGAAAAAGTCAGCATTATTGT
	S.s-R	TTAATCATTATTTTTTCTTCCCTA
RPA	F1	CAAATGGTGGTGTTTCAAACGCAAGGAATT
	F2	TTGACGGCAACATTGTTGAGTCCTTATACAC
	F3	ATGTTTGGAATACGCAGAGCAAGATGGTAG
	R1	Biotin-CATTTCCTAAGTCTCGCACCTCTTTTATCT
	R2	Biotin-TTTGACACTTTTGCAGCTCAGATTCTTGAT
	Probe	FAM-GAGAATGATAGTGATTTGTCGGGAGGGTTA-[THF]-TTGCTACTTTTGATG-PO4

FAM, carboxyfluorescein; THF, tetrahydrofuran.

Reaction condition optimization

DOI: 10.55415/deep-2022-0027.v1

For best primer pair screening, each forward primer and reverse primer were combined in a recommended RPA reaction system using a TwistAmp® RPA nfo kit (TwistDx Limited). Briefly, 2.1 μL of each primer (10 μM), 0.6 μL of probe (10 μM), and 1 μL of template (plasmid *cps2J*-pMD18T or pMD18T at a concentration of 1×10^4 copies/μL), were used in the mixture containing various enzymes and buffer provided in the kit. After 20 min of incubation at 37°C, 5 μL of the amplified products diluted in 95 μL of Tris-buffered saline were used to immerse the sample pad of a Milenia Genline Hybridetect-1 (MGH) strip (Milenia Biotec GmbH, Gieben, Germany) for 3 to 5 min for development. Both test line (T line) and control line (C line) in the strips developing indicated a positive result with amplified product labeled with both FAM and biotin existing and only C line developing indicated a negative result.

To determine the best concentrations of primers and probe, a variety of concentrations of both reverse primer and probe were used to conduct the RPA-LF method as above-described. To determine the best amplification time, amplification products obtained at various amplification time were used for strip development.

Detection limitation evaluation

To evaluate the limit of detection (LOD) of the optimized RPA-LF method in detecting genomic DNA, a serial dilutions of genomic DNA of SS2 from 10^6 to 10^0 copies/μL were used as templates. The LOD was determined to be the concentration

DOI: 10.55415/deep-2022-0027.v1

giving the highest dilution of a positive test result. The evaluation was done in duplicate.

Specificity and sensitivity evaluation

To evaluate the specificity of the method, genomic DNA of *Rickettsia rickettsii*, *Coxiella burnetii*, *Orientia tsutsugamushi*, *Rickettsia heilongjiangensis*, *Rickettsia sibirica*, *Rickettsia prowazekii*, *Staphylococcus aureus*, and *Mycoplasma pneumoniae* were mixed with equal volume of DNA of healthy volunteer and used as templates to conduct the optimized RPA-LF method. DNA of healthy volunteer only was also used as a negative control. All the reactions were done in duplicate.

To evaluate the method's sensitivity, DNA from SS2-infected mouse spleens, with SS2 DNA concentrations around 10^3 to 10^4 copies/μL determined by RT-qPCR, were used as templates to conduct the optimized RPA-LF method.

Results

Construction of positive plasmid

The amplified partial sequence of *cps2j* with 999 bp was successfully linked to pMD18T plasmid to construct a recombinant plasmid *cps2j*-pMD18T, and as shown in Figure 1, the size of PCR amplified product from this recombinant plasmid accorded with the expected size.

DOI: 10.55415/deep-2022-0027.v1

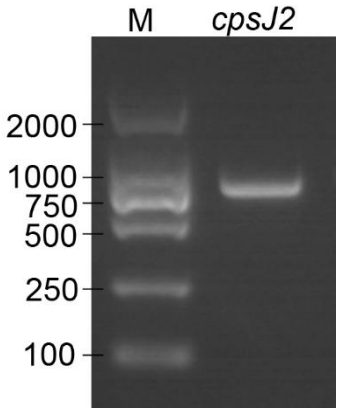

Figure 1 Agarose gel electrophoresis analysis of PCR product amplified from recombinant plasmid *cpsJ2*-pMD18T. M, DNA marker. The size (bp) was indicated on the left.

Primers and probe screening

The best group of forward primer and reverse primer combination was screened. As shown in Figure 2A, both groups of primers F3&R1 and F3&R2 combination, with intensive dark bands on the T lines of the experimental strips (with *cps2j*-pMD18T as template) and no bands on the T lines of the control strips (with pMD18T as template), were considered modest combinations of primers. In the present study, group F3&R1 was used for further analysis.

