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Rapid and visual RPA-CRISPR/Cas12a detection for *Staphylococcus pseudintermedius* and methicillin-resistant *S. pseudintermedius* in clinical samples of dogs and cats

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Abstract

Staphylococcus pseudintermedius can cause severe infections of the skin, ear and other tissues in dogs and cats. Methicillin-resistant *S. pseudintermedius* (MRSP) has recently become more prevalent, posing a severe threat to companion animals and public health. Therefore, rapid and accurate diagnosis of *S. pseudintermedius* and MRSP infections in dogs and cats is essential for timely controlling infections. The development of CRISPR/Cas technology offers an innovative solution for rapid diagnosis. Here, we established an assay combining recombinant polymerase amplification (RPA) and CRISPR/Cas12a. By separately detecting the *spsJ* gene, the specific gene of *S. pseudintermedius*, and the *mecA* gene, the methicillin resistance gene, this method allows for the direct detection of methicillin-susceptible *S. pseudintermedius* (MSSP) and MRSP in clinical samples at 37 °C for a total of 40 min, The results can be directly visualized by the naked eye under blue light. The limits of detection of the RPA-CRISPR/Cas12a assay were 10^3 copies per reaction for the *spsJ* gene and 10^4 copies per reaction for the *mecA* gene. The RPA-CRISPR/Cas12a detection successfully detected and differentiated clinical isolates of MSSP and MRSP without cross-reactivity with other tested bacteria species. The evaluation of the detection performance of RPA-CRISPR/Cas12a with 47 clinical samples (without culture) from dogs and cats showed that the results of detection were 100% consistent with those of clinical culture and colony sequencing, which was more sensitive than PCR. RPA-CRISPR/Cas12a assay can quickly and sensitively detect *S. pseudintermedius* and MRSP in clinical samples without expensive instruments, making it suitable for small veterinary clinics.

Keywords *Staphylococcus pseudintermedius*, Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), Methicillin-susceptible *Staphylococcus pseudintermedius* (MSSP), Recombinase polymerase amplification (RPA), CRISPR/Cas12a

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Introduction

Staphylococcus pseudintermedius, coagulase-positive *Staphylococcus*, is a commensal of the skin and mucosa of dogs and cats, and an opportunistic pathogen [1]. It is the most frequent bacterial pathogen isolated from clinical canine samples, mainly associated with skin and ear infections [2], and can also cause other infections such as wound infections, lung infections, urinary tract infections and osteomyelitis [3]. *S. pseudintermedius* is not a normal colonizer of human skin and mucous, however, it may colonize in humans who are in close contact with dogs. Especially when human immune system is compromised, it may cause disease [4–6]. *S. pseudintermedius* infections in people have been associated with bloodstream infections, abscesses, pneumonia, and septic arthritis, according to research [7–9].

Antibiotic resistance is a significant issue for *S. pseudintermedius* due to the extensive use of antibiotics in canine and feline infections. Methicillin-resistant *S. pseudintermedius* (MRSP) is one of the most commonly reported drug-resistant strains. MRSP was initially reported in Europe in 2006 [10]. In recent years, there has been a global increase in the prevalence of MRSP infection [11]. Reports indicated that 5.8 ~ 32.2% of *S. pseudintermedius* isolated from dogs and cats were methicillin-resistant [12]. Methicillin resistance of MRSP is mediated by the *mecA* gene, which encodes a modified penicillin-binding protein 2a (PBP2a). PBP2a has low affinity to most β-lactam antibiotics [13], resulting in MRSP resistance to almost all β-lactams except ceftaroline and ceftobiprole [14]. MRSP challenges the use of antibiotics in dogs and cats since β-lactam antibiotics are the first line of defense against staphylococcal infections [15]. The *mecA* gene exists on the staphylococcal chromosomal cassette *mec* (SCC*mec*), a mobile element of the bacterial chromosome. SCC*mec* can be transferred between different *Staphylococcus* species, leading to the spread of drug resistance [16]. Therefore, rapid and sensitive detection of *S. pseudintermedius* and MRSP is critical for early diagnosis of *S. pseudintermedius* infections in dogs and cats and the rational use of antibiotics.

In veterinary clinics, *S. pseudintermedius* is usually isolated and identified through phenotype [2]. The antibacterial susceptibility test is detected by oxacillin disc diffusion or broth microdilution [17]. However, these assays are time-consuming (2–3 days), cumbersome, and susceptible to culture conditions. Other molecular-based methods, such as PCR (polymerase chain reaction) [18], multiplex-PCR [19], qPCR (real-time quantitative PCR) [20], and PCR-restriction fragment length polymorphism [21] can directly detect the specific genes of *S. pseudintermedius* and *mecA* genes. These methods can significantly improve detection sensitivity but require expensive equipment and trained personnel. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) provides rapid and highly accurate identification of *S. pseudintermedius* [22, 23]. However, the very high cost of the MALDI-TOF instrument limits its use.

Recently, the discovery of the CRISPR (cluster regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system has offered an innovative solution for rapid diagnosis. The CRISPR system targets DNA guided by RNA [24]. The Cas12a-crRNA (CRISPR RNA) complex specifically recognizes the target DNA and activates the *trans*-cleavage activity of Cas12a to cut single-stranded DNA (ssDNA) indiscriminately [25]. Genetic detection can be performed by designing crRNAs for different target genes and activating *trans*-cleavage activity of Cas12a to cut ssDNA [26]. Several CRISPR/Cas12a-based genetic assays have been developed, including DETECR (DNA endonuclease-targeted CRISPR *trans* reporter) [27] and HOLMES (one-hour low-cost multipurpose highly efficient system) [28]. When CRISPR/Cas12a is combined with isothermal amplification techniques such as recombinase polymerase amplification (RPA) [25, 29] or loop-mediated isothermal amplification (LAMP) [30–32], pathogenic bacteria can be detected in a sensitive and specific manner.

Here, we established the RPA-CRISPR/Cas12a (RPA combined with CRISPR/Cas12a) detection method that can directly, accurately, and sensitively detect *S. pseudintermedius* and MRSP in clinical samples. We selected the *S. pseudintermedius* surface gene (*spsJ* gene) for the specific detection of *S. pseudintermedius* [20, 33] and the *mecA* gene for the specific detection of methicillin resistance. The detection process and principle of RPA-CRISPR/Cas12a were shown in Fig. 1. The RPA-CRISPR/Cas12a detection for *S. pseudintermedius* and MRSP was important for rapid and accurate diagnosis of *S. pseudintermedius* infection and guiding the rational use of antibiotics.

