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ORIGINAL ARTICLE

Rapid Visual Detection of Pathogenic *Streptococcus suis* Type 2 through a 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,*] and Yuexi Li[1,2,3,*]

Abstract

Objective: *Streptococcus suis* serotype 2 (SS2) is an important zoonotic pathogen causing serious disease and even death in pigs and humans. Public health events and economic losses caused by SS2 have prompted widespread concern. Because of the unavailability of vaccines, the development of rapid detection methods for timely diagnosis of SS2 infection or contaminated products, and monitoring of its prevalence in susceptible animals and populations, is required to aid in the prevention and control of SS2 infections.

Methods: Several sets of primers and one probe for a recombinase polymerase amplification (RPA) assay targeting the *cpsJ2* gene were designed and synthesized. Lateral flow (LF) tests in combination with RPA were used to provide visual results. 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 for recognizing SS2 rather than other organisms. The sensitivity was 100%, as evaluated in infected animal samples. The detection could be completed within 20 min and required only constant temperature equipment.

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.

Key words: *Streptococcus suis* serotype 2, recombinase polymerase amplification, lateral flow test, detection, *cpsJ2* gene

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Jun Jiao, Beijing institute of Microbiology and Epidemiology

Reviewed by:
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[#]These authors contributed equally in this research.
***Corresponding authors:**
E-mail: liyxi2007@126.com (YL); hbshen@njmu.edu.cn (HS)

[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

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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, among which *S. suis* serotype 2 (SS2) is the most pathogenic and widespread [2]. SS2 has posed a serious threat to public health as well as the swine breeding industry and has prompted widespread concern [2]. In 1998 and 2005, SS2 caused large-scale

public health events, infecting humans and causing many deaths in Jiangsu province and Sichuan Province in China [3,4]. Currently, because of the lack of available vaccines, the development of rapid detection methods for timely diagnosis of disease or contaminated products, and monitoring of the prevalence of SS2 in susceptible animals and populations, is crucial to prevent and control public health events caused by SS2.

SS2 detection and diagnosis of disease rely on microbial culture, serologic tests and molecular methods [5]. Microbial culturing is labor intensive and time consuming; moreover, serological tests are frequently negative during the acute phase of the disease, because antibodies do not develop until 1–2 weeks after infection [5]. Molecular tests such as conventional polymerase chain reaction (PCR) and nested PCR, coupled with sequencing, are often used to confirm the species; however, this process is inconvenient and time-consuming [6]. Real-time quantitative PCR (RT-qPCR) has been demonstrated to be more useful for detecting endemic pathogens such as SARS-CoV-2. However, this method requires an expensive fluorescence quantitative thermocycler and therefore is not suitable for use in resource-limited areas [7].

Currently, the 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 settings with limited resources. Here, we aimed to develop a simple, fast, sensitive and specific method for detection of SS2 through the combination of RPA and a lateral flow (LF) test.

MATERIALS AND METHODS

Ethics statement and sample preparation

The genomic DNA of SS2 was extracted from strain 05ZYH33, with a 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] was also prepared, with concentrations from 10^5 copies/μL to 10^8 copies/μL.

In addition, DNA from SS2-infected mouse spleens was prepared. Briefly, 4- to 6-week-old female C3H/HeN mice were infected with a sublethal dose of purified SS2 (strain 05ZYH33). The mice were sacrificed, and their spleens were separated on day 3 post infection. The spleen from an uninfected mouse was used as control. DNA from 10 mg of each spleen was purified with a QIAamp Blood and Tissue Mini DNA kit (Qiagen). The concentrations of genomic DNA of SS2 (copies/μL) in these samples were determined with RT-qPCR targeting the *cps2J* gene, as described previously [5,7].

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

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

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 genomic DNA with conventional PCR with a Premix Ex Taq Version 2.0 kit (Takara, Beijing, China). The primer pairs (Table 1) had an annealing temperature of 55°C. The amplified product and the pMD®18T vector (Takara) were ligated according to 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 with a QIAGEN Plasmid Mini Kit (Qiagen). The existence of the target sequence in the recombinant plasmid was confirmed by PCR as described above. The plasmid concentration was determined with a NanoDrop One spectrophotometer, and the number of plasmid copies per μL was calculated.

Three forward and two reverse primers, and one probe for RPA were designed as previously described [10] and

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	TTAATCATTATTTTTTTCTTCCCTA
RPA	F1	CAAATGGTGGTGTTTCAAACGCAAGGAATT
	F2	TTGACGGCAACATTGTTGAGTCCTTATACAC
	F3	ATGTTTGGAATACGCAGAGCAAGATGGTAG
	R1	Biotin-CATTTCCTAAGTCTCGCACCTCTTTTATCT
	R2	Biotin-TTTGACACTTTTGCAGCTCAGATTCTTGAT
	Probe	FAM-GAGAATGATAGTGATTTGTCGGGAGGGTTA-[THF]-TTGCTACTTTTGATG-PO4

FAM, carboxyfluorescein; THF, tetrahydrofuran.

synthesized by the Genscript company (Nanjing, China). The reverse primers and probe were labeled as indicated in Table 1.

