Multiplex PCR assay based on the *citE2* gene and intergenic sequence for the rapid detection of *Salmonella* Pullorum in chickens

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ABSTRACT *Salmonella* is one of the most common Gram-negative pathogens and seriously threatens chicken farms and food safety. This study aimed to establish a multiplex polymerase chain reaction (**PCR**) approach for the identification of different *Salmonella enterica* subsp. *enterica*. The *citE2* gene and interval sequence of SPS4_00301−SPS4_00311 existed in all *S. enterica* subsp. *enterica* serovars by genomic comparison. By contrast, a 76 bp deletion in *citE2* was found only in *Salmonella* Pullorum. Two pairs of special primers designed from *citE2* and interval sequence were used to establish the multiplex PCR system. The optimized multiplex PCR system could distinguish *Salmonella* Pullorum and non-*Salmonella* Pullorum. The sensitivity of the optimized multiplex PCR system could be as low as 6.25 pg/μL and 10^4 colony-forming units (**CFU**)/mL for genomic DNA and *Salmonella* Pullorum cells, respectively. The developed multiplex PCR assay distinguished *Salmonella* Pullorum from 33 different *Salmonella enterica* subsp. *enterica* serotypes and 13 non-target species. The detection of egg samples artificially contaminated with *Salmonella* Pullorum, *Salmonella* Enteritidis, and naturally contaminated 69 anal swab samples showed that results were consistent with the culture method. These features indicated that the developed multiplex PCR system had high sensitivity and specificity and could be used for the accurate detection of *Salmonella* Pullorum in clinical samples.

Key words: *Salmonella* Pullorum, *Salmonella* Enteritidis, multiplex PCR, *citE2*, interval sequence

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INTRODUCTION

Salmonella is an important foodborne pathogen, and more than 2,600 different serovars have been identified so far (Kirk et al., 2015; Geng et al., 2019). *Salmonella* can survive in various environments and hosts, causing up to about 200,000 deaths annually worldwide (Pradhan and Negi, 2019). These pathogenic *Salmonella* often causes infectious diseases that are complex and difficult to control and manage (Park et al., 2014). Among these, *Salmonella enteric* serovar Enteritidis (***S.* Enteritidis**), *Salmonella enteric* serovar Gallinarum biovars Pullorum (***S.* Pullorum**) and Gallinarum (***S.* Gallinarum**) can be found in clean and intact eggs. *S.* Pullorum/Gallinarum are host-specific bacteria, and susceptible hosts include poultry, wild birds, turkeys, ducks, quails, and some guinea fowls (Wilson et al., 2019). Thus, *S.* Gallinarum threatens chicken production or water, whereas *S. Pullorum* can vertical transmission through seed eggs to the progeny (Celis-Estupiñan et al., 2017). These2 pathogens are closely related, but the main epidemiology and pathogenic mechanism of these biovars are completely distinct, which provides a good model for studies of the disease diagnosis and evolutionary processes (Batista et al., 2016; Celis-Estupiñan et al., 2017). *S.* Pullorum/Gallinarum has been basically purified in developed countries but are still widely distributed in chicken farms in most developing countries, such as Asia and Africa, thereby causing serious economic losses with high mortality (Shen et al., 2020; Zhang et al., 2020). Given that the genome is always evolving, the host range of *S.* Pullorum/Gallinarum may widen and is a potential threat to human health. However, there is still a lack of related epidemiologic data of these 2 pathogensin China, which is the first step of formulating effective control strategies. Therefore, a rapid and accurate methodis urgently needed to identify *S.* Pullorum and

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help us make corresponding prevention and control measures.

The traditional serological detection of *Salmonella* identifies O and H antigens by agglutination reaction between the specific antibody and granular antigen, and the serotype of *Salmonella* is determined in accordance with the White−Kauffmann−Le Minor scheme (Issenhuth-Jeanjean et al., 2014). Additionally, *S.* Pullorum/Gallinarum has no motility and have the same O antigen in etiology (Guo et al., 2017), and ornithine decarboxylation is only the main difference in biochemical characteristics (Barrow and de Freitas Neto, 2011). Normally, these methods are time-consuming, laborious, and ineffective in distinguishing some serotypes or biotypes of *Salmonella* especially *S.* Pullorum. However, DNA-based molecular techniques developed for *Salmonella*, such as PCR (Kubo et al., 2020), Multiplex Oligonucleotide Ligation−PCR (Gand et al., 2020), solid-phase PCR (Vinayaka et al., 2020), loop-mediated isothermal amplification combined with lateral flow dipstick (Liu et al., 2019, 2020; Priya et al., 2020), and repetitive sequence-based PCR (Gogoi et al., 2018), have already been described. PCR−restriction fragment length polymorphism (Soler-García et al., 2014), PCR−high−resolution melting (Ren et al., 2017), and enterobacterial repeated intergenic consensus−PCR are also used to identify the serotypes of *S.* Pullorum/Gallinarum (Xu et al., 2018,2020). The main bottle necks of the conventional PCR detection of *S.* Pullorum are due to serotypes of *Salmonella* and production of false-negative results. Given the inherent limitations of these methods, the combination of a variety of diagnostic techniques, repeated operation, and high cost are often needed to obtain results in a timely fashion. Thus, the conventional PCR method is not suitable for the routine clinical diagnosis of *Salmonella*. By contrast, the developed multiplex PCR method constitutes a highly efficient, systematic, economical, and simple tool for diagnostic *Salmonella* species and can be used to classify for the serotyping of the target gene in a single reaction (Zhou et al., 2020; Wilwet et al., 2021).