Results

Establishment and optimization of RPA reaction system

Two sets of forward and reverse primers were initially designed for *the spsJ* and *mecA* genes, respectively. When the primer pairs RPA-spsJ-F1/R2 (*spsJ* gene) and RPA-mecA-F2/R2 (*mecA* gene) participated in RPA using plasmids DNA containing *spsJ* or *mecA* as template, the amplified products had the strongest bands with less non-specific amplification (Fig. 2A, D). Therefore, these primer pairs were selected as the optimal primers for RPA. To determine the best reaction temperature, RPA was performed at 37 °C, 39 °C, and 42 °C using the best primer pairs. The bands of the amplified products were all quite strong regardless of the difference in

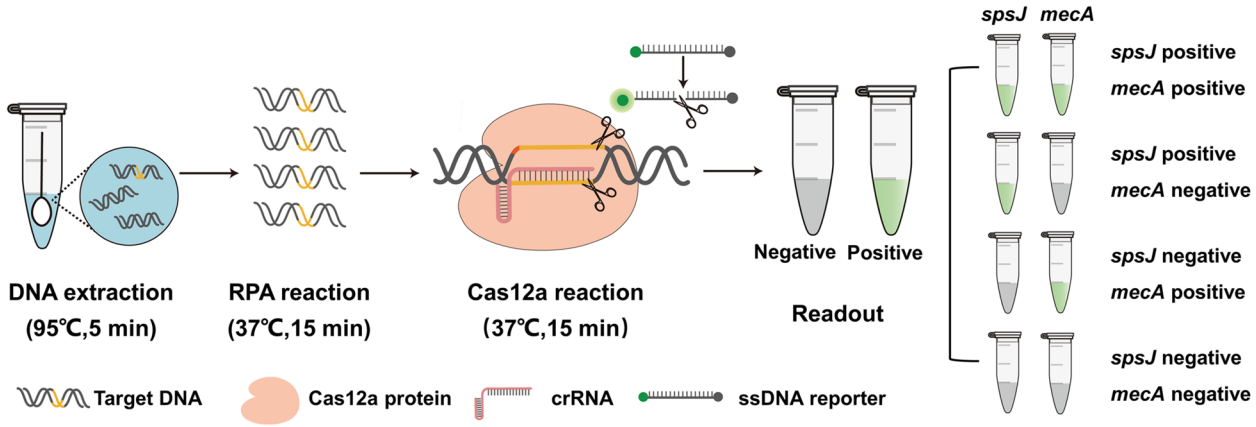

Fig. 1 The workflow of RPA-CRISPR/Cas12a detection of *S. pseudintermedius* and MRSP in clinical samples. Genomic DNA was quickly extracted from clinical samples of dogs and cats using DNA extraction buffer. The *spsJ* gene and *mecA* genes were amplified by RPA, and the products were transferred to the CRISPR/Cas12a detection system comprising specific crRNAs targeting the *spsJ* and *mecA* genes. The Cas12a-crRNA complex bound to the target DNA activated the *trans*-cleavage activity of Cas12a and cleaved the ssDNA reporter labeled with FAM and BHQ1 at either end. The product produced an intense fluorescence signal at a specific wavelength. The fluorescence can be detected by a fluorescence microplate reader or observed directly by the naked eye under blue light as green fluorescence. The diagnosis was MRSP when both *spsJ* and *mecA* genes were positive; MSSP when *spsJ* gene was positive but *mecA* gene was negative. The diagnosis was *S. pseudintermedius* negative and other *mecA* positive *Staphylococcus* when *spsJ* was negative but *mecA* was positive. The diagnosis was *S. pseudintermedius* negative when both *spsJ* and *mecA* were negative. The entire reaction can be finished in 40 min at 37 °C

temperature (Fig. 2B, E). Since the CRISPR/Cas12a reaction temperature was 37 °C and there was no need to change the temperature between the RPA and CRISPR/Cas12a reactions, we decided to use that temperature for the RPA reaction. To evaluate the effect of reaction time on RPA amplification yield, RPA was performed for 10, 15, 20, and 25 min, respectively. Figure 2C and F showed that increasing reaction time led to higher yield of amplified products. RPA products of different times were involved in the CRISPR/Cas12a reaction. It was observed that when RPA was performed for 15 min, it triggered high-intensity endpoint fluorescence in the CRISPR/Cas12a reaction (Fig. S1). To shorten the overall reaction time, 15 min was chosen as the amplification time for RPA.

Optimization of CRISPR/Cas12a reaction system and establishment of RPA-CRISPR/Cas12a detection

The CRISPR/Cas12a reaction system relies on crRNA to guide Cas12a cleavage efficiency. The crRNA sequence was screened, and the concentration of crRNA and Cas12a was optimized. Three crRNAs were designed for the *spsJ* and *mecA* genes, respectively, and the crRNAs were employed in the CRISPR/Cas12a reaction. Figures 3A, B showed that crRNA-spsJ2 and crRNA-mecA3 had the best efficiency in the CRISPR/Cas12a reaction with the highest fluorescence intensity at the same time. The concentration of crRNA and Cas12a was then optimized. The highest endpoint fluorescence intensity was observed at crRNA concentration of 2 μM (Fig. 3C, D), and Cas12a concentration of 30 nM (Fig. 3E, F).

To establish the RPA-CRISPR/Cas12a detection system, the RPA-CRISPR/Cas12a reaction was performed under the optimal RPA and Cas12a reaction conditions using *spsJ* or *mecA* plasmids as templates, respectively. In Fig. 4A, B, the products produced obvious fluorescence signals, emitting green fluorescence under blue light in the presence of target genes. The fluorescence intensity did not increase significantly in absence of the target gene, and there was no obvious fluorescence visible to the naked eye (Fig. 4B). This indicated that we have successfully established the RPA-CRISPR/Cas12a method for detecting the *spsJ* gene and *mecA* gene. It was evaluated that when the ratio of the endpoint fluorescence intensity of the sample to be tested to that of the negative control was greater than two (S/N > 2), significant fluorescence of the sample to be tested could be observed by the naked eye, and the fluorescence intensity differed significantly from that of the negative control.

