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Rapid detection of zoonotic *Streptococcus suis* serotype 2 and 14 by enzyme-activated probe fluorescence quantitative PCR method

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Abstract

Streptococcus suis serotypes 2 and 14 are the most common zoonotic strains, but previous identification methods made distinguish these two serotypes from other S. suis serotypes difficult. To effectively prevent and control them, there is an urgent need for a highly sensitive and specific method to identify these two serotypes. In this study, a fluorescent probe was designed for the single nucleotide polymorphism site at *cpsK* 483 of *Streptococcus suis* type 2 and type 14 compared with other serotypes, and an enzyme-activated probe quantitative PCR (EA-probe qPCR) method was established for the detection of *Streptococcus suis* type 2 and type 14 by combining with the specific hydrolysis characteristics of the RNase H2 enzyme. The results showed that the optimal probe concentration for this method was 0.5 μM and the optimal RNase H2 enzyme concentration was 25 mU.This method showed no reactivity with genomic DNA from *Streptococcus suis* strains 1/2, 5, 7, 9, 23, 28, 29, and 31, confirming its high specificity. And its sensitivity can reach 18.4 CFU. In addition, 19 clinical strains of *Streptococcus suis* type 2 or type 1/2 were tested. The results showed 100% agreement with the gene sequencing method. In conclusion, this method can meet the needs of accurate laboratory testing of *Streptococcus suis* serotypes 2 and 14 and has value for clinical prevention.

Keywords *Streptococcus suis* serotypes 2 and 14, Single-nucleotide polymorphism, EA-probe qPCR, Rapid detection, RNase H2 enzyme

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Introduction

Streptococcus suis (SS) is a Gram-positive bacterium responsible for the streptococcal disease with a high mortality rate in pigs. Most of the SS isolates obtained from sick pigs belonged to serotype 1, 1/2, 9 and 14, out of the 29 porcine streptococcal serotypes [1]. Additionally, SS is a significant zoonotic pathogen resulting in clinical symptoms such as meningitis, septicemia, endocarditis, and endophthalmitis in humans [2]. Human cases of SS infection were first identified in Denmark in 1968 [3] and have since been reported in various Asian countries, including China, Thailand and Vietnam, as well as in Western countries such as the Netherlands [4]. In Asia, human SS infections are typically associated with exposure to diseased pigs or contaminated pork products [5]. In contrast, in Western countries, human infections are generally sporadic [6]. Among the 29 SS serotypes, 11 serotypes have been reported to be linked with human infection cases, including serotypes 1, 2, 4, 5, 7, 9, 14, 16, 21, 24, and 31 [7, 8]. SS serotype 2 is the most prevalent in human infections, accounting for 93.4% of cases, followed by type 14 at 5.2% [9], underscoring the critical need for epidemiological surveillance of these serotypes. Therefore, a rapid, accurate, and sensitive typing assay for SS serotype 2 and 14 is crucial for evaluating their potential public health risks.

Traditional serological identification methods are limited in the detection of SS. The slide agglutination test is time-consuming and results are less reliable due to its subjective nature. In addition, the standard diagnostic sera required for this method are difficult to be prepared and expensive. In recent years, PCR has been widely used for the detection of SS due to its high sensitivity, specificity, and reproducibility [10–13]. However, due to the high degree of homology between the *cpsK* genes of SS serotype 1/2 and 2 or 1 and 14, conventional PCR-based assays were unable to distinguish between these serotypes. PCR methods based on single nucleotide polymorphisms (SNP) sites have been established for the differential detection of SS serotype 1/2 from 2 and serotype 1 from 14, but the method requires sophisticated dedicated instrumentation, i.e. polymerase chain reaction-restriction fragment length polymorphisms, high-resolution melting curves and mismatch amplification mutation detection [14–16].

In this study, we developed an enzyme-activated fluorescence quantitative polymerase chain reaction (qPCR) method for detecting the SNP at position 483 of the *cpsK* gene in SS serotypes 2 and 14. Utilizing the precise recognition abilities of the RNase H2 enzyme on the nucleotide SNPs, it enables rapid, accurate, and sensitive typing of SS serotypes 2 and 14. This convenient method meets the requirements for high-throughput laboratory detection and can be used for both differential detection and epidemiological investigations.