In the study, we establish a multiplex PCR system for the simultaneous detection of *S.* Pullorum from *S. enterica* subsp. *enterica*. The region of difference (**ROD**) of *citE2*, a 76 bp deletion, has been demonstrated to exist in all *S.* Pullorum strains, but not in non−*S.* Pullorum bacteria. Specifically, the intergenic sequence between SPS4_00301 and SPS4_00311 has been present in *S. enterica* subsp. *enterica*. Two pairs of primers based on the *citE2* and the intergenic sequence of SPS4_00301 −SPS4_00311 are exploited to establish the multiplex PCR system identifying *S. enterica* subsp. *enterica* strains. This rapid and sensitive multiplex PCR assay is applied to distinguish the genomic DNA of *Salmonella* from pure cells, artificially spiked egg samples, and clinical samples. Collectively, such results demonstrate that this promising method will be useful for the accurate detection of viable *S.* Pullorum and *S.* Enteritidis in chickens.

MATERIALS AND METHODS

Bacterial Strains

A collection of 46 bacterial strains, including 6 strains of *S.* Pullorum, 27 strains of other serotypes of non-*S.* Pullorum (belong to *S. enterica* subsp. *enterica*) and 13 strains of non-*Salmonella* strains, were used (Table 1). *S.* Pullorum (CVCC 530) and *S.* Enteritidis (ATCC 4931) were used as standard strains to optimize and establish the multiplex PCR method. All glycerol strains were stored at −80°C in a refrigerator until use.

Bacterial Culture and Genomic DNA Extraction

All *Salmonella* strains were cultivated in Luria−Bertani broth medium at 37°C for 12 to 14 h with shaking at 180 rpm. All non-*Salmonella* strains were routinely cultured overnight under suitable conditions and medium. The bacterial culture medium of overnight cultures (5 mL) were centrifuged at 10,000 rpm for 1 min, and the supernatant was removed as much as possible. Then, the genomic DNA from each bacteria was extracted via the TIANamp Bacteria DNAKit (Tiangen-Biotech, Beijing, China). The DNA of some *Salmonella* samples was extracted as template through the boiling method. The concentration and purity of DNA were quantified using the $A_{260/280}$ obtained by the Biodrop spectrophotometer (BioDrop, Cambridge, England). The DNA solution was subsequently packed and stored at −20°C prior to use.

Bioinformatics Analysis and Designing Primers

To detect *S.* Pullorum and non-*S.* Pullorum by the multiplex PCR assay, we compared the differences of genome sequences. The *citE2* gene and intergenic sequence between SPS4_00301 and SPS4_00311 of *S.* Pullorum (GenBank accession No: LK931482.1) were each analysed between *S.* Pullorum and *Salmonella* from the National Center for Biotechnology Information (**NCBI**). The *citE2* gene and the intergenic sequence between SPS4_00301 and SPS4_00311 were evaluated individually in the search database nucleotide collection (nr/nt) by using the Megablast from the Basic Local Alignment Search Tool (**BLAST**). All aligned target sequences in the NCBI GenBank must be present. Two pairs of specific primers for the *citE2* gene and intergenic sequence of SPS4_00301−SPS4_00311 were designed using the Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA). The nucleotide sequences of 2 primer pairs used for multiplex PCR were as follows: *citE2*-F, 5′-TCGACATCGCCACCTCCAG-3′; *citE2*-R, 5′-CGGCAATCACCTCATACAT-5′; SPS4_00301 −SPS4_00311-F, 5′-GCACGCGACGTTCAAATC TG-3′; and SPS4_00301−SPS4_00311-R, 5′-GAC GGTCACACCAAATAAGC-3′.

Table 1. List of the bacteria strains used in this study and the results of the multiplex PCR system.