Sensitivity of RPA-CRISPR/Cas12a detection

The sensitivity of the RPA-CRISPR/Cas12a detection system was evaluated using *spsJ* and *mecA* plasmid DNA (10^6–10^0 copies / μL) as templates and compared with PCR. As shown in Fig. 5A, for *spsJ*, the detection can detect at least 10^3 copies of *spsJ* plasmids. For *mecA*, the system can detect 10^4 copies of *mecA* plasmids (Fig. 5B). Therefore, for the *spsJ* gene and *mecA* gene, the limits of detection (LOD) of RPA-CRISPR/Cas12a were 10^3

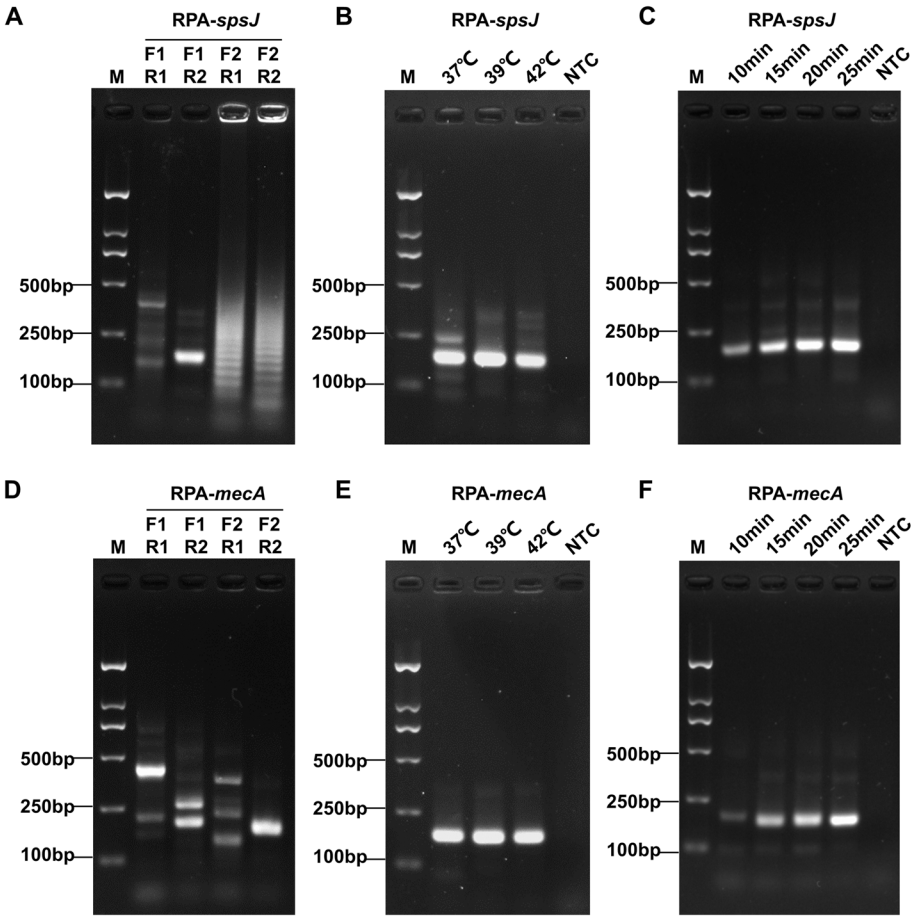

Fig. 2 Optimization of RPA reaction conditions. Optimization of RPA primer pairs (**A**), reaction temperature (**B**), and reaction time (**C**) for detection of the *spsJ* gene. Optimization of RPA primer pairs (**D**), reaction temperature (**E**), and reaction time (**F**) for detection of the *mecA* gene. 10^4 copies of the *spsJ* plasmid (containing part of the *spsJ* gene) and 10^4 copies of the *mecA* plasmid (containing part of the *mecA* gene) were used as templates for RPA. M, marker. NTC, no-target control

copies and 10^4 copies, respectively. For PCR, the bands were obvious when there were 10^5 copies of plasmids in the reaction system, but they were difficult to see when there were 10^4 copies (Fig. 5C, D). The LOD of PCR was 10^5 copies per reaction, indicating that the RPA-CRISPR/Cas12a detection was more sensitive.

Specificity of RPA-CRISPR/Cas12a detection

To evaluate the specificity of RPA-CRISPR/Cas12a for detecting *spsJ* and *mecA* genes, 10 MRSP strains, 10 MSSP strains, 6 *Staphylococcus* strains of non-*S. pseudintermedius* and 7 non-*Staphylococcus* strains were used in the reaction. The specific information of the strains was listed in Table S1. Only when *S. pseudintermedius* participated in the reaction did the fluorescence intensity of the RPA-CRISPR/Cas12a reaction for detecting the *spsJ* gene increase significantly and did not cross-react with other species (Fig. 6A). Similarly, only when *Staphylococcus* carrying the *mecA* gene participated in the reaction did the fluorescence intensity of RPA-CRISPR/Cas12a reaction for detecting the *mecA* gene increase significantly (Fig. 6B). These results showed that the RPA-CRISPR/Cas12a detection had great specificity, no cross-reactivity, and could diagnose *spsJ* gene and *mecA* gene specifically. This method allowed specific detection of MRSP and MSSP.

Detection of clinical samples

To evaluate the effectiveness of the RPA-CRISPR/Cas12a detection system in clinical samples, forty-seven clinical samples of dogs and cats were collected, including 21 skin samples ($n=21$) and 26 ear canal samples ($n=26$). The clinical samples were cultured, and PCR was performed on DNA extracted from cultured colonies to identify the *spsJ* and *mecA* genes and the products were sequenced, and this result was taken as the "gold standard". Among all the clinical samples collected, MRSP was isolated and identified from

