A one-step closed-tube enzyme-activated blocked probe assay based on SNP for rapid detection of *Salmonella* Pullorum

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ABSTRACT *Salmonella* enterica serovar Gallinarum biovars Pullorum (*S.* Pullorum) is an infectious bacterial pathogen in the poultry industry that causes systemic pullorum disease. This disease causes great losses in terms of the clinical production and quality of chicken products in breeding farms. However, an acknowledged usable rapid detection method for its specific identification has not been reported, and it is generally difficult to distinguish from fowl typhoid caused by *Salmonella* enterica serovar Gallinarum biovars Gallinarum. The development of a specific and rapid detection method for this pathogen is therefore needed. In the present study, we targeted the single-nucleotide mutation position 237 of the *S.* Pullorum rfbS gene to develop an enzyme-activated blocked probe for its clinical rapid detection. The method displayed robust specificity and reproducibility, and it achieved minimal detection limits of 21 copies/μL of copy number and 4.53 pg/μL of genomic DNA. Compared with traditional identification and PCR methods, this method performed better for the detection of 100 clinical actual samples and without false negative results. The entire process can be accomplished in a 1-step closed-tube operation, overcomes the difficulties currently associated with *S.* Pullorum detection, and provides a specific and rapid method with broad application potential for SNP detection.

Key words: *Salmonella* Pullorum, SNP, enzyme-activated blocked probe (EA probe), rapid detection

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INTRODUCTION

Salmonella enterica serovar Gallinarum biovars Pullorum (*S.* Pullorum) causes pullorum disease, an acute infectious disease of poultry that can result in huge losses (Shah et al., 2005; Hu et al., 2019). The key to preventing and controlling pullorum disease is precise diagnosis and removal of infected birds (Christensen et al., 1993). However, it is easily confused with fowl typhoid caused by *Salmonella* enterica serovar Gallinarum biovars Gallinarum (*S.* Gallinarum) in the clinic. These diseases share similar clinical symptoms in chicks and mature fowl, hence it is difficult to distinguish them based only on superficial symptoms (Shivaprasad, 2000; Barrow et al., 2011). Traditional identification methods for *S.* Pullorum are time-consuming and labor-intensive, taking several day to obtain results. Moreover, the results of serologic typing methods are often indistinguishable owing to nonspecific reactions and a lack of sensitivity. Because *S.* Pullorum and *S.* Gallinarum belong to White-Kauffmann-LeMinor scheme serogroup D, they display "O" antigens 1, 9 and 12 and exhibit high cross-reactivity with each other and other serogroup D serovars such as *Salmonella* enterica serovar Enteritidis (*S.* Enteritidis) (Gast, 1997; Proux et al., 2002; Issenhuth-Jeanjean et al., 2014; Ren et al., 2017; Ma

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et al., 2018). Accordingly, the development of a highly specific rapid detection method for *S.* Pullorum is immediately needed.

Various methods have been published for rapid *S.* Pullorum detection, but they either cannot distinguish *S.* Pullorum from *S.* Gallinarum or the target gene is located on a plasmid and easily misdetected. Although some methods use SNP for a more specific detection of *S.* Pullorum, these methods are based on PCR amplification. The electrophoresis process is troublesome, and tubes must be opened, which can result in contamination (Shah et al., 2005; Cheraghchi et al., 2014; Xiong et al., 2016; Xiong et al., 2017; Xiong et al., 2018). Therefore, it would be useful to have an accurate, closed-tube, 1-step assay available for rapid *S.* Pullorum detection.

The cycling probe method is a real-time PCR approach that can be applied to identify SNP using a cycling probe with the ability to distinguish a single-base mutation. This method has been used for the detection of SNP and certain pathogens using a CycleavePCR Starter Kit (Takara Bio) (Yatabe et al., 2006; Yabutani et al., 2009; Suzuki et al., 2010; Hou et al., 2011; Suzuki et al., 2011; Liu et al., 2014; Nan et al., 2016). However, we found that the 5'−3′ exonuclease activity of Taq DNA polymerase may interfere with the ability of the strictly designed short probe to recognize the SNP. Based on this knowledge, we propose a new enzyme-activated blocked probe (**EA-probe**) method to resolve these problems.

In the present study, we successfully developed an EA-probe method that is better than the cycling probe technique, and established a 1-step closed-tube rapid detection method for the clinical specific identification of *S.* Pullorum as per the nucleotide at position 237 of the rfbS gene sequence. The assay overcomes our inability to rapidly and specifically detect *S.* Pullorum, and it has broad application potential for SNP rapid detection.