DOI: 10.55415/deep-2022-0027.v1

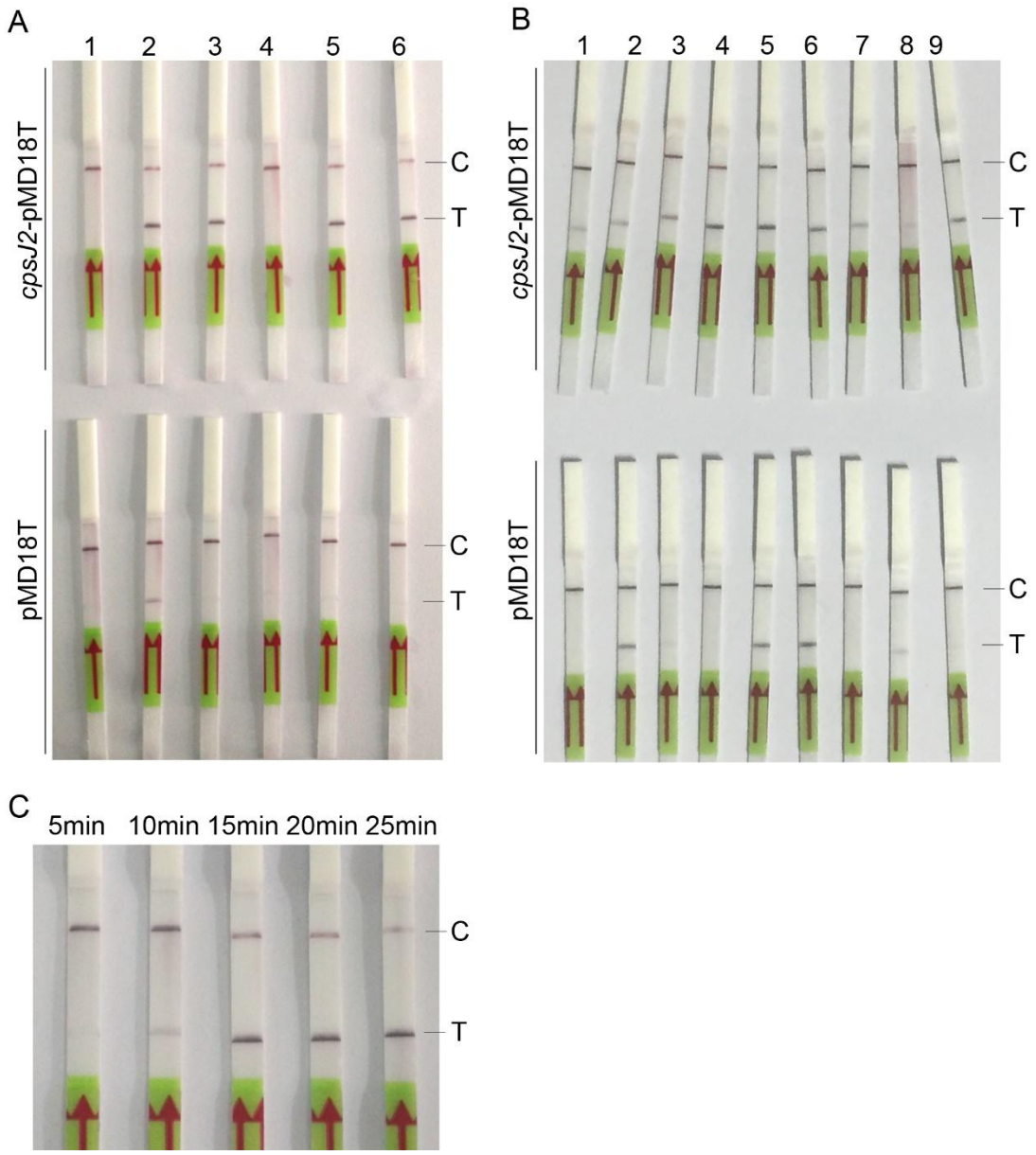

Figure 2 Optimization of the RPA-LF assay. A, screening of the best primers from 6 groups of primer combinations. Primer combinations from 1 to 6 in (A) were indicated in Table 2 and recombinant plasmid *cpsJ2*-pMD18T and plasmid pMD18T were used as experiment and control template, respectively. B, screening of the best concentrations of reverse primer R1 and probe. Concentration combinations from 1 to 9 in (B) were indicated in Table 3. C, optimization of the amplification time. The amplification time was as indicated. C, control line; T, test line.