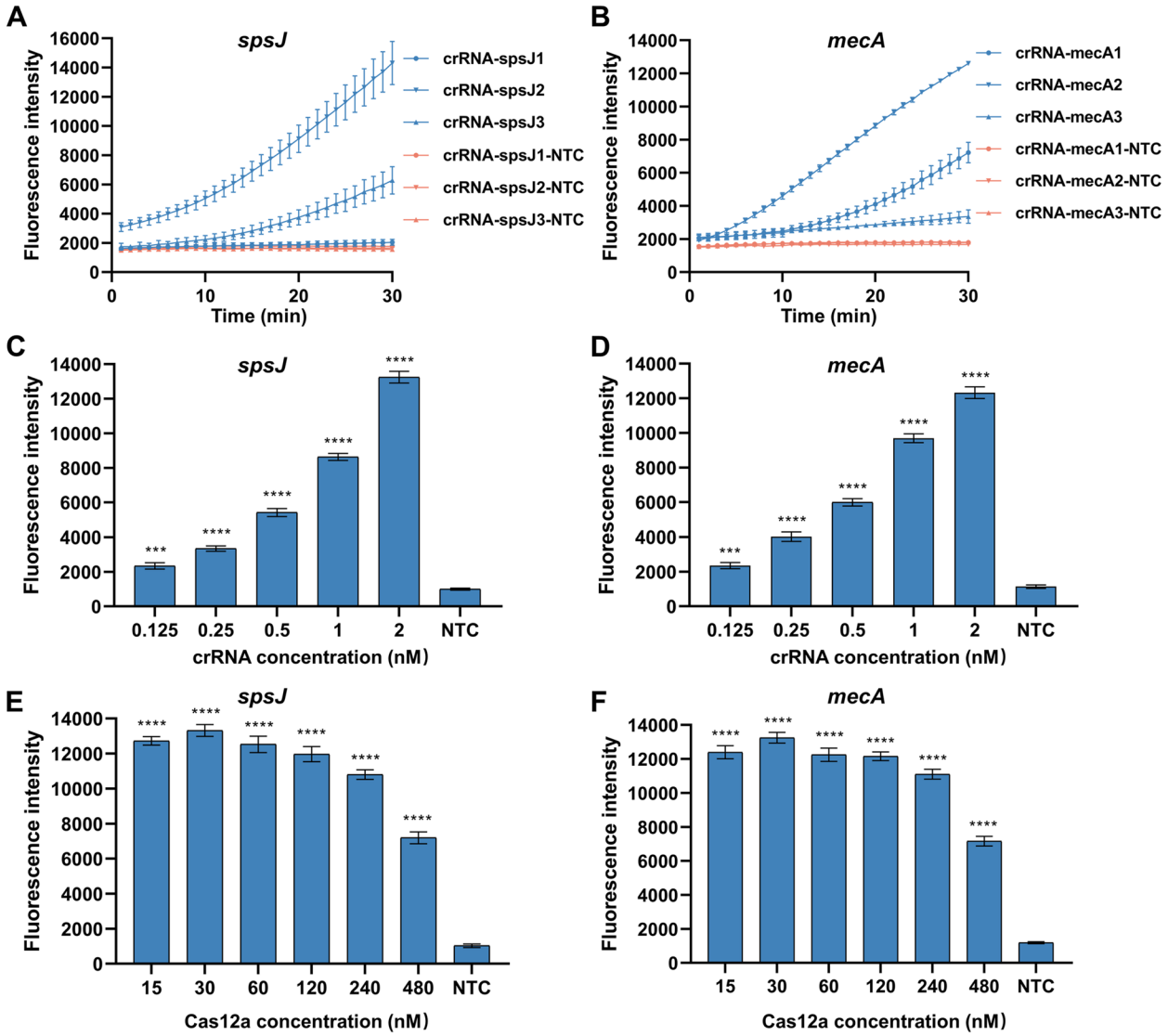

Fig. 3 Optimization of the Cas12a reaction system. Optimization of the crRNA for the *spsJ* gene (**A**) and the *mecA* gene (**B**). Optimization of crRNA concentration for the *spsJ* gene (**C**) and the *mecA* gene (**D**). Optimization of Cas12a concentration for the *spsJ* gene (**E**) and the *mecA* gene (**F**). The purified product of PCR of *spsJ* plasmid or *mecA* plasmid was used as the substrate of Cas12a reaction, and the concentration of substrate was 6.25 nM. NTC, no-target control. Data were represented as mean ± standard deviation (SD) ($n = 3$ technical replicates). All data were compared with NTC for significant difference. **** $p < 0.0001$; *** $p < 0.001$

samples No. 1-27, MSSP was isolated only from samples No. 28-40, and *S. pseudintermedius* and the *mecA* gene were not isolated from samples No. 41-47 (Table S2). The RPA-CRISPR/Cas12a assay and direct PCR were used in the crude genomic DNA of clinical samples. When RPA-CRISPR/Cas12a was used to detect the *spsJ* gene, the fluorescence intensity of clinical samples No. 1-40 matched our predefined standard of positive results of S/N > 2. When detecting the *mecA* gene, the fluorescence intensity of clinical samples No. 1-27 matched the standard of positive results of S/N > 2, and other tests matched the standard of negative results of S/N < 2. As a result, RPA-CRISPR/Cas12a correctly detected 27 MRSP samples, 13 MSSP samples, and 7 *S. pseudintermedius* negative samples (Fig. 7A, B). Direct PCR correctly detected 25 MRSP clinical samples, 13 MSSP samples, and 7 *S. pseudintermedius* negative samples, MRSP was incorrectly identified as MSSP in samples 2 and 23 (Fig. S2). The coincidence rate between RPA-CRISPR/Cas12a test results and gold standard was 100%, while the coincidence rate of direct PCR with gold standard was 95.7% (Table 1).

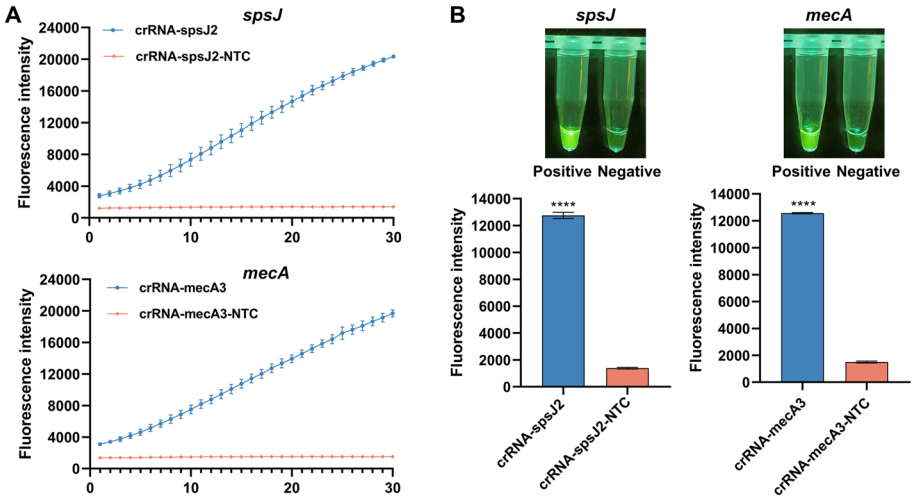

Fig. 4 Establishment of RPA-CRISPR/Cas12a detection. Real-time fluorescence intensity (**A**) and endpoint fluorescence intensity (**B**) of the *spsJ* and *mecA* genes were detected by RPA-CRISPR/Cas12a. NTC, no-target-control. Data were expressed as mean ± SD ($n = 3$ technical replicates). All data were compared with NTC for significant difference. **** $p < 0.0001$