MATERIALS AND METHODS

Bacterial Strains

The following 95 bacterial strains were used in specificity tests (Supplementary Table 1): various *Salmonella* serovars (57 strains of *S.* Pullorum, 5 strains of *S.* Gallinarum, 5 strains of *S.* Enteritidis, *Salmonella* Choleraesuis, *Salmonella* Saintpaul, *Salmonella* Indiana, *Salmonella* Agona, *Salmonella* Meleagridis, *Salmonella* Goldcoast, *Salmonella* Infantis, *Salmonella* Mbandaka, *Salmonella* Havana, *Salmonella* Kentucky, *Salmonella* Cerro, *Salmonella* Typhimurium, *Salmonella* Corvallis, *Salmonella* Derby, *Salmonella* Rissen, *Salmonella* London, *Salmonella* Weltevreden, *Salmonella* Albany, and *Salmonella* Panama) and 9 non-*Salmonella* species (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Campylobacter coli*, *Escherichia coli*, *Listeria monocytogenes*, *Riemerella anatipestifer*, *Listeria iuanuii*, and *Shigell*a). All were purchased as standard strains or samples isolated from various markets in Guangzhou, which had been previously identified and stored at −40°C by the laboratory of the Veterinary College, South China Agricultural University, Guangzhou, China.

Bacterial Culture and Genomic DNA Extraction

The stored strains were recovered on xylose lysine tergitol-4 agar or brain heart infusion agar (Huankai Microbiology Technology Corporation, Guangdong, China) at 37°C overnight. Colonies were transferred to Luria Bertani broth or brain heart infusion broth and cultured at 37°C with shaking at 180 rpm for 16 h. Genomic DNA was extracted using a TIANamp Bacteria DNA Kit (TIANGEN Biochemical Technology Corporation, Beijing, China) or via the boiling method described previously (Youn et al., 2016) and stored at −20°C until analysis.

Construction of Standard Plasmids

Based on the rfbS gene sequence of *S.* Pullorum (GenBank: LK931482.1) and *S.* Gallinarum (GenBank: AF442573), primers for constructing plasmids were designed and synthesized. Full-length *S.* Pullorum and *S.* Gallinarum rfbS genes were amplified by PCR and ligated into the T-vector at 16°C for 8 h after sequence verification. Vectors were subsequently transformed into *E. coli* DH5α cells and plasmid was extracted using a commercial kit (Omega Inc.) as per the manufacturer's instructions, confirmed via DNA sequencing, and stored at −20°C until analysis.

Primer Design

Primers and probes were designed as per the *S.* Pullorum rfbS gene sequence (GenBank: LK931482.1) and the nucleotide at position 237 (Figure 1) using Primer Premier 5 (Table 1). All primers and probes were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) and stored at −20°C until use.

Establishment of the Basic Reaction System

The EA-probe method involved a basic reaction system containing 0.5U of Deep Ocean (exo-) DNA polymerase, 1 × PCR buffer, 8 mmol $MgSO_4$ (ABclonal Technology, China), 1.2 mmol dNTP mixture (Takara Bio, Dalian, China), 0.1 U/μL RNase H2 Enzyme (Integrated DNA Technologies), 2.5 μL of DNA sample, 0.2 μmol primers and blocking probe (Sangon Biotech), made up to 25 μL with deionized water. The reaction procedure was as follows: prdenaturation at 95°C for 2 min, and then, the following 40 cycles were performed, 95°C for 20 s, 55°C for 20 s, and 72°C for 40 s. Amplification was performed using a CFX96 Touch Real-time PCR Detection System (Bio-Rad). Signal acquisition was performed in FAM mode. Standard plasmids for *S.* Pullorum and *S.* Gallinarum were used for detection to

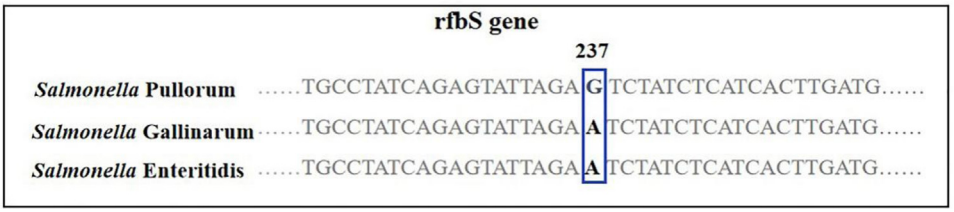

Figure 1. SNP at position 237 of the rfbS gene in the *Salmonella* D serogroup.

establish a basic reaction system. Simultaneously, the same detection template was amplified by PCR using rfbS gene primers (Table 1), sequenced by Sangon Biotech after electrophoresis, and the results were compared with those of the basic reaction system.

Optimization of the Basic Reaction System

Different annealing temperatures (53.0°C, 53.3°C, 54.0°C, 55.0°C, 56.2°C, 57.2°C, 57.7°C, and 58.0°C) and dosage of probe (1, 7.5, 5, and 2.5 pmol) were tested to establish the optimum conditions for the basic reaction system.