Materials and methods

Bacterial culture

SS standard strains (serotype 1/2, serotype 2, serotype 5, serotype 7, serotype 9, serotype 14, serotype 23, serotype 28, serotype 29, and serotype 31) and 19 clinical strains of serotype 1/2 or 2 identified by Multiplex PCR are kept at the laboratory in Guangdong Animal Health Institute [17].

Genomic DNA extraction

All SS strains were grown about 12 h in tryptic soy broth (TSB) medium (Difco) at 37℃. The details of genomic DNA extraction are as follows: The bacteria pellet was collected after centrifugation at 13,523 rcf for 2 min. Then, it was resuspended in PBS and centrifuged again at 13,523 rcf for 4 min. This process was repeated three times. The washed bacterial pellet was resuspended in 100 µL of double-distilled water (dd-H_2O). The suspension was boiled at 100℃ for 5 min, then centrifuged at 13,523 rcf for 2 min. The supernatant was stored at -20℃ and used as template DNA for amplification.

Primer design

Specific primers and probes were designed according to the SS *cpsK* gene sequence (GenBank: AF118389) by using the Primer5 software. Probes were targeted to the 483rd position of the *cpsK* gene. All oligonucleotide primers and probes (Table 1) were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Basic EA-probe qPCR reaction system

The reaction mixture for EA-probe qPCR consisted of 10 µL Taq HS Mix (Takara), 0.2 µM primers P1 and P2, 25 mU RNase H2 enzyme (Integrated DNA Technologies) and 0.4 µM probe. Then, 1 µL of the DNA sample was added, and the final volume was adjusted to 25 µL with dd-H_2O. A Light Cycler 480 (Roche) was employed to perform EA-probe qPCR, following a program with an

Table 1 Sequences of primers and probes for EA-PCR

Primers and probes	Sequences (5′-3′)	Length (bp)
P1	AGCGGCAGGAATGTTTGTAA	20
P2	CATAGAGCAAGCGATAAGTGAAGTA	25
Probe	TGGTGGCC**T**(FAM)G**G**(RNA)AA**T**(BHQ1)AAACTCT-C3 Spacer	21

Note The bold base indicated it was modified as the manner in the bracket.

initial denaturation step at 95℃ for 2 min, 40 cycles of denaturation step of at 95℃ for 20 s, an annealing step of at 55℃ for 20 s, and an extension step at 72℃ for 40 s.

Optimization of the EA-probe qPCR reaction system

The optimal probe dose was determined by performing an EA-probe qPCR test with different concentrations including 0 μM, 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM, 0.5 μM, 0.6 μM, and 0.7 μM. Similarly, the RNase H2 enzyme was set at various doses with 0 mU, 5 mU, 10 mU, 20 mU, 25 mU, 30 mU, and 35 mU. The optimization was performed with SS serotype 2 DNA.

Sensitivity test

Colony-forming units (CFU) of SS serotype 2 culture were determined by using colony-counting agar plates. In detail, the culture was 10-fold diluted by using sterile distilled PBS. Then, the 10-fold serial-dilution were respectively spread on tryptic soy broth (TSB) medium at 37℃. After 24 h of incubation, colonies were counted. Meanwhile, genomic DNA of each 10-fold serial-dilution was extracted by boiling at 100°C for 5 min. Then, 1μL dilution was directly used as the template for the EA-probe qPCR.

All SS strains were grown about 12 h in tryptic soy broth (TSB) medium (Difco) at 37℃. The details of genomic DNA extraction are as follows: The bacteria pellet was collected after centrifugation at 13,523 rcf for 2 min. Then, it was resuspended in PBS and centrifuged again at 13,523 rcf for 4 min. This process was repeated three times. The washed bacterial pellet was resuspended in 100 μL of double-distilled water (dd-H_2O). The suspension was boiled at 100℃ for 5 min, then centrifuged at 13,523 rcf for 2 min. The supernatant was stored at -20℃ and used as template DNA for amplification.

Specificity test

To assess the specificity of the EA-probe qPCR method, genomic DNA was extracted from SS standard strains (serotypes 1/2, 5, 7, 9, 23, 28, 29, and 31) as previously described, and then the qPCR was performed using the optimized reaction mixture.

Application of the EA-probe qPCR method in clinical detection

In our laboratory, 19 clinical SS strains were identified as serotype 1/2 or 2 using Multiplex PCR [11]. To evaluate the clinical detection capability of the EA-probe qPCR method, these clinical SS strains were tested. Concurrently, the sequencing results of the 483rd position of the *cpsK* gene, amplified using the P1/P2 primer pair, were compared with results obtained using the EA-probe qPCR method.