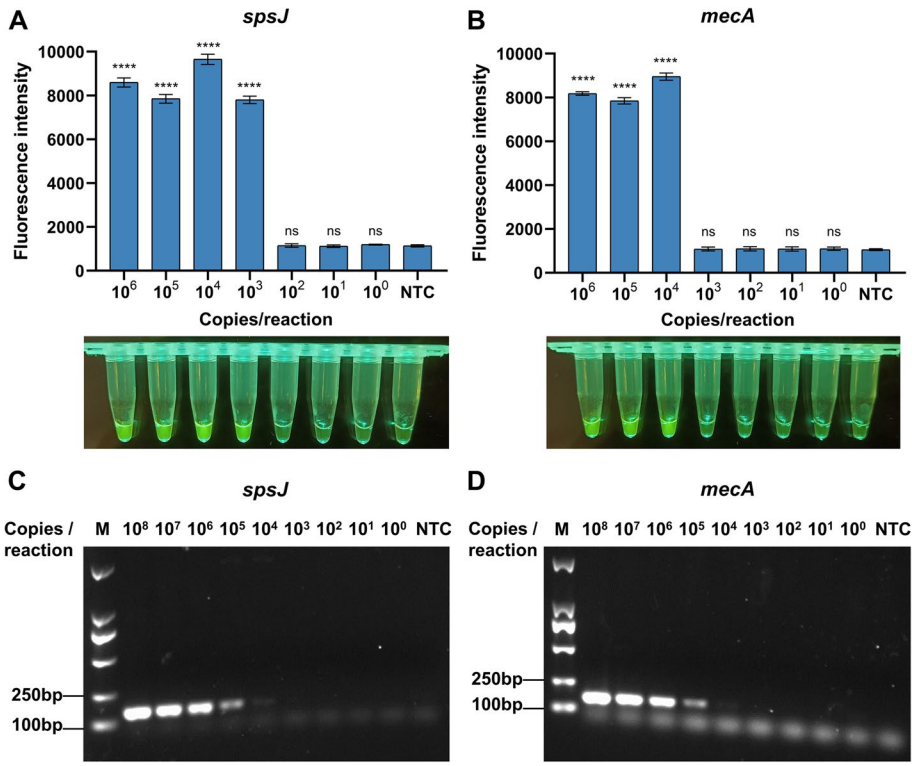

Fig. 5 Sensitivity evaluation of RPA-CRISPR/Cas12a detection and PCR. Sensitivity evaluation of RPA-CRISPR/Cas12a detection of *spsJ* gene (**A**) and *mecA* gene (**B**). Sensitivity evaluation of PCR of *spsJ* gene (**C**) and *mecA* gene (**D**). The serial 10-fold dilution of *spsJ* and *mecA* plasmids (10^6–10^0 or 10^8–10^0 copies/reaction) was used as templates to evaluate the sensitivity of RPA-CRISPR/Cas12a detection and PCR. The sensitivity evaluation results of RPA-CRISPR/Cas12a detection were shown by the endpoint fluorescence intensity and endpoint fluorescence photography. The sensitivity of PCR was shown by agarose gel electrophoresis. M, marker. NTC, no-target control. Data were expressed as mean ± SD ($n = 3$ technical replicates). All data were compared with NTC for significant difference. **** $p < 0.0001$; ns, not significant

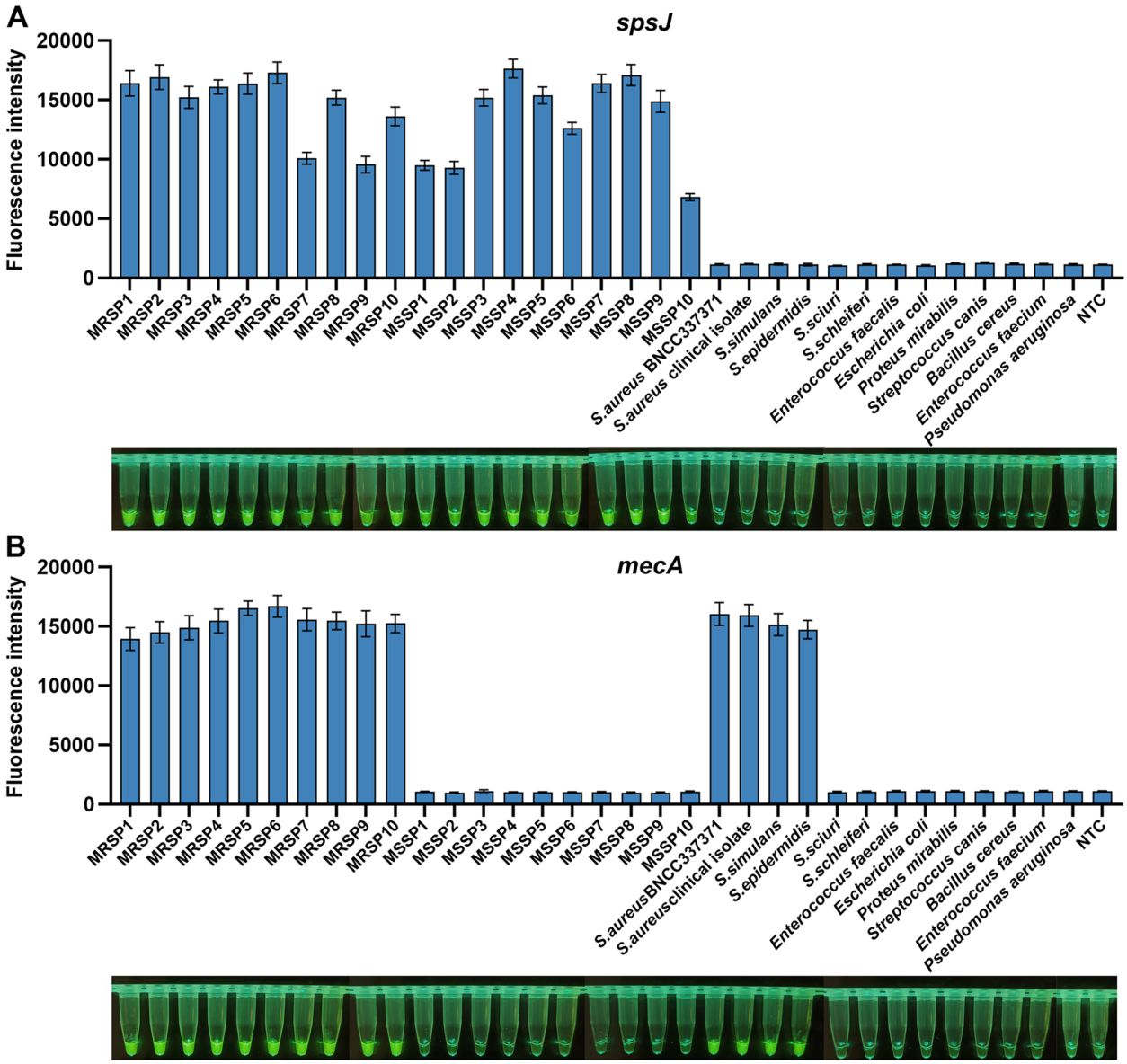

Fig. 6 Specificity evaluation of the RPA-CRISPR/Cas12a detection. The endpoint fluorescence intensity and endpoint fluorescence photography of the RPA-CRISR/Cas12a reaction for the detection of the *spsJ* gene (**A**) and the *mecA* gene (**B**). NTC, no-target control. Data were expressed as mean ± SD ($n = 3$ technical replicates)