Comparison of Different Methods

The cycling probe method was performed as per the instructions supplied with the CycleavePCR Reaction Mix Kit (Takara Bio). Reactions contained 2.5 μL DNA template, 1 × CycleavePCR reaction mixture (including TaKaRa Ex Taq HS, dNTP mixture, Mg^{2+}, and Tli RNaseH II), cycling probe (5 μmol/L), primers (10 μmol/L), and deionized water to 25 μL. The reaction procedure was performed as per the kit instructions. High concentrations of *S.* Pullorum and *S.* Gallinarum standard plasmids were used as detection templates. The cycling probe PCR method was compared with the EA-probe method established in this study using a polymerase without exonuclease activity and TaKaRa Ex Taq HS polymerase with exonuclease activity.

Sensitivity Test

The purified recombinant plasmids were 10-fold serially diluted with deionized water and concentrations between 2.1 copies/μL to 2.1 × 10^6 copies/μL were tested. Meanwhile, genomic DNA from *S.* Pullorum strain ATCC9120 was 10-fold serially diluted from 45.3 ng/μL to 4.53 fg/μL in deionized water. All samples were analyzed in triplicate, and the results were used to establish respective standard curves. The lowest concentration of DNA (lower limit) that could be detected by our EA-probe method was thereby determined.

Specificity Test

The specificity of the EA-probe method was assessed using genomic DNA from 95 bacterial strains (Supplementary Table 1), including 57 strains of *S.* Pullorum and 38 strains of various *Salmonella* serovars and non-*Salmonella* pathogens (*S. aureus, P. aeruginosa, C. jejuni, C. coli, E. coli, L. monocytogenes, R. anatipestifer, L. iuanuii*, and *Shigell*a).

Reproducibility Test

Standard plasmids were diluted from 21 copies/μL to 2.1 × 10^6 copies/μL and used as templates to carry out EA-probe tests under the optimized reaction conditions. Three repetitions were performed in triplicate for analysis of intra-assay and interassay variability, and the CV was calculated.

Application of the EA-Probe Method

A total of 100 samples were randomly collected from a poultry farm in Guangzhou, China, in accordance with the Chinese National Standards (GB4789.4-2016), comprising 25 anal swabs, 25 liver samples, and 50 embryos. Buffered peptone water was used for preenrichment of all samples at 37°C for 8 h. Bacterial cultures were inoculated into Selenite Cystine broth and cultured at 37°C for 18 h. Subsequently, bacterial broth was streaked onto *Salmonella Shigella* agar and incubated at 37°C for 14 h. A *Salmonella* biochemical identification kit was then used for serotyping via rapid agglutination (Huankai Microbiology Technology Corporation, Guangdong, China). Samples after preenrichment in buffered peptone water overnight were extracted by the boiling method and used as detection templates to evaluate the actual application performance of our method on natural-occurring *Salmonella* isolates. Meanwhile, using a previously reported method (Xu et al., 2018), PCR primers targeting the *S.* Pullorum ipaj gene were synthesized (Sangon Biotech) to analyze and compare the same samples (Table 1). Traditional identification method (GB4789.4-2016) for *S.* Pullorum detection was then compared with the PCR method and the EA-probe method.

RESULTS

Development and Optimization of the Basic Reaction System

Position 237 is a guanine in the *S.* Pullorum rfbS gene sequence, but in *S.* Gallinarum and *S.* Enteritidis, it is an adenine. Other *Salmonella* serotypes and non-*Salmonella* pathogens do not generally contain this gene or

Table 1. Primer used in this study.

Primer name	Sequence (5′–3′)	Length (bp)
Forward primer	CGAACCTGCAACAGCTTTAATAGAAAGC	28
Reverse primer	CTCGTATTTGGTGGCAGTGATGTTC	25
EA probe	CTTATGCCTATCAGAGTATT(FAM)AGAG (RNA base) TCTAT(BHQ)CTG-C3 Spacer	32
Cycling probe	(FAM)-TATTAGAG (RNA base) TCT-Eclipse	11
rfbsF	TCACGACTTACATCCTACTTCGAAAGT	27
rfbsR	CTGCTATATCAGCACAACTATACATCAATTCAT	33
ipaJ F	TACCTGTCTGCTGCCGTGA	19
ipaJ R	ACCCTGCAAACCTGAAATC	19

very different (Figure 1). Based on this feature, we targeted position 237 of the *S.* Pullorum rfbS gene to design a reporter dye and quencher-modified mutation discrimination blocked probe with a ribonucleotide insertion based on the original real-time PCR primer sets. When the ribonucleotide perfectly matches with the mutant site, the hydrolytic mechanism of the RNase H2 enzyme is activated, releasing the quencher and blocking to generate an amplified signal, and extension carries on. Conversely, a mismatched base will not produce a signal (Figure 2A). Thus, we called it enzyme-activated blocked probe. The use of polymerases without exonuclease activity avoids the probe binding to the wild-type template when only 1 base is mismatched that can be nonspecifically cleaved by traditional Taq DNA polymerases to generate interfering fluorescent signals. Therefore, our method can specifically detect the mutation site. We successfully established the basic reaction system by testing standard plasmids to confirm the feasibility of this method (Figure 2B). Only the *S.* Pullorum standard plasmids generated amplification curves, whereas the *S.* Gallinarum standard plasmid was not detected. We simultaneously amplified the same plasmid template using traditional PCR methods and rfbS primers, and two 721-bp bands were observed after gel electrophoresis, corresponding to the length of the rfbS gene (Figure 2C). Sequencing results after gel recovery showed that the 2 bands were consistent with *S.* Pullorum and *S.* Gallinarum rfbS genes with an SNP at position 237 (Figure 2D). These results further prove that traditional PCR methods cannot achieve the required distinction, whereas the EA-probe method can be used effectively to detect mutant template, while the wild-type template cannot be detected. Thus, the basic reaction system was successfully established.