Reaction condition optimization

For optimal primer pair screening, each forward primer and reverse primer was combined in the recommended RPA reaction system with 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 the various enzymes and buffers 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 was used to immerse the sample pad of a Milenia Genline Hybridetect-1 (MGH) strip (Milenia Biotec GmbH, Gieben, Germany) for 3–5 min for development. Development of both the test (T) line and control (C) line in the strips indicated a positive result with amplified product labeled with both FAM and biotin, whereas development of only the C line indicated a negative result.

To determine the best primer and probe concentrations, we tested a variety of concentrations of reverse primers and probes with the RPA-LF method as described above. To determine the best amplification time, we used the amplification products obtained at various amplification times in strip development.

Detection limitation evaluation

To evaluate the limit of detection (LOD) of the optimized RPA-LF method in detecting genomic DNA, we used serial dilutions of genomic DNA of SS2 from 10^6 to 10^0 copies/μL as templates. The LOD was determined as the highest dilution concentration showing a positive test result. The evaluation was performed in duplicate.

Specificity and sensitivity evaluation

To evaluate the specificity of the method, we mixed genomic DNA of *Rickettsia rickettsii*, *Coxiella burnetii*, *Orientia tsutsugamushi*, *Rickettsia heilongjiangensis*, *Rickettsia sibirica*, *Rickettsia prowazekii*, *Staphylococcus aureus* and *Mycoplasma pneumoniae* with an equal volume of DNA from a healthy volunteer. The DNA was used as a template for testing the optimized RPA-LF method. Only DNA from the healthy volunteer was used as a negative control. All reactions were performed in duplicate.

To evaluate the method's sensitivity, we used DNA from SS2-infected mouse spleens, with SS2 DNA concentrations of approximately 10^3 to 10^4 copies/μL, determined by RT-qPCR, as a template in the optimized RPA-LF method.

RESULTS

Construction of positive plasmid

The amplified partial sequence of *cps2j* with 999 bp was successfully ligated into pMD18T plasmid to construct the recombinant plasmid *cps2j*-pMD18T. As shown in Fig 1, the size of the PCR amplified product from this recombinant plasmid was consistent with the expected size.

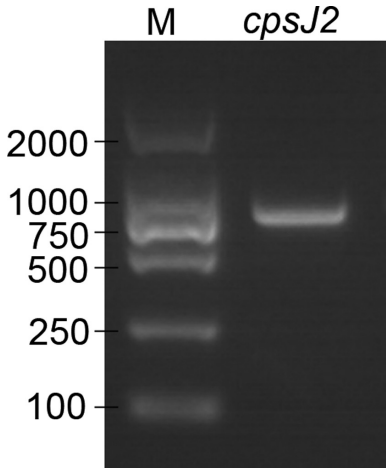

FIGURE 1 | Agarose gel electrophoresis analysis of PCR products amplified from the recombinant plasmid *cpsJ2*-pMD18T. M, DNA marker. The size (bp) is indicated at left.

Primer and probe screening

The best combination of forward primer and reverse primers was screened. As shown in Fig 2A, primer groups F3 & R1 and F3 & R2 showed intensely dark bands on the T lines of the experimental strips (with *cps2j*-pMD18T as a template) and no bands on the T lines of the control strips (with pMD18T as a template). Group F3 & R1 was used for further analysis.

Optimization of the RPA-LF detection method

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

Detection limit

The LOD of the RPA-LF method was evaluated with genomic DNA of SS2. As shown in Fig 3A, the LOD of the RPA-LF in the detection of SS2 was 100 copies/reaction or lower (concentrations of between 10 and 100 copies/reaction were not further evaluated).

Specificity and sensitivity evaluation

Genomic DNA from several unrelated bacteria was used to evaluate the specificity. As shown in Fig 3B, the T lines did not develop in detection of DNA from *R. heilongjiangensis*, *R. sibirica*, *O. tsutsugamushi*, *C. burnetii*, *S. aureus*, *R. rickettsii*, *M. pneumoniae* and human nasopharyngeal swab samples, whereas the T line did develop in detection of DNA of SS2.

The sensitivity was evaluated with SS2-infected mouse samples. As shown in Fig 3C, all nine SS2-infected mouse samples were recognized with the RPA-LF method, thus indicating a sensitivity of 100% in detecting mouse samples.