Discussion

S. pseudintermedius can cause serious infections of the skin, ear, and other tissues. It is the primary cause of canine pyoderma, which can also lead to otitis externa, urinary tract infections, and respiratory infections [3]. Due to the inappropriate use of antibiotics and the lack of new antibiotics development, the rate of methicillin resistance in *S. pseudintermedius* has rapidly increased [34], which poses a challenge to the use of antibiotics. Therefore, rapid and reliable diagnosis of *S. pseudintermedius* and MRSP is critical for early diagnosis and treatment of diseases. Traditional methods for diagnosing *S. pseudintermedius* and drug resistance based on the isolation and culture of bacteria and antibacterial susceptibility test are time-consuming, cumbersome, and unsuitable for rapid clinical diagnosis. Although PCR-based methods can rapidly and sensitively diagnose specific genes and methicillin resistance genes of *S. pseudintermedius*, they are not suited for on-site detection since they rely on laboratory conditions, equipment, and trained personnel.

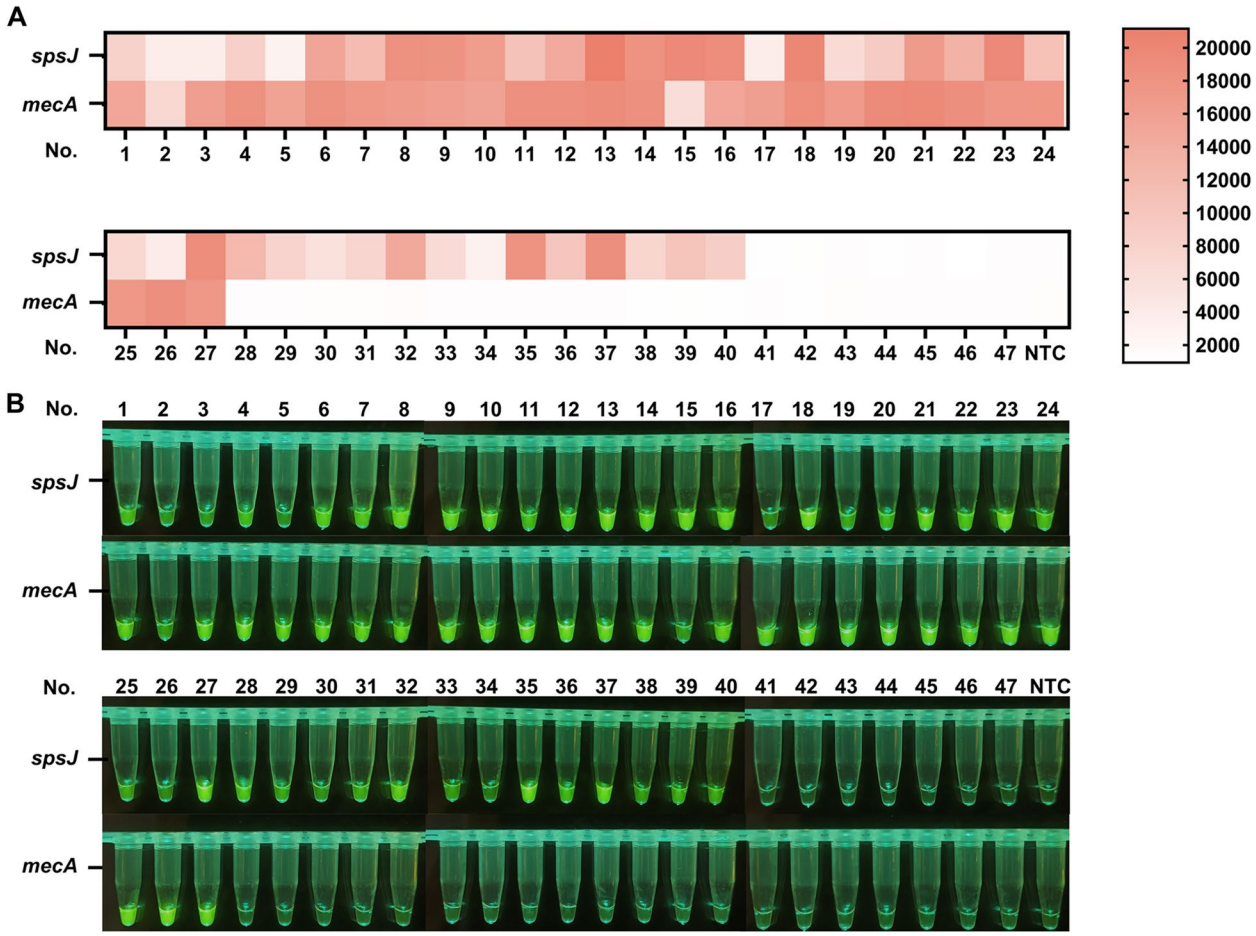

Fig. 7 Application of RPA-CRISPR/Cas12a detection in 47 clinical samples. (**A**) Endpoint fluorescence intensity of the *spsJ* gene and *mecA* gene in clinical samples was detected separately employing RPA-CRISPR/Cas12a detection. Different shades of rad indicated positive results, and white indicated negative results. (**B**) Endpoint fluorescence photography of the *spsJ* and *mecA* genes in clinical samples employing RPA-CRISPR/Cas12a detection. NTC, no-target control

Table 1 Evaluation of RPA-CRISPR/Cas12a detection for detecting *spsJ* and *mecA* genes in clinical samples

		Bacterial Culture + PCR (Gold Standard)				Coincidence Rate (CR)
		MRSP	**MSSP**	***S. pseudintermedius* (-)**	**Total**	
RPA-CRISPR/Cas12a	MRSP	27	0	0	27	100%
	MSSP	0	13	0	13	
	S. pseudintermedius (-)	0	0	7	7	
	Total	27	13	7	47	
Direct PCR	MRSP	25	0	0	25	95.7%
	MSSP	2	13	0	15	
	S. pseudintermedius (-)	0	0	7	7	
	Total	27	13	7	47	

Forty-seven clinical isolates for evaluation of RPA-CRISPR/Cas12a detection. CR = [both MRSP + both MSSP + both *S. pseudintermedius* (-)] / total samples × 100%.