The performance of basic reaction system is subsequently improved by optimization. The experimental results showed that when the annealing temperature was 56.2°C, this method achieved the highest amplification efficiency. In addition, a low dosage of probe was sufficient for the reaction to proceed efficiently (Figure 3A). Thus, from the perspective of overall performance and cost reduction, we selected 2.5 pmol as the optimal reaction dosage (Figure 3B).

Comparison of Different Methods

To analyze the SNP detection ability of the EA-probe method, we used a high concentration *S.* Pullorum and *S.* Gallinarum standard plasmid as detection template and compared detection by the 3 methods (the EA-probe method established in this study using a polymerase without exonuclease activity, the EA-probe method using Taq DNA polymerase with exonuclease activity, and the cycling probe PCR method using Taq DNA polymerase with exonuclease activity).

The EA-probe method established in this study using a polymerase without exonuclease activity only detected a fluorescence signal from the *S.* Pullorum template, while a high concentration *S.* Gallinarum template did not result in an amplification curve (Figure 4A). By contrast, the EA-probe method and the cycling probe PCR method using Taq DNA polymerase with exonuclease activity not only generated an amplification curve from *S.* Pullorum template but also exhibited a low-level fluorescence signal from a high concentration *S.* Gallinarum template (Figure 4B and Figure 4C). Thus, the EA-probe method established in this work specifically detected mutant type template without interference from high-concentration wild-type template.

Sensitivity Test Results

The sensitivity test results showed that plasmid concentrations from 21 copies/μL to 2.1×10^6 copies/μL were successfully detected, and the detection limit was 21 copies/μL. The standard curve fitted the equation $y = -3.0124x + 38.78$ ($R^2 = 0.9944$), confirming a good linear relationship between copy number and cycle threshold (Figure 5A).

Sensitivity was also evaluated by analyzing serially diluted genomic DNA from *S.* Pullorum (ATCC9120). The results showed that concentrations from 4.53 pg/μL to 45.3 ng/μL were successfully detected. This revealed that at least 4.53 pg/μL of bacterial genomic DNA was required to identify *S.* Pullorum using this method. The standard curve fitted the equation $y = -4.1553x + 37.957$ ($R^2 = 0.9976$). Thus, there was also a strong correlation between genomic DNA concentration and cycle threshold (Figure 5B). Therefore, unknown samples could be quantified using the aforementioned standard equation.

Specificity Test Results

The results showed that only 57 *S.* Pullorum stains could be detected without interference from the 5 *S.* Gallinarum stains and the 5 *S.* Enteritidis stains. Meanwhile, the other 28 *Salmonella* serovars and non-

Salmonella strains did not generate amplification curves too (Figure 6 and Supplementary Table 1), demonstrating that the developed EA-probe method displayed excellent specificity.

Reproducibility Test Results

CV were determined for the EA-probe method established in this study; the intra-assay range was 0.03 to 1.27% and the interassay range was 0.82 to 3.37%. All results were lower than 5%, demonstrating that this method is stable and has good reproducibility (Table 2).

Application of the EA-Probe Method

To evaluate the effect of the established method in actual sample analysis, we tested 100 isolates comprising unknown serovars from anal swabs, liver samples, and embryos derived from a naturally contaminated chicken farm. The results showed that 71 samples (21 anal swabs, 15 livers, and 35 embryos) gave consistent detection results by all 3 methods; the number of positive results was 2, 5, and 22 for anal swabs, liver samples, and embryos, respectively, while the number of negative results was 19, 10, and 13, respectively. A total of 23 samples (2 anal swabs, 8 liver samples, and 13 embryos) tested positive using 2 molecular biological methods but not using the traditional identification method. Only 3 samples (2 liver and 1 embryo) tested positive using the traditional identification method and the EA-probe method established in this work but not using the PCR method. Moreover, 3 samples (2 anal swab and 1 embryo) only tested positive using the EA-probe method but not the other 2 methods (Table 3).

DISCUSSION

Pullorum disease has been almost eradicated from many developed countries in North America and Europe. However, it is still contagious and leads to serious economic losses to commercial poultry industries in many developing countries, and seriously affects the production and quality of chicken products. There is no effective true sense rapid detection method for *S.* Pullorum now. In the present work, we successfully developed a specific and rapid detection method for *S.* Pullorum using an EA-probe approach.