No.	Analyte	Source	Serogroup	The multiplex PCR results (333 bp/257 bp/167 bp)[d]		
				citE2 citE2[d]	ROD	SPS4_00301- SPS4_00311
			Salmonella			
1	*S.* Pullorum	CVCC 530[a]	D	−	+	+
2	*S.* Pullorum	CVCC 1791	D	−	+	+
3	*S.* Pullorum	CVCC 1799	D	−	+	+
4	*S.* Pullorum	CVCC 535	D	−	+	+
5	*S.* Pullorum	ATCC 9120[b]	D	−	+	+
6	*S.*Pullorum	ATCC 9120	D	−	+	+
7	*S.* Enteritidis	ATCC 4931	D1	+	−	+
8	*S.* Typhimurium	ATCC 13311	B	+	−	+
9	*S.* Typhimurium	CMCC50115[c]	B	+	−	+
10	*S.* Typhimurium	CVCC541	B	+	−	+
11	*S.* Paratyphi	Laboratory stock	B	+	−	+
12	*S.* Kentucky	Laboratory stock	/	+	−	+
13	*S.* Paratyphoid	Laboratory stock	B	+	−	+
14	*S.* Heidelberg	Laboratory stock	/	+	−	+
15	*S.* Enteritidis	ATCC 13076	D	+	−	+
16	*S.*Choleraesuis	ATCC 10708	D	+	−	+
17	*S.* Enteritidis	ATCC 4931	D	+	−	+
18	*S.* Enteritidis	Laboratory stock	D	+	−	+
19	*S.* Choleraesuis	ATCC 10708	C1	+	−	+
20	*S.* Dublin	Laboratory stock	D1	+	−	+
21	*S.* Indiana	ATCC 51959	/	+	−	+
22	*S.* Oranienburg	ATCC 9239	B	+	−	+
23	*S.* Hadar	ATCC 51956	E	+	−	+
24	*S.* Newport	ATCC 6962	C2	+	−	+
25	*S.* Paratyphi A	ATCC 9150	/	+	−	+
26	*S.* Madelia	Laboratory stock	/	+	−	+
27	*S.* Kaapstad	Laboratory stock	/	+	−	+
28	*S.* Kentucky	Laboratory stock	/	+	−	+
29	*S.* Dublin	Laboratory stock	/	+	−	+
30	*S.* Enteritidis	Laboratory stock	/	+	−	+
31	*S.* Westhampton	ATCC 9712	/	+	−	+
32	*S.* Saintpaul	ATCC 19430	/	+	−	+
33	*S.* Typhi	Laboratory stock	D	+	−	+
			Non-*Salmonella*			
1	*Escherichia coli* O157:H7	Laboratory stock	/	−	−	−
2	*Escherichia coli* O157:H7	CMCC 44828	/	−	−	−
3	*Listeria monocytogenes*	ATCC 19111	/	−	−	−
4	*Shigella flexneri*	ATCC 13932	/	−	−	−
5	*Staphylococcus aureus*	ATCC 25931	/	−	−	−
6	*Aeromonas hydrophila*	CMCC 26001	/	−	−	−
7	*Bacillus subtilis*	ATTC 9372	/	−	−	−
8	*Campylobacter jejuni*	ATCC 63501	/	−	−	−
9	*Pseudomonas aeruginosa*	ATCC 27853	/	−	−	−
10	*Escherichia coli*	CICC 10783	/	−	−	−
11	*Staphylococcus aureus*	ATCC 6538	/	−	−	−
12	*Enterococcus faecalis*	ATCC 29212	/	−	−	−
13	*Yersinia pseudotuberculosis*	ATCC 908	/	−	−	−

[a]CVCC, China Veterinary Culture Collection Center, China.
[b]ATCC, American Type Culture Collection, USA.
[c]CMCC, National Center for Medical Culture Collections, China.
[d]+, positive; −, negative.

Multiplex PCR Assay

Different annealing temperatures (52.5°C−61.5°C) and ratios of 2 primer pairs (*citE2*: SPS4_00301 −SPS4_00311; 0.2:1, 0.4:1, 0.8:1, 1:1, 1:0.2, 1:0.4, and 1:0.8) were used to optimize and establish the multiplex PCR system. The multiplex PCR amplifications were set up using a total volume of 25 μL containing 12.5 μL 2 × ES Taq Master Mix (No: RR902, Takara Bio, Dalian, China), 10 μM each of the *citE2* F/R primers, 10 μM each of the SPS4_00301−SPS4_00311F/R primers, 1.0 μL genomic DNA, and sterile double-distilled water (**DDW**). The multiplex PCR conditionsin the assay were performed as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 40 s, annealing at 58°C for 30 s, and extension step at 72°C for 30 s. The final extension was at 72°C for 10 min, and repeated three times. The amplified products were separated with 1.5% agarose gel electrophoresis and observed under ultraviolet light.