MRSP, methicillin-resistant *S. pseudintermedius*; *MSSP*, methicillin-sensitive *S. pseudintermedius*; *S. pseudintermedius* (-), *S. pseudintermedius* negative

The RPA used in this study is an isothermal amplification technique that uses the activity of biological enzymes to amplify nucleic acids at isothermal conditions of 37 ~ 42 °C without complex instrumentation [35]. The target amplification products can be obtained within 5 ~ 20 min, allowing portable and rapid nucleic acid detection. However, RPA can tolerate mismatches, primers and templates can mismatch up to nine base pairs [36–38], causing non-specific amplification and false positive in RPA which lower the accuracy of the results. CRISPR/Cas12a detection has high specificity provided by the complementarity between crRNA and target DNA. When there is a base mismatch between crRNA and target DNA, especially in the seed region, the cleavage activity of Cas12a will be significantly reduced [24, 39]. The specificity of the detection can be significantly increased by combining RPA with CRISPR/Cas12a. When we investigated the specificity of detection of *S. pseudintermedius* using RPA-CRISPR/Cas12a, we found that when using RPA alone, the RPA products of *Staphylococcus* other than *S. pseudintermedius* also showed non-specific bands after agarose gel electrophoresis, but when using CRISPR/Cas12a to detect the RPA products, only *S. pseudintermedius* amplification products produced bright green fluorescence, indicating the high specificity of our RPA-CRISPR/Cas12a detection method.

In this study, we developed a rapid and accurate method for detecting *S. pseudintermedius* and MRSP. This method was more sensitive than PCR and provided visual results at a constant temperature of 37 °C for 40 min without the use of expensive instruments. The RPA-CRISPR/Cas12a had LOD of 10^3 copies for the *spsJ* gene and 10^4 copies for the *mecA* gene, which was higher than that of PCR at 10^5 copies. We verified its high specificity to specifically identify MRSP and MSSP strains by testing *Staphylococcus* strains and non-*Staphylococcus* strains. Additionally, we verified its capacity to identify *S. pseudintermedius* and MRSP in the crude genomic DNA of clinical samples. The coincidence rate between the results of RPA-CRISPR/Cas12a detection in 47 clinical samples and the gold standard was 100%, while that of PCR was 95.7%. These results indicated that RPA-CRISPR/Cas12a can more sensitively and specifically detect *S. pseudintermedius* and MRSP in clinical samples than PCR. This assay has the potential to be used in small veterinary clinics due to the simplicity of the instrumental requirement. To carry out the assay, the operator only needs an isothermal device (or use body heat for 37 °C incubation) and a blue light machine, which are low-cost. In addition, this assay could be further integrated into a lateral flow strip test or a microfluidic system.

We only used RPA-CRISPR/Cas12a to directly detect the skin and ear canal samples of dogs and cats, and no samples from other sources were detected. Because RPA is tolerant to common PCR inhibitors such as 15–20% milk, 20 g/L hemoglobin, 4% (V/V) ethanol, 0.5 U heparin, serum, 1.25% urine, and a certain concentration of background DNA [35]. In the future, we can broaden the usage of this detection by using it to detect other kinds of clinical samples, such as blood, synovial fluid, urine, lung lavage fluid, etc.

However, this detection has some limitations. Firstly, RPA reagents are available from TwistDx™, but they are expensive. RPA reagents are now available in liquid form which allows for greater flexibility in reaction conditions. We still obtained good results despite reducing the 50 μL reaction system specified in the instructions to 12.5 μL which significantly lowered the detection cost. Secondly, although *S. pseudintermedius* is the most common bacterial pathogen isolated from clinical samples of dogs [2], they also carry other *Staphylococcus* such as *S. schleiferi* and *S. aureus* which can also carry the *mecA* gene [40]. When we directly test clinical samples with positive results for *spsJ* and *mecA* genes, the *S. pseudintermedius* in clinical samples could be MSSP and the *mecA* gene may be carried by other *Staphylococcus*, yielding inaccurate results. Although this has not yet occurred in any of the collected clinical samples, and there was a 100% coincidence rate between the data produced from this approach and the gold standard, the result should be interpreted with caution due to the limited clinical samples studied. In the follow-up, we should collect a large number of clinical samples to evaluate the effect of this method for direct detection of clinical samples.

Conclusion

In this study, we combined RPA and CRISPR/Cas12a to establish a rapid and accurate assay for detecting *S. pseudintermedius* and MRSP in clinical samples from dogs and cats. With simple instrumentation, easy-to-read results, and high sensitivity and specificity, the entire reaction can be finished within 40 min. We demonstrated the method's applicability to clinical testing with 47 clinical samples. This method can be used for diagnosing *S. pseudintermedius* and MRSP infections in dogs and cats to guide the rational use of antibiotics.

Materials and methods

Materials and reagents

The *S. aureus* BNCC337371 (methicillin-resistant) used in this study was purchased from BeNa Culture Collection (Henan, China), and all other strains involved in this study were preserved in our laboratory (National Key Laboratory of Veterinary Public Health and Safety,

College of Veterinary Medicine, China Agricultural University, Beijing, China) The specific information of the strains was listed in Table S1. *Francisella novicida* Cas12a (FnCas12a) was pre-expressed and purified in our lab as previously described [41]. TwistAmp® Liquid Basic kit was purchased from TwistDX (Cambridge, UK). HiScribe™ T7 High Yield RNA Synthesis kit, NEBuffer™ r3.1, and DNase I were provided by New England BioLabs (Beijing, China). E.Z.N.A. MicroElute RNA Clean-up kit was purchased from Omega Bio-Tek (Georgia, USA). RNase inhibitor was purchased from Takara Biomedical Technology Co. (Beijing, China). TIANgel Purification kit was purchased from Tiangen Biotech Co (Beijing, China). The synthesis of primers and ssDNA reporter, as well as the gene sequencing, were completed by Sangon Biotech (Shanghai, China). Tecan Infinite 200 PRO was purchased from Tecan Trading AG (Männedorf, Switzerland). Blue LED transilluminator was purchased from Shanghai Life Lab Biotech Co. (Shanghai, China).

Culture of bacteria and extraction of genome

The bacteria were inoculated on Columbia Blood Agar (Aobox, Beijing, China) and cultured for 24 h at 37 °C. A single colony was placed in 45 μL DNA extraction buffer (50 mM NaOH), heated at 95 °C for 5 min, and added 5 μL of 1 M Tris–HCl (pH 8.0). The mixture was centrifuged at 12,000 g for 1 min. and the supernatant was the extracted crude genomic DNA of strains. The similar method was used to extract the DNA from clinical samples. Briefly, swabs for collecting clinical samples were placed in 180 μL of DNA extraction buffer and heated at 95 °C for 5 min, then 20 μL of Tris–HCl was added and centrifuged at 12,000 g for 1 min, The supernatant was crude genomic DNA of clinical samples.

Design and synthesis of PCR primers, RPA primers, crRNAs, and positive recombinant plasmids

The *spsJ* gene and the methicillin resistance gene *mecA* gene of *S. pseudintermedius* (GenBank access: CP031561) were used as target genes to design PCR primers (PCR-spsJ-F/R, PCR-mecA-F/R) and RPA primers [RPA-spsJ-F/R (1, 2), RPA-mecA-F/R (1, 2)]. The specific crRNA (crRNA-spsJ1-3, crRNA-mecA1-3) primers were designed according to the amplification sequences of PCR and RPA primers (Table S3).