The lack of an ideal molecular target has proved a great hindrance to the rapid detection of *S.* Pullorum. The ipaj gene was used as a detection target by one group, but it is located on a plasmid that not all *S.* Pullorum strains carry, hence misdetection is an issue (Xu et al., 2018). Some other *S.* Pullorum detection method failed to identify a specific detection target, and *S.* Pullorum and *S.* Gallinarum could not be completely differentiated (Xiong et al., 2016; Xiong et al., 2017). In this approach, researchers decided to target a single-nucleotide mutation site for detection. As per previous research, *S.* Pullorum, *S.* Gallinarum, and *S.* Enteritidis belong to serogroup D, and the rfbS gene is a unique sequence with an SNP site in this serogroup, whereas other serotypes and pathogens lack this gene or harbor

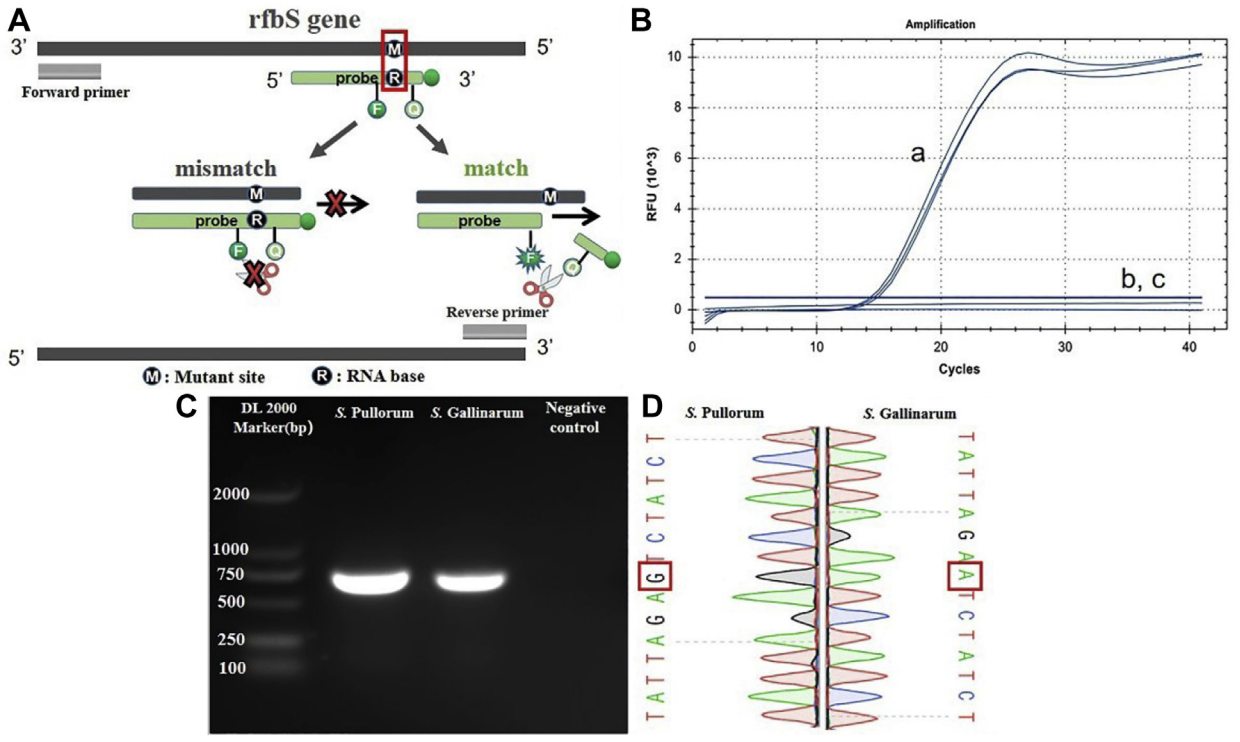

Figure 2. Developing the basic EA-probe reaction system. (A) Reaction principle. (B) Basic reaction system for the EA-probe method; (a) *Salmonella* Pullorum standard plasmid, (b) *Salmonella* Gallinarum standard plasmid, (c) Negative control. (C) Simultaneous electrophoresis of the same template. (D) Electrophoretic band sequencing results. Abbreviation: EA-probe, enzyme-activated blocked probe.