Genomic DNA was extracted as described above to confirm whether the signal of the multiplex PCR could be detected for cross-reaction among different combinations of primer pairs (*citE2* and SPS4_00301−SPS4_00311) and templates of *Salmonella* cells (*S.* Pullorum and *S.* Enteritidis). The PCR system and conditions were conducted in accordance with the PCR protocol. Bacterial tests were performed and repeated in triplicate.

Sensitivity and Specificity of the Multiplex PCR Assay

The genomic DNA of *S.* Pullorum was diluted 10 times serially from 62.5 ng/μL to 6.25 fg/μL with sterile DDW. The overnight culture of *S.* Pullorum was washed 3 times with sterile DDW, and the final concentrations of *S.* Pullorum cells were adjusted from 4×10^6 CFU/mL to 5×10^3 CFU/mL with sterile DDW. The sensitivity of multiplex PCR was evaluated under the optimized reaction conditions in triplicate. Finally, 1 μL of each dilution was used as template for the multiplex PCR detection.

The specificity of the multiplex PCR assay based on the *citE2* and SPS4_00301−SPS4_00311 primers were conducted using genomic DNA from 33 *Salmonella* (belong to *S. enterica* subsp. *enterica*) and 13 non-*Salmonella* strains. Cultures were adjusted to 10^6 CFU/mL and confirmed using plate counts for 6 times. The DNA template was obtained by direct boiling method and subjected to simple centrifugation for 3 min at 5,000 rpm. The supernatant (1 μL) was analyzed for the multiplex PCR assay, and sterile DDW was used as blank control. These experiments were independently repeated in triplicate.

Artificial Contamination of Egg for the Multiplex PCR Assay

A total of 100 fresh eggs were purchased from a local supermarket (Xinxiang, China) and used to evaluate whether the multiplex PCR detection of *S.* Pullorum (CVCC 530) *and S.* Enteritidis (ATCC 4931) artificially contaminated eggs. All eggs were checked to ensure the *Salmonella*-free according to the standard culture method (GB/T4789.4−2016) depicted in Figure 1 (China National Food Safety Standard, 2016). Briefly, take 3 eggs, each egg was placed into a homogeniser and stirred into the homogenate to ensure uncontaminated samples of target bacteria. Then, the homogenate (25 mL) was added into 225 mL buffered peptone water (**BPW**; Hopebio-Technology, Shangdong, China) to obtain 1:10 as the culture medium of *Salmonella*. After the OD_{600} value of *S.* Pullorum culture was adjusted to 1, its serial 10-fold dilutions with sterile DDW were from 10^6 CFU/mL to 10° CFU/mL. Simultaneously, each dilution was carried out using xylose−lysine−desoxycholate agar (Hopebio-Technology) plates through the plate count method to determine the actual concentration of *S.* Pullorum. Ten fold serial dilutions (1 mL) were thoroughly added into 9 mL culture medium of *Salmonella* and incubated at 37°C with shaking at 180 rpm for 0, 2, 6,10, and 12 h, respectively. Thereafter, 1 mL enrichment culture of each dilution was collected by centrifugation at 10,000 rpm for 1 min. DNA was extracted by using the boiling method, and 1 μL of each supernatant was used for the multiplex PCR under the same conditions. These results of the multiplex PCR assay of all samples were compared with those of the culture method. Non-inoculated egg with *Salmonella* was tested as the negative template. All experiments were independently repeated in triplicate.

Application of the Multiplex PCR Assay

To ensure the effect of the multiplex PCR assay, we tested the genomic DNA of 69 clinical samples from the feces of naturally contaminated chicken farms and 3 negative samples of smears from chickens (from which *Salmonella* was not isolated) in Xinjiang Province, China. Chickens were preliminary screened by *S.* Pullorum/Gallinarum Serum Plate Agglutination Test Polyvalent Antigen (Zhonghai Biotech, Beijing, China). Finally, 69 chicken anal swab samples were identified as *S.* Pullorum strains by using the culture method (GB/T4789.4−2016). All clinical samples were tested using the multiplex PCR assay described above after 12 h enrichment at 37°C in BPW. The results of multiplex PCR for known *S.* Pullorum strains were compared with those ofthe culture method in this assay.

RESULTS

Sequence Alignment Analysis and Salmonella Detection of the Multiplex PCR Assay

Bioinformatics analysis showed that *citE2* was conserved, repeatable, and existed in all *S. enterica* subsp. *enterica* strains after searching in the BLASTn program of Web BLAST (Supplementary S1). The *citE2* sequence comparison showed that the 76 bp fragment of the *citE2* gene in all *S.* Pullorum strains might be absent compared with that in non-*S.* Pullorum. The intergenic sequence between SPS4_00301 and SPS4_00311 had 100% homology with all *S. enterica* subsp. *enterica* strains after searching in the BLASTn program of Web BLAST (Supplementary S2). In this study, the specific primer from the *citE2* gene was designed for *S.* Pullorum, and the intergenic sequence primer of SPS4_00301−SPS4_00311 was designed as a molecular marker for the identification of all *S. enterica* subsp. *enterica* strains (Figure 2A). The fragment sizes of multiplex PCR were 167 and 257 bp for *S.* Pullorum and 167 and 333 bp for *S.* Enteritidis (Figure 2B).