To prepare crRNAs, full-length crRNA primers carrying the T7 promoter were annealed and employed as RNA transcription templates. To synthesize crRNAs, transcription was performed overnight at 37 °C using T7 High Yield Transcription kit. After transcription, the DNA templates were digested with DNase I. E.Z.N.A. MicroElute RNA Clean-up kit was used to purify the crRNAs.

To create the *spsJ* and *mecA* recombinant plasmids, the *spsJ* and *mecA* genes of *S. pseudintermedius* were employed as the target genes. Partial sequences containing the target sequences of the *spsJ* and *mecA* genes were constructed into pUC57 backbone plasmids. Table S4 showed the partial sequences of *spsJ* and *mecA* contained in the recombinant plasmids.

PCR and RPA reaction

Each PCR reaction (25 μL) contained 12.5 μL 2×TIANGE® Taq PCR Mix, 1 μL of each forward and reverse primers (10 μM), 1 μL of DNA template, and 9.5 μL DNase/RNase-free deionized water. The reaction conditions were as follows: predenaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 20 s. and final extension at 72 °C for 5 min. The reaction product was purified using TIANgel Purification kit.

The RPA was performed using the TwistAmp® Liquid Basic kit according to the instructions, with might modifications. Each RPA reaction (12.5 μL) contained a premix of 6.25 μL of 2×Reaction Buffer, 0.9 μL of dNTPs (25mM), 1.25 μL of 10×E-mix, 0.6 μL each of forward and reverse RPA primers, 0.625 μL of 20×Core Reaction mix, 0.5 μL of DNA templates, 1.15 μL of DNase/RNase-free deionized water, and 0.625 μL of MgOAc was added at the end. The system mixture was incubated at 37 °C for 15 min to obtain the amplified products. Confirmation of the amplification product using agarose gel electrophoresis required that the RPA reaction product be heated at 65 °C for 10 min.

Establishment of RPA-CRISPR/Cas12a detection

CrRNA-spsJ1-3 and crRNA-mecA1-3 were used in CRISPR/Cas12a reactions. Each CRISPR/Cas12a reaction (20 μL) contained 30 nM Cas12a, 2 μM crRNA, 1 μL RPA amplification product, 500 nM ssDNA, 0.5 μL RNase inhibitor, 1.5 μL NEB r3.1 buffer, and DNase/RNase-free deionized water replenished the total volume to 20 μL. The system was placed in fluorescence microplate reader (Tecan Infinite 200 PRO), and the real-time fluorescence intensity was detected at 37 °C (excitation wavelength, 480 nm; emission wavelength, 520 nm). Alternatively, the reaction system was placed at 37 °C for 15 min, the reaction products were placed under blue light, the fluorescence was observed with the naked eye and photos were taken with a camera, and the endpoint fluorescence intensity of the reaction for 15 min was measured using fluorescence microplate reader.

Sensitivity of RPA-CRISPR/Cas12a detection system

The *spsJ* and *mecA* plasmids were extracted, purified and quantified by Nanodrop 2000C (Thermo Fisher Scientific, Waltham, MA, USA). The copy number was calculated, and the plasmids were diluted to a concentration of 10^6–10^0 copies per μL in a 10-fold gradient using DNase/RNase-free deionized water. The plasmids with different copy numbers were used as templates for the RPA-CRISPR/Cas12a detection.

Specificity of RPA-CRISPR/Cas12a detection system

To verify the specificity of the RPA-CRISPR/Cas12a assay for detecting *spsJ* and *mecA* genes, genomic DNA extracted from 10 MRSP strains, 10 MSSP strains, 6 *Staphylococcus* strains of non-*S. pseudintermedius* and 7 non-*Staphylococcus* strains using DNA extraction buffer were used for RPA-CRISPR/Cas12a reaction. The specific information of the strains was listed in Table S1.

Detection of clinical samples

We collected 47 clinical samples from China Agricultural University Veterinary Teaching Hospital (Beijing, China), including 21 samples from the skin and 26 samples from the ear canal. All clinical samples were collected with the consent of pet owners. Clinical samples were obtained by repeatedly wiping the lesion site or collecting the lesion secretions using sterile swabs. As gold standards, the sample swabs were inoculated on MSA and cultured at 37 °C for 24 h. PCR reactions were performed on yellow colonies on MSA using PCR-spsJ-F/R and PCR-mecA-F/R primers. After that, PCR products were sequenced and compared on the NCBI website to identify whether the clinical samples were cultured with MRSP or MSSP (culture + PCR). If both *spsJ* and *mecA* genes were detected in the colonies, the colonies were considered as MRSP and the corresponding clinical samples contained MRSP. If only the *spsJ* gene but not the *mecA* gene was detected, the colonies were considered MSSP, and the corresponding clinical samples contained MSSP. If no colonies were cultured or the *spsJ* gene was not detected from the colonies, the corresponding clinical samples did not contain *S. pseudinterm*edius. This result was used as the gold standard. To evaluate the effect of the RPA-CRISPR/Cas12a detection system in clinical samples, sample swabs were placed in DNA extraction buffer to extract crude genomic DNA which was directly detected by RPA-CRISPR/Cas12a and direct PCR.

Statistical analysis

All experimental results were shown as mean average with standard deviation (SD) with triplicates, and data were processed by one-way ANOVA using GraphPad Prism 8.0. $^{***}P < 0.001$ was considered to indicate a statistically significant difference.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44280-023-00021-z.

Additional file 1: Fig. S1. Endpoint fluorescence intensities of RPA products involved in Cas12a reactions at different reaction times. **Fig. S2.** Results for direct detection of the *spsJ* and *mecA* genes in 47 clinical samples by direct PCR. **Table S1.** Bacterial strains used in specificity of RPA-CRISPR/Cas12a detection. **Table S2.** Gold standard, RPA-CRISPR/Cas12a and direct PCR results of 47 clinical samples. **Table S3.** The primer sequences used in this study. **Table S4.** The *spsJ* and *mecA* gene sequences contained in the recombinant plasmids.

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Authors' contributions

P.G., D.Z., and Y.Z. conceived and designed the experiments; S.S. provided the methodology and discussion. P.G. performed the experiments and analyzed the data; P.G. and Y.Z. wrote the manuscript; Y.Z. and D.Z. participated in critical review of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Declarations

Ethics approval and consent to participate

The study protocol was approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Committee (Issue No. AW11503202-2–5).

Competing interests

There is no conflict of financial interests.

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