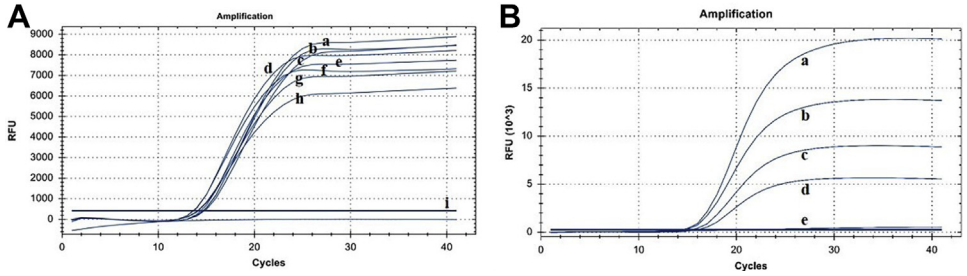

Figure 3. Optimization of the basic EA-probe reaction system. (A) Optimization of annealing temperature; (a) 53.3°C, (b) 55.0°C, (c) 54.0°C, (d) 57.2°C, (e) 57.7°C, (f) 56.2°C, (g) 53.0°C, (h) 58.0°C, (i) negative control. (B) Optimization of cycling probe usage; (a) 1 pmol, (b) 7.5 pmol, (c) 5 pmol, (d) 2.5 pmol, (e) negative control. Abbreviation: EA-probe, enzyme-activated blocked probe.

a very different gene (Luk et al., 1993). There are regular mutations in the rfbS gene at position 237, which is a guanine in *S.* Pullorum but an adenine in other strains. Therefore, SNP at position 237 provide a potentially robust molecular target for the specific detection of *S.* Pullorum.

Initially, we tried to use the previously reported cycling probe method to analyze this mutation site. However, we found that this method required strict probe design conditions and suffered from length limitations. In addition, we found that for high concentrations of wild-type templates, there were low-level interference signals. This may be because even when the cycling probe is short, it has only a single-base mismatch, hence it will inevitably bind to wild-type template. In addition, the traditional Taq DNA polymerase has 5'−3′ exonuclease activity and may also cleave the mismatch probe to generate an interference signal. Therefore, we developed the EA-probe method by designing blocked probes and using a polymerase lacking exonuclease activity. The probe design is easier and is not limited by length, and the probe with a ribonucleotide insertion can induce a fluorescence signal only when the ribonucleotide perfectly matches with the mutant site, hence the specificity is stronger and wild-type templates no longer produce low-level signal interference.

Therefore, using the unique *S.* Pullorum target and the highly specific EA-probe method, the basic reaction system was established, and only *S.* Pullorum could be detected, whereas *S.* Gallinarum and *S.* Enteritidis did not produce amplification curves. Moreover, the conventional PCR method using the same plasmid template is unable to effectively distinguish these 2 serotypes, which further highlights the outstanding usefulness of