Optimization and Effectof the Multiplex PCR Assay

After the multiplex PCR system was generated, the annealing temperature and the combination ratio of 2 pairs of primers were optimized. As shown in Supplementary S3A and S3B, no significant difference was observed in the annealing temperature of *citE2* and SPS4_00301−SPS4_00311 primers at 52.5°C−61.5°C. Supplementary S3C demonstrates that the different annealing temperatures of multiplex PCR were 55.3°C, 56.4°C, 57.6°C, 58.7°C, 59.8°C, and 60.7°C and that the

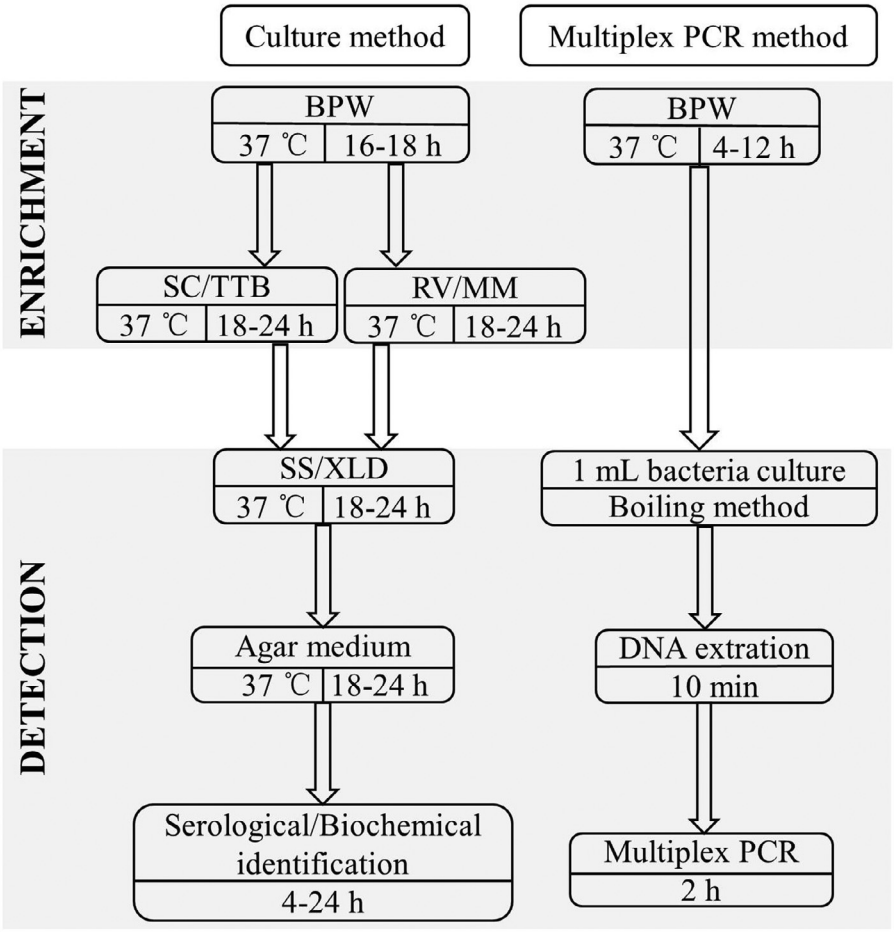

Figure 1. Comparison of the culture method and multiplex PCR for *Salmonella* spp. detection in a fecal sample. Abbreviations: BPW, buffered peptone water; MM, Rappaport−Vassiliadis Medium; RV, Rappaport Vassiliadis; SC, selenite cystine broth; *Salmonella*−*Shigella* agar; TTB, tetrathionatebroth base; XLD, Xylose Lysine Desoxycholate Medium.

electrophoretic bands were relatively bright and had no significant difference. Thus, the optimal conditions of multiplex PCR were as follows: annealing temperature of 60°C and ratio of 2 pairs of primers (*citE2* primers: SPS4_00301−SPS4_00311 primers) of 1:1 (Supplementary S3D). These conditions were applied to amplify the simplex and multiple templates of *Salmonella* (*S.* Pullorum and *S.* Enteritidis, respectively) to verify whether single and mixed primer pairs, respectively, could be used in a multiplex PCR system. The results of agarose gel electrophoresis showed that the amplified fragments of 167, 257, and 333 bp were obtained from *S.* Pullorum and *S.* Enteritidis. The amplicon lengths of *S.* Pullorum were 167 and 257 bp, and the product sizes of *S.* Enteritidis were 167 and 333 bp (Figure 3). Results showed that the mixed primer pairs could be specifically and effectively applied to the detection system of *S.* Pullorum and non-*S.* Pullorum.