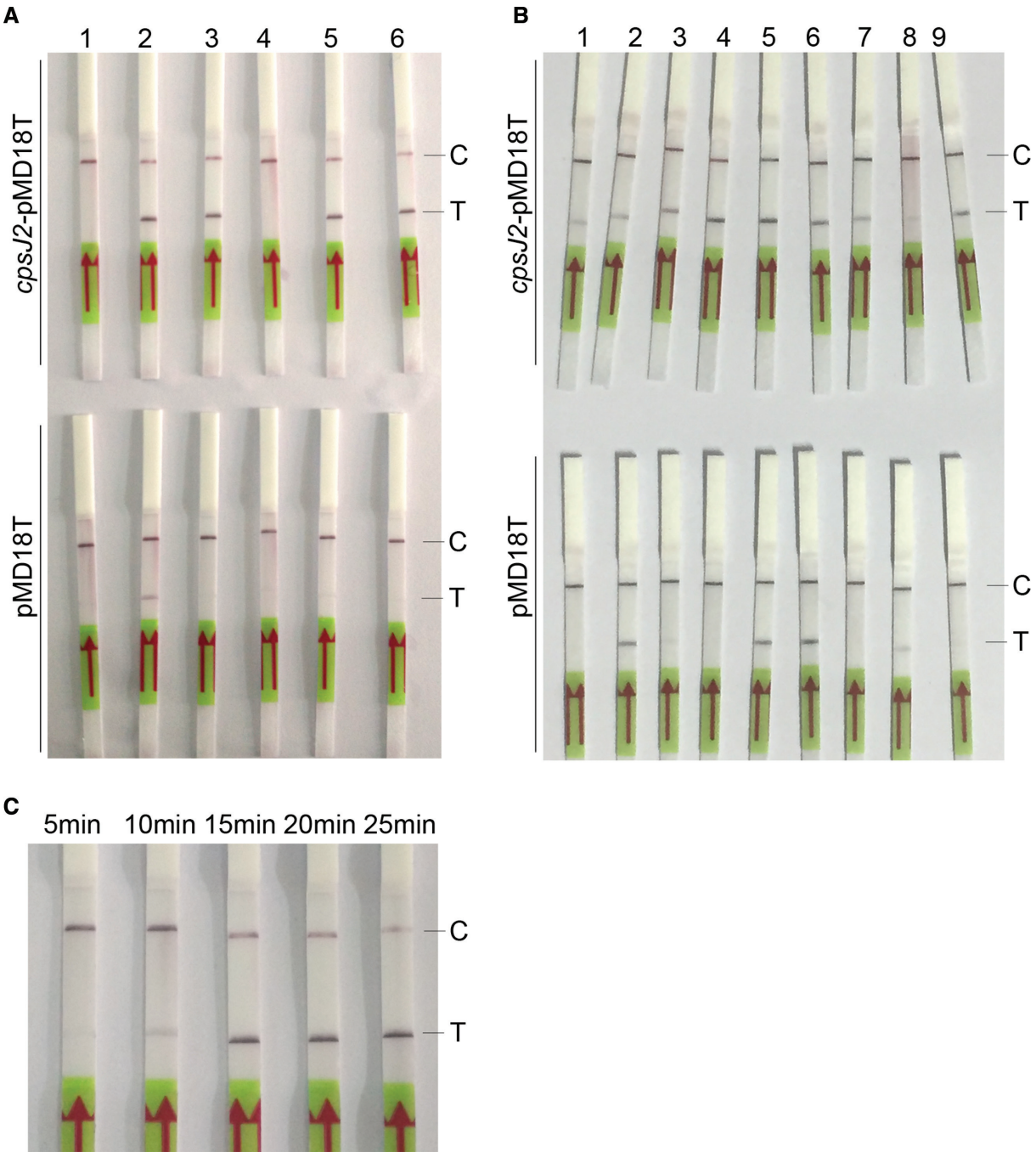

FIGURE 2 | Optimization of the RPA-LF assay. A, screening of the best primers from six primer combinations. Primer combinations 1–6 in (A) are indicated in Table 2, and recombinant plasmid *cpsJ2*-pMD18T and plasmid pMD18T were used as experimental and control templates, respectively. B, screening of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

DISCUSSION

SS2 is an important zoonotic pathogen that not only causes serious economic losses in the pork industry through swine streptococcosis but also threatens public health through its rapid spread and high mortality rates, particularly in developing countries [2,5,11-13]. Therefore, the development of a rapid detection method suitable for use in resource-limited areas is highly important to control this zoonosis in epidemic areas.