DOI: 10.55415/deep-2022-0027.v1

Table 2 Performances of various combinations of primers in RPA-LF assay.

Group No.	Combinations	Results
1	F1 & R1	-
2	F2 & R1	-
3	F3 & R1	++
4	F1 & R2	-
5	F2 & R2	+
6	F3 & R2	++

-, bad result; +, good result; ++, excellent result.

Table 3 Results of various of concentrations of reverse primer R1 and probe in the RPA-LF assay.

Group No.	Concentrations (R1&probe, μM)	Results
1	10 & 10	+
2	5 & 10	-
3	2.5 & 10	+
4	10 & 5	++
5	5 & 5	-

DOI: 10.55415/deep-2022-0027.v1

6	2.5 & 5	-
7	10 & 2.5	+
8	5 & 2.5	-
9	2.5 & 2.5	+

-, bad result; +, good result; ++, excellent result.

Optimization of RPA-LF detection method

Concentrations of primer and probe as well as the amplification time were optimized. As shown in Figure 2B, group 4, with 10 μM of R1 combined with 5 μM of probe performed the best result. In Figure 2C, an amplification time of 15 min led to similar result with longer time of amplification, and was determined to be used in the optimized method.

Detection limit

The LOD of RPA-LF method was evaluated using genomic DNA of SS2. As shown in Figure 3A, the LOD of the RPA-LF in detection of SS2 was 100 copies/reaction or lower considering that concentrations between 10 to 100 copies/reaction were not further evaluated.