Results

Establishment and optimization results of basic reaction system

The fundamental reaction system of EA-probe qPCR was employed to evaluate the generation of fluorescent signals utilizing SS serotype 2 and serotype 14 DNA as templates. In contrast, the negative control did not produce any fluorescent signal (Fig. 1A). The optimal probe dose was determined by varying the probe concentration from 0 μM to 0.7 μM. When the probe concentration was 0.5 μM, amplification efficiency was good, with no significant difference compared to higher concentrations (Fig. 1B). A probe concentration of 0.5 μM was therefore selected as the optimal. An optimization test was also conducted on the RNase H2 enzyme. The results showed that an RNase H2 enzyme concentration of 25 mU had the best reaction and amplification efficiency (Fig. 1C). Therefore, 25 mU RNase H2 enzyme was selected as the optimal dose. Finally, the optimized reaction mixture for EA-probe qPCR consisted of 10 μL Taq HS Mix, 0.2 μM primers P1 and P2, 25 mU RNase H2 enzyme, and 0.5 μM probe.

Sensitivity test results

After plate counting of SS serotype 2 at a concentration of 1.84×10^5 CFU, 1 μL of each sequential 10-fold gradient dilution was used as a template for the EA-probe qPCR assay, following the optimal reaction conditions to identify the minimum detection limit of the EA-probe qPCR method. The results showed that the EA-probe qPCR had a sensitivity as low as 18.4 CFU (Fig. 2). Although low amplification signals emerged when gradient dilution templates were lower than 18.4 CFU (Ct > 35), they could not be distinguished from the negative group. The Cycle Threshold (Ct) refers to the number of PCR cycles required for the fluorescence signal to exceed the set threshold during qPCR. Hence, we set the cut-off value of this method at Ct < 35.

Specificity test results

The results are shown in Fig. 3, where genomic DNA from SS serotypes 2 and 14 produced a fluorescent signal, while genomic DNA from eight standard SS strains did not. Thus, the EA-probe qPCR method is highly specific.

Evaluation of EA-probe qPCR by testing clinical SS strains

Sequencing of 19 clinical SS strains revealed that three strains contained T nucleotides at position 483 of the *cpsK* gene, while sixteen strains had G nucleotides. This indicates that three of these strains belong to SS serotype 1/2, while sixteen of them belongs to SS serotype 2. The DNA extraction from these strains followed previously described methods [18]. The EA-probe qPCR results showed a fluorescent signal for 16 strains and no fluorescent signal for three strains. The results of the EA-probe