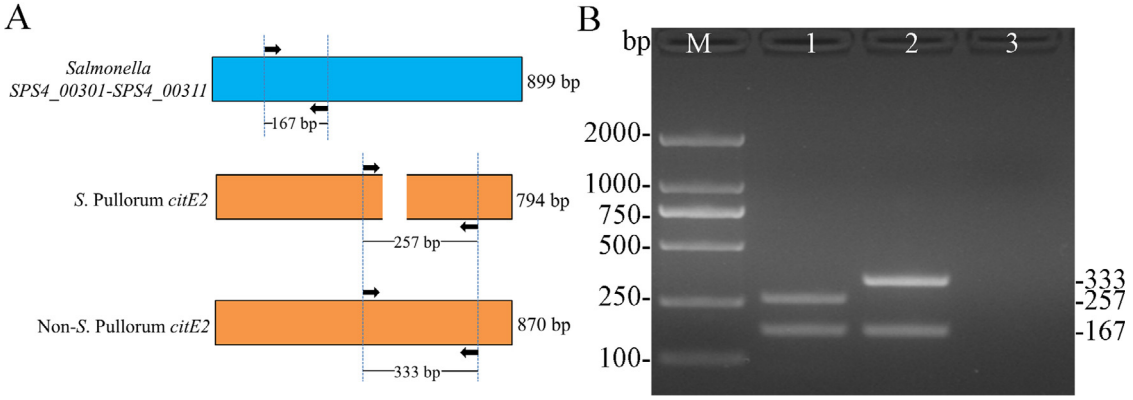

Figure 2. Overview of the multiplex PCR assay for the detection of *S.* Pullorum. (A) Intergenic sequence between SPS4_00301 and SPS4_00311 and *citE2* gene existing in all *Salmonella enterica* subsp. *enterica* serovars and highly conserved ROD of the *citE2* of *S.* Pullorum among *S. enterica* subsp. *enterica* serovars. The intergenic sequence and *citE2* were applied to design primers, and black arrows show the size of the amplified fragment. (B) Multiplex PCR results using genomic DNA from *Salmonella* strains. M: Takara DL2000 DNA marker (No: 3427A, Takara, Dalian, China), Lane 1: *S.* Pullorum, Lane 2: *S.* Enteritidis, Lane 3: negative control (sterile DDW).

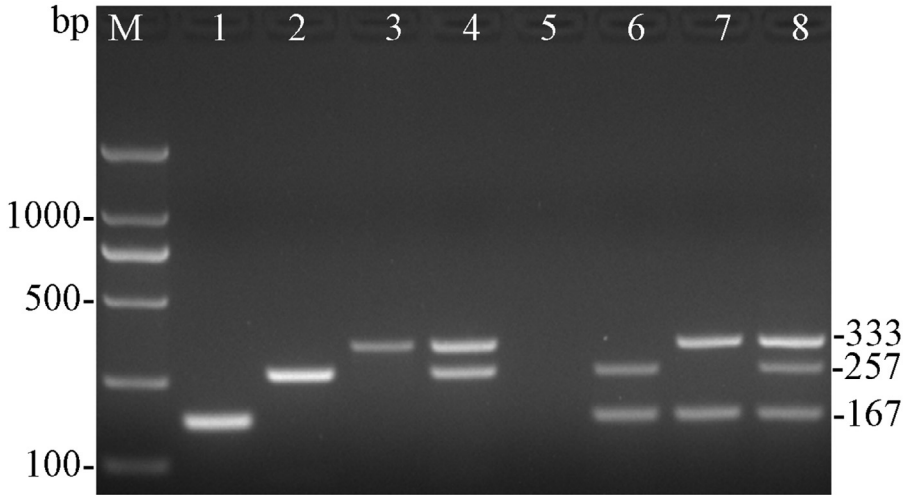

Figure 3. Effect of PCR and multiplex PCR assays for detecting *S.* Pullorum and *S.* Enteritidis. Lane M: Takara DL2000 DNA marker, Lane 1: *S.* Pullorum with SPS4_00301−SPS4_00311 primers, Lane 2: *S.* Pullorum with *citE2* primers, Lane 3:*S.* Enteritidis with *citE2* primers, Lane 4: Two target bacteria with *citE2* primers, Lane 5: negative control (sterile DDW) with mixed primer pairs, Lane 6: *S.* Pullorum with mixed primer pairs, Lane 7: *S.* Enteritidis with mixed primer pairs, and Lane 8: Two target bacteria with mixed primer pairs.