DOI: 10.55415/deep-2022-0027.v1

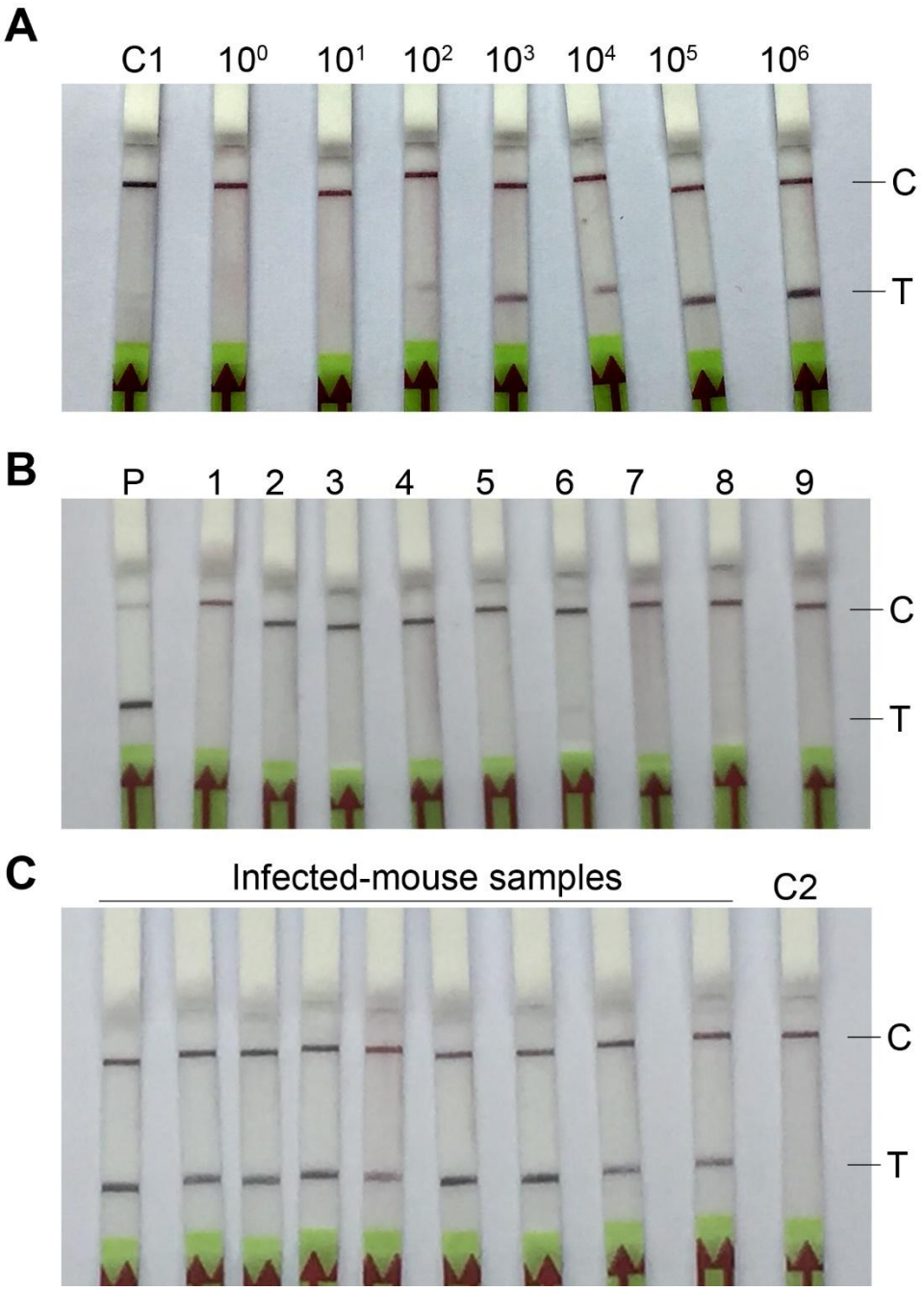

Figure 3 Detection limit (A), specificity (B), and sensitivity evaluation of the method. The concentrations of various dilutions of SS2 genomic DNA were indicated in (A). Strips 1 to 9 in (B) used DNA of healthy volunteer mixed with genomic DNA of *Rickettsia heilongjiangensis*, *Rickettsia sibirica*, *Staphylococcus aureus*, *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Orientia tsutsugamushi*, *Mycoplasma pneumoniae*, *Coxiella burnetii*, and distilled water as templates, respectively. P, positive control

DOI: 10.55415/deep-2022-0027.v1

using healthy volunteer DNA mixed with genomic DNA of SS2 as template; C1, negative control using distilled water as template; C2, negative control using DNA extracted from uninfected mouse spleen as template. The other 9 strips in (C) used DNA extracted from 9 SS2-infected mouse spleens as templates.

Specificity and sensitivity evaluation

Genomic DNA of several unrelated bacteria were used to evaluate the specificity. As shown in Figure 3B, the T lines didn't develop when detecting DNA of *R. heilongjiangensis*, *R. sibirica*, *O. tsutsugamushi*, *C. burnetii*, *St. aureus*, *R. rickettsii*, *M. pneumoniae* and DNA from human nasopharyngeal swab sample, while the T line did develop in detecting DNA of SS2.

The sensitivity was evaluated with SS2-infected mouse samples. As shown in Figure 3C, all the 9 SS2-infected mouse samples were recognized by the RPA-LF method, indicating its sensitivity was 100% in detecting mouse samples.

Discussion

SS2 is an important zoonotic pathogen that not only causes serious economic losses in the pork industry by causing swine streptococcosis, but also imposes threats on public health due to its rapid spread and high mortality rates, especially in developing countries [2, 5, 11-13]. In this sense, development of rapid detection method suitable for use in source-limited areas is of great importance to control this zoonosis in these epidemic areas.

DOI: 10.55415/deep-2022-0027.v1

In recent years, isothermal amplification methods have been developed quickly due to their fast amplification, convenient operation and low instrument requirement compared with the conventional PCR or RT-qPCR. Zhang et al. developed loop-mediated isothermal amplification (LAMP)-based detection assays that could complete the visual detection in 48 minutes with naked eyes [5]. Recently, Jiang et al. developed real-time RPA for detection SS2 within 20 min, while a fluorescence detection instrument was still in need [14]. Here in the present study, the developed RPA-LF method seems to conquer the disadvantages of both methods mentioned above, by completing the detection within 20 min without any expensive or sophisticated instrument like fluorescence detection machine. The results can be judged by naked eyes. Compared with the conventional molecular methods, the established method is more suitable for use in the field and in some resource-limited medical units.

Limitations also exist in the present study. Due to resource constraints, only limited genomic DNA of unrelated pathogens were collected to evaluate the specificity, and no clinical samples were available in the present study. Also, the method is supposed to be unable to distinguish type 2 and type 1/2, for the target *cps2J* gene is shared by both types, which also existed in the former developed methods targeting this gene [5, 14]. Still, more genomic DNA from various phylogenic-related and similar symptom-causing pathogens as well as clinical

DOI: 10.55415/deep-2022-0027.v1

samples are needed to evaluate the specificity and sensitivity of the established method.