In recent years, isothermal amplification methods have been rapidly developed because of their benefits of fast amplification, convenient operation and low instrument requirements, in contrast to conventional PCR or RT-qPCR. Zhang et al. have developed loop-mediated isothermal amplification-based detection assays that enable visual detection with the naked eye within 48 minutes [5]. Recently, Jiang et al. have developed a real-time RPA for detection of SS2 within 20 min, although a fluorescence

TABLE 2 | Performance of various combinations of primers in RPA-LF assays.

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

–, poor result; +, good result; ++, excellent result.

TABLE 3 | Results of various of concentrations of reverse primer R1 and probe in RPA-LF assays.

Group No.	Concentrations (R1 and probe, μM)	Results
1	10 & 10	+
2	5 & 10	–
3	2.5 & 10	+
4	10 & 5	++
5	5 & 5	–
6	2.5 & 5	–
7	10 & 2.5	+
8	5 & 2.5	–
9	2.5 & 2.5	+

–, poor result; +, good result; ++, excellent result.

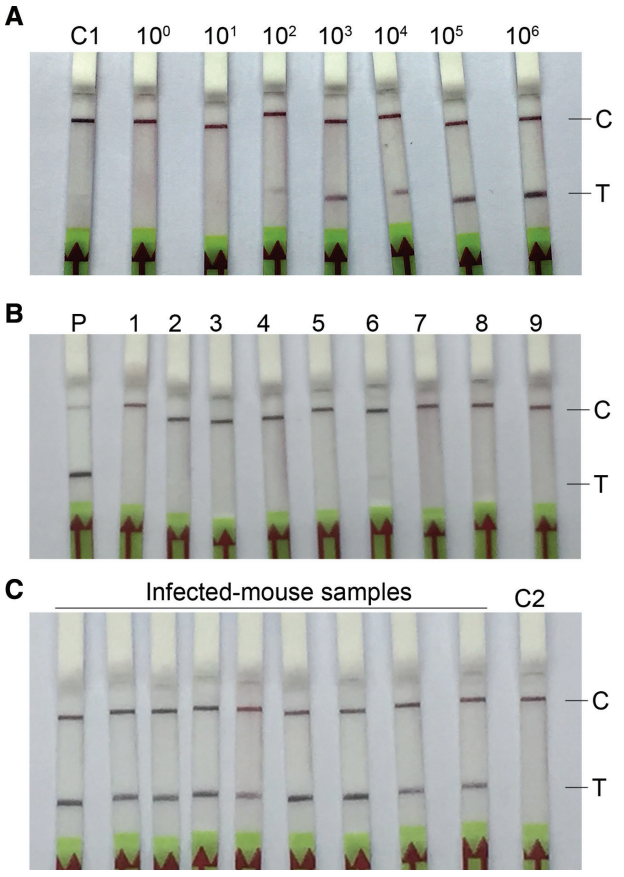

FIGURE 3 | Detection limit (A), specificity (B) and sensitivity evaluation of the method. The concentrations of various dilutions of SS2 genomic DNA are indicated in (A). Strips 1–9 in (B) used DNA from a healthy volunteer mixed with genomic DNA of *Rickettsia heilongjiangensis*, *Rickettsia sibirica*, *Staphylococcus aureus*, *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Orientia tsutsugamushi*, *Mycoplasma pneumoniae*, *Coxiella burnetii*, and distilled water as templates. P, positive control comprising healthy volunteer DNA mixed with genomic DNA of SS2 as a template; C1, negative control with distilled water as a template; C2, negative control with DNA extracted from uninfected mouse spleen as a template. The other nine strips in (C) used DNA extracted from nine SS2-infected mouse spleens as templates.

detection instrument is necessary [14]. Here, our developed RPA-LF method appears to overcome the disadvantages of both methods described above, by enabling detection within 20 min without any expensive or sophisticated instrument, such as a fluorescence detection machine. The results can be assessed by the naked eye. Compared with conventional molecular methods, our method is more suitable for use in the field and in resource-limited medical units.

This study has several limitations. Because of resource constraints, only limited genomic DNA of unrelated pathogens was collected to evaluate the specificity, and no clinical samples were available in the present study. In addition, the method is unable to distinguish type 2 from type 1/2, because both types share the target *cps2J* gene; this drawback also exists in the previously developed methods targeting this gene [5,14]. More genomic DNA from various phylogenetically related and pathogens causing similar symptoms, as well as clinical samples, are needed to evaluate the specificity and sensitivity of the established method.

CONCLUSION

In conclusion, this study is the first to establish a rapid visual detection method based on an RPA and LF test for SS2. The method has high sensitivity and specificity, and does not require sophisticated instruments; therefore, it has potential for use in field conditions and resource-limited areas. This work provides a promising and alternative tool for future clinical detection and surveillance of SS2 infections.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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