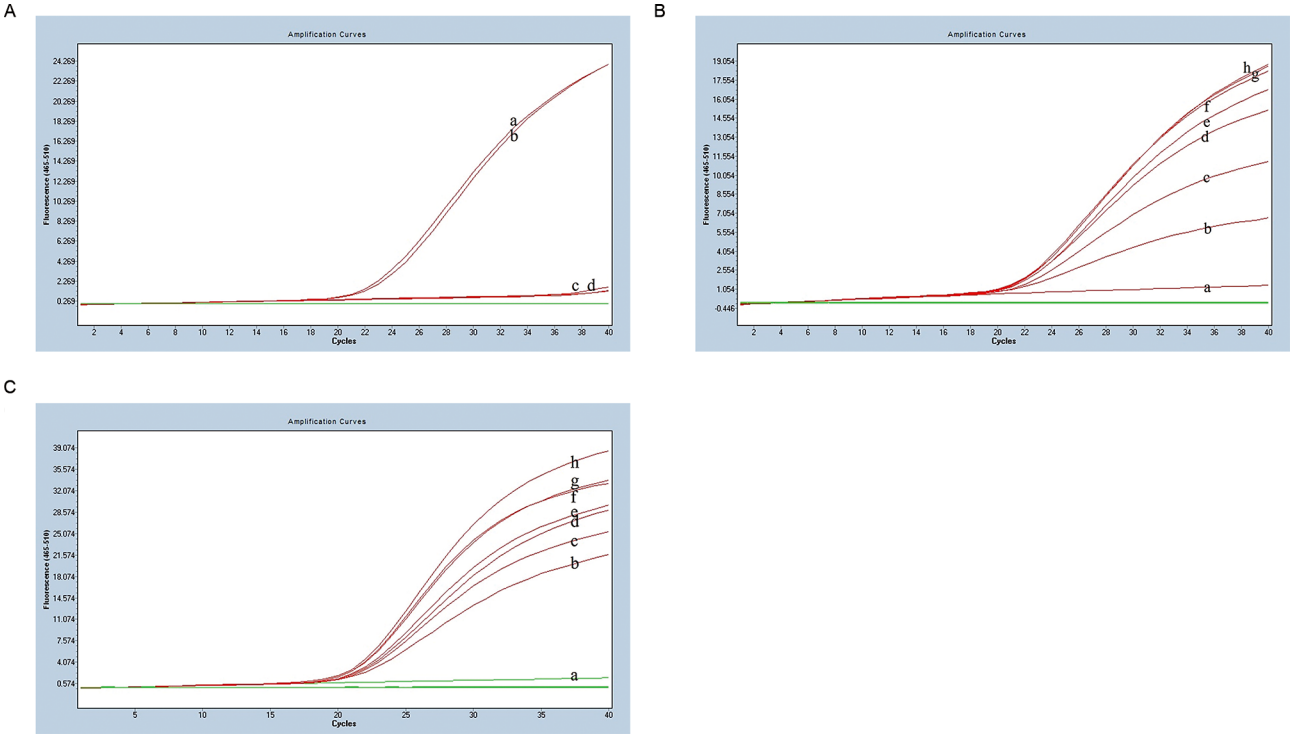

Fig. 1 Establishment of the reaction system and the results of the optimization: (**A**) Results of establishing the basic reaction system. (a) SS serotype 2 genomic DNA, (b) SS serotype 14 genomic DNA, (c) and (d) Negative control. (**B**) Optimization results of probe concentration for the EA-probe qPCR Method. Probe concentration: (a) 0 μM, (b) 0.1 μM, (c) 0.2 μM, (d) 0.3 μM, (e) 0.4 μM, (f) 0.5 μM, (g) 0.6 μM, (h) 0.7 μM. (**C**) Optimization results of RNase H2 enzyme concentration for EA-probe qPCR Method. RNase H2 enzyme dosage: (a) 0 mU, (b) 5 mU, (c) 10 mU, (d) 15 mU, (e) 20 mU, (f) 25 mU, (g) 30 mU, (h) 35 mU

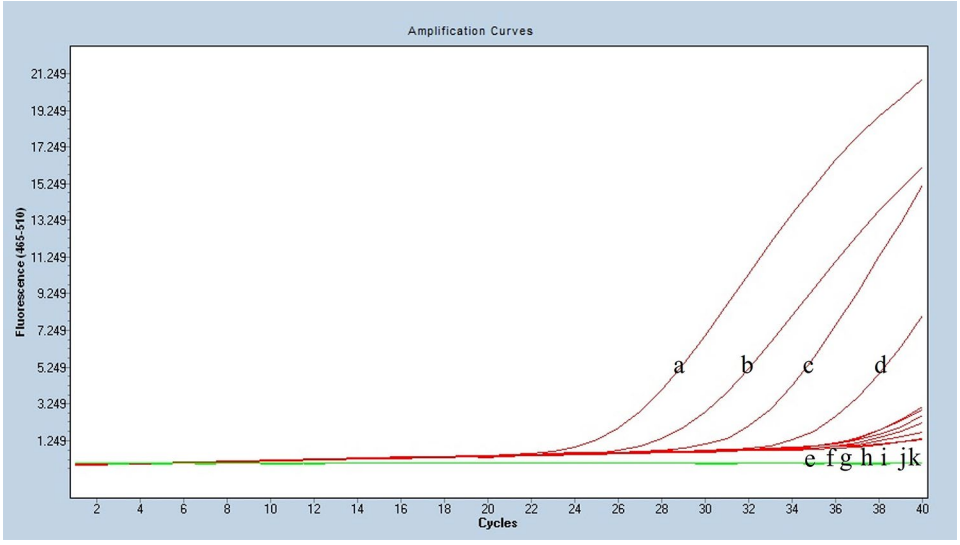

Fig. 2 Sensitivity test results: The EA-probe qPCR method was used to detect genomic DNA from SS serotype 2 at the following diluted concentrations: (a) 1.84×10^{4} CFU, (b) 1.84×10^{3} CFU, (c) 1.84×10^{2} CFU, (d) 1.84×10^{1} CFU, (e) 1.84×10^{0} CFU, (f) 1.84×10^{-1} CFU, (g) 1.84×10^{-2} CFU, (h) 1.84×10^{-3} CFU, (i), (j) and (k) Negative control