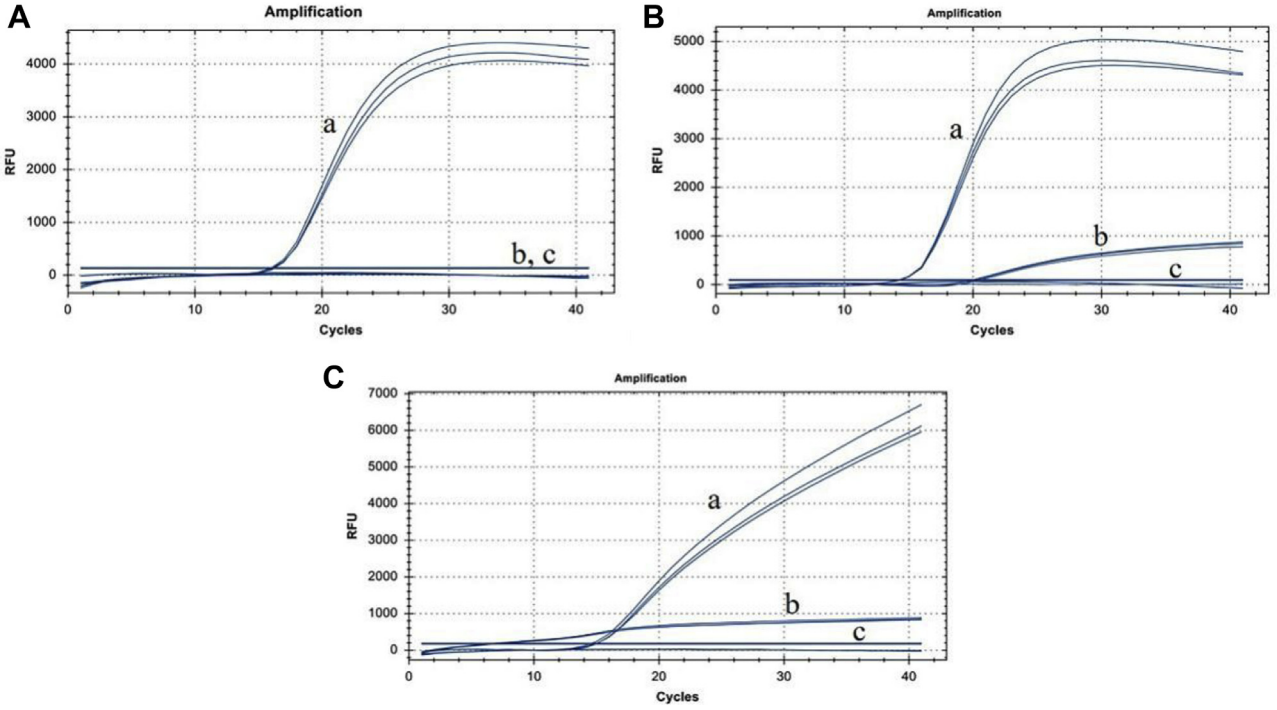

Figure 4. Comparison of the EA-probe method with other methods. (A) The EA-probe method established in this study (using a polymerase without exonuclease activity); (a) *Salmonella* Pullorum standard plasmid, (b) *Salmonella* Gallinarum standard plasmid, (c) negative control. (B) The EA-probe method (using Taq DNA polymerase with exonuclease activity); (a) *Salmonella* Pullorum standard plasmid, (b) *Salmonella* Gallinarum standard plasmid, (c) negative control. (C) Cycling probe method (using Taq DNA polymerase with exonuclease activity); (a) *Salmonella* Pullorum standard plasmid, (b) *Salmonella* Gallinarum standard plasmid, (c) negative control. Abbreviation: EA-probe, enzyme-activated blocked probe.

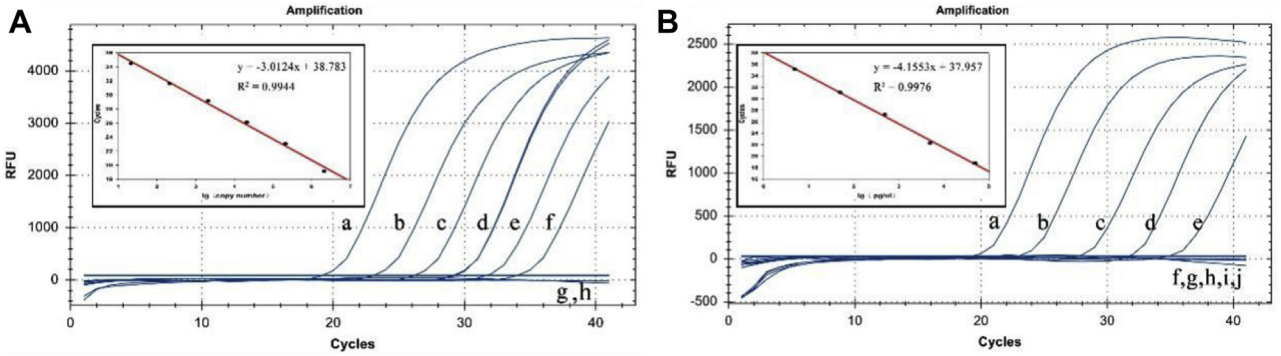

Figure 5. Sensitivity test results. (A) EA-probe analysis of plasmid concentrations. Diluted *Salmonella* Pullorum plasmid was used at the following concentrations: (a) 2.1×10^6 copies/μL, (b) 2.1×10^5 copies/μL, (c) 2.1×10^4 copies/μL, (d) 2,100 copies/μL, (e) 210 copies/μL, (f) 21 copies/μL, (g) 2.1 copies/μL, (h) negative control. (B) Cycling probe analysis of genomic DNA. Diluted *Salmonella* Pullorum genomic DNA was used at the following concentrations: (a) 45.3 ng/μL, (b) 4.53 ng/μL, (c) 453 pg/μL, (d) 45.3 pg/μL, (e) 4.53 pg/μL, (f) 453 fg/μL, (g) 45.3 fg/μL (h) 4.53 fg/μL, (i) 0.453 fg/μL, (j) negative control. Abbreviation: EA-probe, enzyme-activated blocked probe.

recognizing the single-nucleotide mutant of our method. Furthermore, unlike allelic PCR and PCR-RFLP methods for single-nucleotide mutation detection (Kwon et al., 2000; Park et al., 2002), our method does not require special restriction sites or opening tubes for electrophoresis, which risks aerosol contamination, hence our method has broader application potential.

We subsequently assessed genomic DNA from 95 bacterial strains to further examine the feasibility of this method. Only 57 strains of *S.* Pullorum generated specific amplification curves, whereas other *Salmonella* serovars and non-*Salmonella* strains could not be detected. Thus, our method solves the problems of traditional slide agglutination approaches that require visual judgment that make it difficult to distinguish strains, as well as other rapid detection methods with suboptimal specificity reported in the past. Our method can specifically identify *S.* Pullorum.

In addition to strict specificity requirements, great sensitivity is essential to avoid misdetection in practical applications. The sensitivity test results revealed a detection limit of 21 copies/μL for our method, which is close the polymerase chain reaction-high resolution melting method reported previously (34 copies/μL) (Ren et al., 2017) and 100 times more sensitive than the PCR method. In addition, the detection limit for genomic DNA was 4.53 pg/μL. Reproducibility tests indicated that our method was highly stable, with a CV within the acceptable range (<5%).

In addition to its excellent performance, our method also saved detection time. Unlike the time-consuming traditional identification method that involves multiple steps for preproliferation, selective enrichment, biochemical testing, and serological typing, which often takes several day to yield results, our method can be accomplished under closed-tube conditions with a 1-step reagent addition in about 1.5 h. Unlike conventional PCR, there are no complicated operations after the amplification reaction, which greatly improves the *S.* Pullorum detection efficiency, facilitating rapid detection.