Specificity of the Multiplex PCR

A total of 33 strains of *S. enterica* subsp. *enterica* and 13 strains of non-*Salmonella* were tested to determine the specificity of the multiplex PCR assay. The amplicons of 167 and 257 bp size fragments were clearly generated on 1.5% agarose gels for *S.* Pullorum, and 2 bands with sizes of 167 and 333 bp were observed in 1.5% agarose gels for other *Salmonella* (Figure 4 and Table 1). By contrast, non-*Salmonella* species and blank control did not have the specific band, indicating no cross amplification with other primers. Overall, we speculated that the multiplex PCR showed excellent specificity for the detection of *S.* Pullorum and non-*S.* Pullorum.

Sensitivity of the Multiplex PCR Assay

The genomic DNA concentration of *S.* Pullorum was aseptically diluted 10-fold from 62.5 ng/μL to 6.25 fg/μL as a template and tested using the multiplex PCR

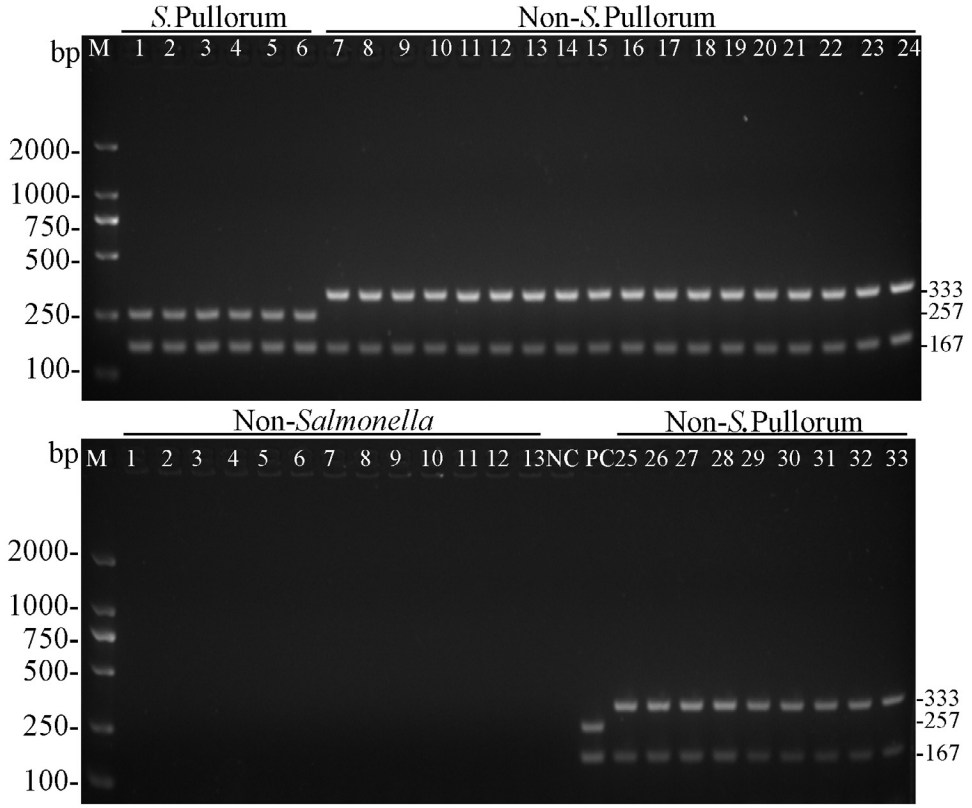

Figure 4. Specificity of a multiplex PCR assay for the detection of *S.* Pullorum. Lane M: Takara DL2000 DNA marker, NC: negative control (sterile DDW), PC: positive control (*S.* Pullorum CVCC 530). All strain details are shown in Table 1.

assay to assess the diagnostic sensitivity of the multiplex PCR system. The detection limit of the PCR method could reach at least 6.25 pg/μL genomic DNA (Figure 5A). Pure *S.* Pullorum cells were diluted from 4×10^6 CFU/mL to 5×10^3 CFU/mL. Results showed that the purpose fragment (as low as 10 CFU per reaction) was still detected (Figure 5B).

Multiplex PCR Assay Evaluationin Artificially Contaminated Egg Samples

The different concentrations of *S.* Pullorum and *S.* Enteritidis from 10^6 CFU/mL to 10° CFU/mL were used to examine the detection sensitivity of the multiplex PCR assay for artificially contaminated egg samples. As shown in Figure 6, the results of multiplex PCR with different enrichment time points corresponding to different concentrations of *S.* Pullorum and *S.* Enteritidis were detected. At initial inoculation concentrations of 10^6 and 10^5 CFU/mL per reaction, positive signals could be identified without enrichment. The limits of detection of the multiplex PCR assay in egg were 10^4 CFU/mL after 2 h enrichment, 10^1CFU/mL after 6 h enrichment, and 10° CFU/mL after 10 h and 12 h enrichment, respectively. In addition, the detection limits of *S.* Pullorum and *S.* Enteritidis were similar to that of 10^4 CFU/mL viable *S.* Pullorum pure cells in this multiplex PCR assay. Results indicated that the multiplex PCR was experimentally sufficient for the target pathogen in artificially contaminated egg samples.