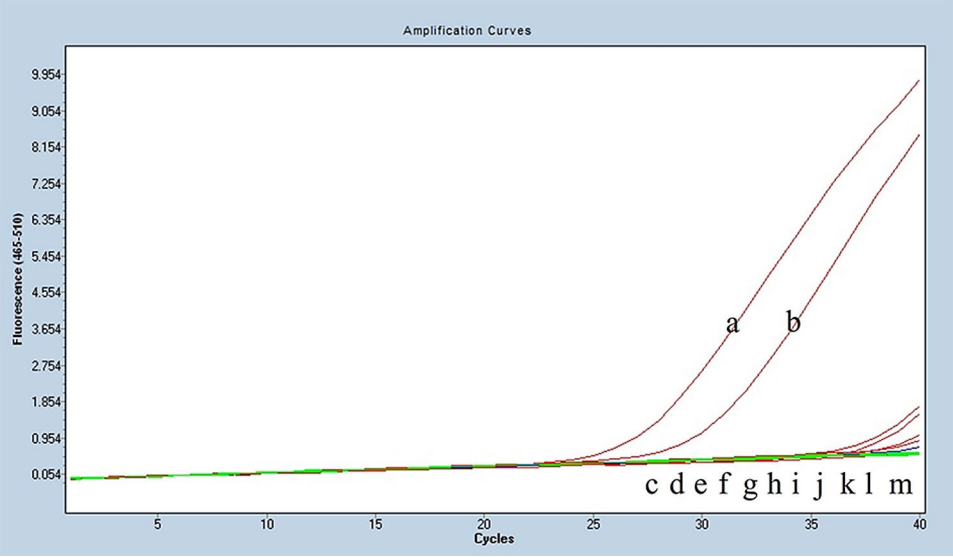

Fig. 3 Specificity test results: The EA-probe qPCR method was conducted with genomic DNA from (a) SS serotype 2, (b) serotype 14, (c) serotype 1/2, (d) serotype 5, (e) serotype 7, (f) serotype 9, (g) serotype 23, (h) serotype 28, (i) serotype 29, (j) serotype 31, (k), (l), and (m) Negative control

Table 2 Evaluation of EA-probe qPCR by testing clinical SS strains

Number	*Streptococcus suis* strains	Multiplex PCR	Locus 483 of the *cpsK* gene by sequencing	Serotypes	EA-probe qPCR
1	GZ-156	Serotypes 2 or 1/2	G	2	+
2	GZ-894	Serotypes 2 or 1/2	T	1/2	-
3	GZ-1016	Serotypes 2 or 1/2	G	2	+
4	GZ-746	Serotypes 2 or 1/2	G	2	+
5	GZ-759	Serotypes 2 or 1/2	G	2	+
6	GZ-766	Serotypes 2 or 1/2	G	2	+
7	GZ-783	Serotypes 2 or 1/2	G	2	+
8	GZ-1018	Serotypes 2 or 1/2	T	1/2	-
9	GZ-804	Serotypes 2 or 1/2	G	2	+
10	GZ-820	Serotypes 2 or 1/2	G	2	+
11	GZ-834	Serotypes 2 or 1/2	G	2	+
12	GZ-840	Serotypes 2 or 1/2	G	2	+
13	GZ-905	Serotypes 2 or 1/2	G	2	+
14	GZ-953	Serotypes 2 or 1/2	G	2	+
15	GZ-964	Serotypes 2 or 1/2	G	2	+
16	GZ-975	Serotypes 2 or 1/2	T	1/2	-
17	GZ-1084	Serotypes 2 or 1/2	G	2	+
18	GZ-1079	Serotypes 2 or 1/2	G	2	+
19	GZ-1089	Serotypes 2 or 1/2	G	2	+

Note (+) Positive, (–) Negative

qPCR method and the sequencing method were in agreement (Table 2).