The performance of our EA-probe method was outstanding when assessing actual samples. Test results using real samples showed that among 100

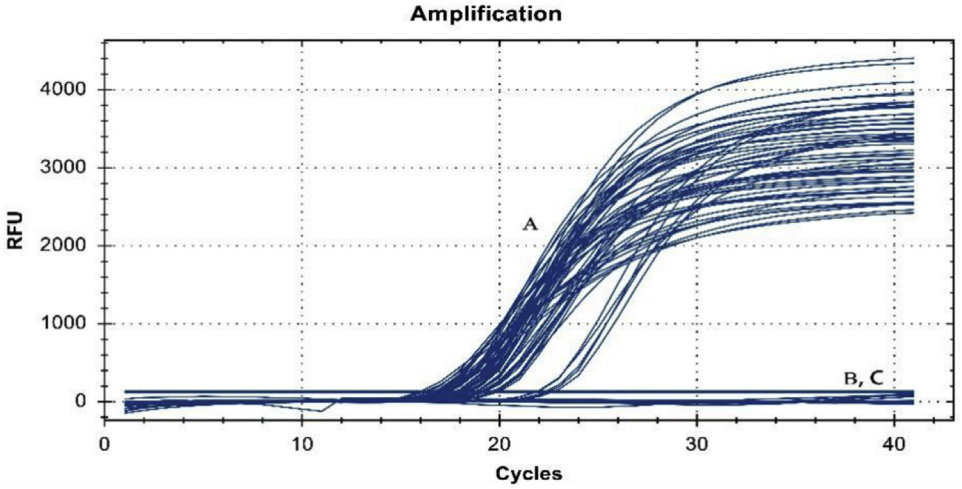

Figure 6. Specificity test results. (A) 57 strains of *Salmonella* Pullorum. (B) 38 strains of other serotypes of *Salmonella* and non-*Salmonella* species. (C) Negative control.

Table 2. Reproducibility of the EA-probe method established in this study.

Plasmid concentration (copies/μL)	Intra-assay reproducibility		Interassay reproducibility	
	Results (mean ± SD)	CV (%)	Results (mean ± SD)	CV (%)
2.1×10^6	19.14 ± 0.08	0.42	19.67 ± 0.66	3.37
2.1×10^5	23.07 ± 0.01	0.03	23.07 ± 0.19	0.82
2.1×10^4	26.14 ± 0.03	0.12	26.60 ± 0.52	1.94
2.1×10^3	29.17 ± 0.06	0.19	29.64 ± 0.55	1.85
2.1×10^2	31.61 ± 0.01	0.03	32.31 ± 1.00	3.10
2.1×10^1	34.49 ± 0.44	1.27	34.46 ± 0.78	2.26

Abbreviation: EA-probe, enzyme-activated blocked probe.

samples, 71 gave results identical to traditional identification and PCR methods (Xu et al., 2018). Of the remaining samples, 23 samples were only detected by the 2 molecular biological methods but not by the traditional identification method. Three samples were missed by the PCR method, but the other 2 methods yielded positive results, and 3 samples were only detected using our method. This may be because the 2 molecular biological methods are more sensitive than traditional identification methods. Furthermore, compared with the PCR method, our cycling probe method has a lower detection limit, making it less likely to produce false negative results in actual use.

CONCLUSION

In this study, we successfully developed an EA-probe method for the specific discrimination of *S.* Pullorum, solving the long-standing *S.* Pullorum rapid detection problem. This is the first practicable improved real-time PCR method capable of specific *S.* Pullorum detection. Our 1-step, closed-tube rapid detection method has high specificity, sensitivity, reproducibility, and rapidity and avoids interference from *S.* Gallinarum and *S.* Enteritidis. Thus, it has broad application prospects for *S.* Pullorum epidemiologic research and SNP clinical detection.

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Ethical Approval: Herein, we detected *Salmonella* Pullorum in dead poultry samples. There were no live animals used in this research. The sample collection process complied with the relevant recognized standards, and did not involve animal experiments.

DISCLOSURES

The authors declare no conflicts of interest.

Table 3. Comparison of testing real samples using different methods.

Sources	Traditional identification method	PCR method	EA-probe method	Number of samples (total = 100)
	+	+	+	2
	−	−	−	19
Anal swab (25)	−	+	+	2
	−	−	+	2
	+	+	+	5
	−	−	−	10
Liver (25)	−	+	+	8
	+	−	+	2
	+	+	+	22
	−	−	−	13
Embryo (50)	−	+	+	13
	+	−	+	1
	−	−	+	1
Reference	GB4789.4-2016	(Xu et al., 2018)	This study	

Abbreviations: EA-probe, enzyme-activated blocked probe; +, positive for *Salmonella* Pullorum; −, negative for *Salmonella* Pullorum.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.11.007.

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