Conclusion

In conclusion, this study is the first to establish rapid and visual detection method based on RPA and LF test for SS2. The method, with high sensitivity and specificity and without need of sophisticated instrument, has potential for use in the field and resource-limited areas. This work provides a promising and alternative tool for future application in clinical detection and surveillance of SS2 infections.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by grants from Medical Science and Technology Project (19SWAQ04 and 2020QN06357).

References

1. Hlebowicz M, Jakubowski P, Smiatacz T. Streptococcus suis Meningitis: Epidemiology, Clinical Presentation and Treatment. Vector borne and zoonotic diseases. 2019;19(8):557-62; doi: 10.1089/vbz.2018.2399.

2. Xia X, Qin W, Zhu H, Wang X, Jiang J, Hu J. How Streptococcus suis serotype 2 attempts to avoid attack by host immune defenses. J Microbiol Immunol Infect. 2019;52(4):516-25; doi: 10.1016/j.jmii.2019.03.003.

3. Tang J, Wang C, Feng Y, Yang W, Song H, Chen Z, et al. Streptococcal toxic shock syndrome caused by Streptococcus suis serotype 2. PLoS Med. 2006;3(5):e151; doi: 10.1371/journal.pmed.0030151.

DOI: 10.55415/deep-2022-0027.v1

4. Feng Y, Zhang H, Wu Z, Wang S, Cao M, Hu D, et al. Streptococcus suis infection: an emerging/reemerging challenge of bacterial infectious diseases? Virulence. 2014;5(4):477-97; doi: 10.4161/viru.28595.

5. Zhang J, Zhu J, Ren H, Zhu S, Zhao P, Zhang F, et al. Rapid visual detection of highly pathogenic Streptococcus suis serotype 2 isolates by use of loop-mediated isothermal amplification. J Clin Microbiol. 2013;51(10):3250-6; doi: 10.1128/jcm.01183-13.

6. Marois C, Bougeard S, Gottschalk M, Kobisch M. Multiplex PCR assay for detection of Streptococcus suis species and serotypes 2 and 1/2 in tonsils of live and dead pigs. J Clin Microbiol. 2004;42(7):3169-75; doi: 10.1128/jcm.42.7.3169-3175.2004.

7. Nga TV, Nghia HD, Tu le TP, Diep TS, Mai NT, Chau TT, et al. Real-time PCR for detection of Streptococcus suis serotype 2 in cerebrospinal fluid of human patients with meningitis. Diagn Microbiol Infect Dis. 2011;70(4):461-7; doi: 10.1016/j.diagmicrobio.2010.12.015.

8. Piepenburg O, Williams C, Stemple D, Armes N. DNA detection using recombination proteins. PLoS biology. 2006;4(7):e204.

9. Y Q, Y S, J R, W S, Q Y, X L, et al. Development of a rapid and visual detection method for Rickettsia rickettsii combining recombinase polymerase assay with lateral flow test. PloS one. 2018;13(11):e0207811; doi: 10.1371/journal.pone.0207811.

10. Qi Y, Yin Q, Shao Y, Li S, Chen H, Shen W, et al. Rapid and Visual Detection of Coxiella burnetii Using Recombinase Polymerase Amplification Combined with Lateral Flow Strips. Biomed Res Int. 2018;2018:6417354; doi: 10.1155/2018/6417354.

11. Boyle B, Vaillancourt K, Bonifait L, Charette SJ, Gottschalk M, Grenier D. Genome sequence of the swine pathogen Streptococcus suis serotype 2 strain S735. J Bacteriol. 2012;194(22):6343-4; doi: 10.1128/jb.01559-12.

12. Mai NT, Hoa NT, Nga TV, Linh le D, Chau TT, Sinh DX, et al. Streptococcus suis meningitis in adults in Vietnam. Clin Infect Dis. 2008;46(5):659-67; doi: 10.1086/527385.

13. Takeuchi D, Kerdsin A, Pienpringam A, Loetthong P, Samerchea S, Luangsuk P, et al. Population-based study of Streptococcus suis infection in humans in Phayao Province in northern Thailand. PLoS One. 2012;7(2):e31265; doi: 10.1371/journal.pone.0031265.

14. Jiang X, Zhu L, Zhan D. Development of a recombinase polymerase amplification assay for rapid detection of Streptococcus suis type 2 in

DOI: 10.55415/deep-2022-0027.v1

nasopharyngeal swab samples. Diagn Microbiol Infect Dis. 2022;102(2):115594; doi: 10.1016/j.diagmicrobio.2021.115594.