Discussion

SS has posed a serious economic impact on the pig industry. Both SS serotypes 2 and 14 are significant human and veterinary pathogens that endanger the general public's health. Serotyping methods for SS are essential for controlling and prevention of zoonotic disease outbreaks. Therefore, rapid, specific, and sensitive detection methods for SS serotypes 2 and 14 are urgently needed. We designed an RNase H2 enzyme-activated probe fluorescence quantitative PCR method based on the specific SNP site in the *cpsK* gene of SS serotype 2 and 14, proposing a novel detection method for these serotypes. Previous studies have shown that the SNP at *cpsK* position 483 in SS serotypes 2 and 14 is an ideal molecular target that can be used to differentiate between types 1/2 from 2 and between types 1 from 14 [19]. In this study, we developed an enzyme-activated probe-fluorescence quantitative PCR (EA-probe qPCR) method based on the SNP at position 483 in the SS serotypes 2 and 14 *cpsK*

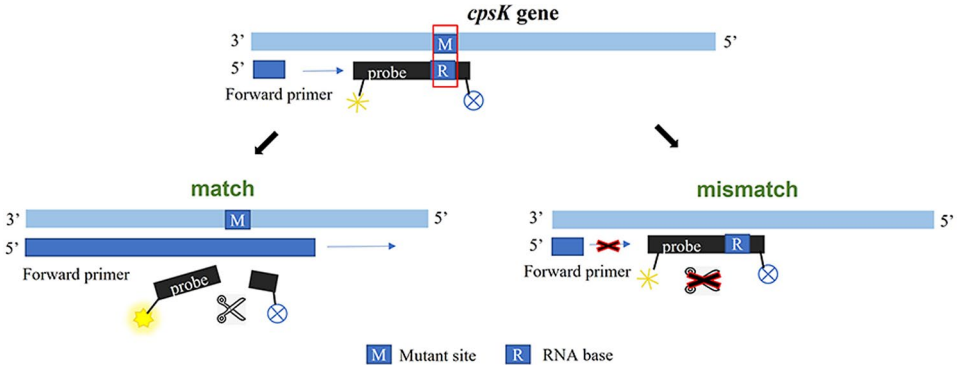

Fig. 4 Principle of EA-probe qPCR Method. The scissors represent the hydrolytic activity of RNase H2. The left side of the R nucleotide contains the quencher dye, while the right side contains the reporter dye.

gene. This method utilizes a ribonucleotide insertion and a probe modified with FAM fluorescent and BHQ1 quenching motifs, combined with the specific hydrolytic properties of RNase H2. Based on the principle of RNase H2, G deoxyribonucleotides at the 483 site in the probe is replaced with a ribonucleotide. When the ribonucleotide in the probe pairs complementarily with the deoxyribonucleotide at the corresponding site in the target gene, RNase H2 hydrolyses the paired site, causing the FAM fluorescent motif to separate from the BHQ1 quenching moiety and the generating a fluorescent signal. Conversely, the special Taq DNA Polymerase used in this study lacks exonuclease activity, so it cannot cleave and the FAM fluorescent motifs in the enzyme-activated probes like the work principle of Taq-Man probes. If the probe is mismatched with the amplification template, the ribonucleotide cannot pair complementarily with the corresponding deoxyribonucleotide, preventing RNase H2 from hydrolyzing it and resulting in no fluorescent signal (Fig. 4).

This method significantly enhances the detection of SNPs through the specificity of the probes. PCR-restriction fragment length polymorphism method was a choice for the differentiation of SS serotypes 1/2 versus 2 and 1 versus 14, but this method requires gel electrophoresis. In contrast, our EA-probe qPCR method does not require opening the cap to read the results. It is not only easy to be perform but also avoids aerosol contamination. Additionally, EA-probe qPCR results are interpreted similarly to those of TaqMan probe based qPCR, avoiding the subjective instability associated with high-resolution melting curves analysis. In recent years, whole-genome sequencing (WGS) has emerged as an alternative for differentiation SS serotypes [20]. However, it requires specialized laboratory equipped with sequencers. To address this limitation, we developed a probe based LAMP method, which is well-suited for field and on-site detection, to rapid detect SS serotype 2 and 14 [18]. Compared to the probe-based LAMP method, our current EA-probe qPCR method is more suitable for routine diagnostic laboratories and high throughput detection. Moreover, this method has a low limit of detection of 18.4 CFU, making it sufficiently sensitive for routine detection.

In conclusion, the EA-probe qPCR method developed for SS serotypes 2 and 14 is easy to perform and suitable for rapid, high-throughput analysis in routine diagnostic laboratories. Therefore, this method shows great potential for differentiating zoonotic SS infections.

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Author contributions
YS, JM and MZ carried out the experiment and drafted the manuscript. PC, ZB, and DY participated in the experiments. CL, SZ, YL, KZ and ZJ participated in the analysis of the data. CX and HG conceived the idea and design the study. All authors have read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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