Application of the Multiplex PCR Assayin Fecal Samples

A total of 69 typical anal swab samples from chicken farms were simultaneously subjected tothe multiplex PCR assay to test the diagnostic efficiency of this assay for the detection of *S.* Pullorum. As shown in Figure 7, results demonstrated that 69 bacterial samples amplified 2 expected bands of 167 and 257 bp for *S.* Pullorum, and no PCR product was observed in 3 negative samples. The multiplex PCR method was excellent and consistent with the culture method, indicating that the assay could be used for the clinical diagnosis of *S.* Pullorum.

DISCUSSION

S. Pullorum can spread horizontally and vertically. Once these pathogens occur, they can hardly be eliminated (Liu et al., 2019). Therefore, the accurate and rapid diagnosis of pathogens is significant for the control and eradication of *S.* Pullorum. At present, the detection of pathogenic bacteria depends on culture-based techniques and biochemical identification, which are time-consuming, have long detection cycle and low sensitivity, and cannot meet the needs of social development (Blanco and de Tuesta, 2018). Thus, the accurate and simple detection method should be developed for the serotype diagnosis of *S.* Pullorum.

The conventional PCR assay has been successfully established for the diagnosis and identification of *Salmonella*. At the same time, these techniques did still need to develop an improved method for the identification and detection of target bacteria (Park et al., 2014; Babu et al., 2021). Many studies reported that multiplex PCR is widely used for identifying microorganisms due to the advantages of high efficiency, system, economy, and simplicity, but can't detected for the poor template (Yang et al., 2013, 2020; Quick et al., 2017; Zhou et al., 2020). Strikingly, the design and quantity of multiplex PCR primers are important for the effective amplification of the target gene sequence. Herein, our assay was applied successfully to improve this problem by designing two sets of primers with different target sequences derived from *S.* Pullorum. Our PCR method can remarkably improve the detection specificity and eliminate false-positive results. This design may extend the application of PCR for genotyping, evolutionary history of host adaptation, and bacterial biology (Dobrindt and Hacker, 2001; Thomson et al., 2008; Shen et al., 2020).

Our established method could successfully solve the difficulties on the basis of the diagnostic marker of the *citE2* gene and intergenic sequence between SPS4_00301 and SPS4_00311 for *S.* Pullorum. A previous study indicated that *citE2* is a subunit of bacterial citrate lyase in bacterial energy metabolism and has

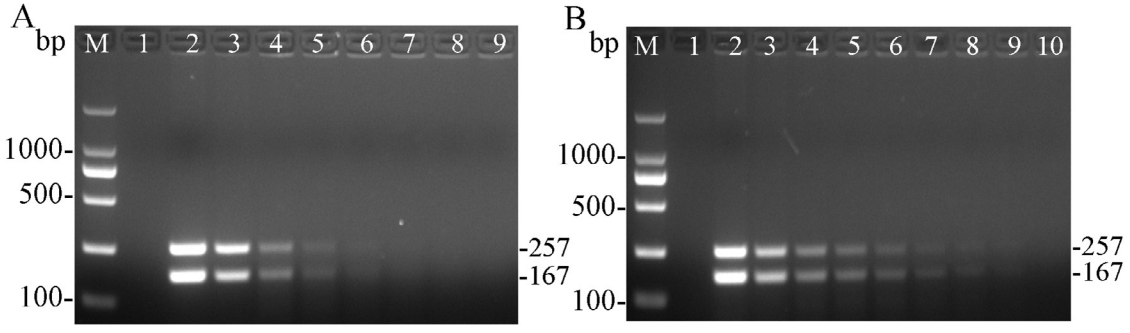

Figure 5. Limit of detection of multiplex PCR assays for *S.* Pullorum. Lane M: Takara DL2000 DNA marker, Lane 1: negative control (sterile DDW). (A) PCR for the detection of *Salmonella* DNA samples. Lanes 2–9: 62.5 ng/μL, 6.25 ng/μL, 625 pg/μL, 62.5 pg/μL, 6.25 pg/μL, 625 fg/μL, 62.5 fg/μL, and 6.25 fg/μL, respectively. (B) PCR for the detection of *S.* Pullorum cells. Lanes 2–10: 4×10^6, 2×10^6, 1×10^6, 1×10^5, 8×10^4, 4×10^4, 2×10^4, 1×10^4, and 5×10^3 CFU/mL, respectively.

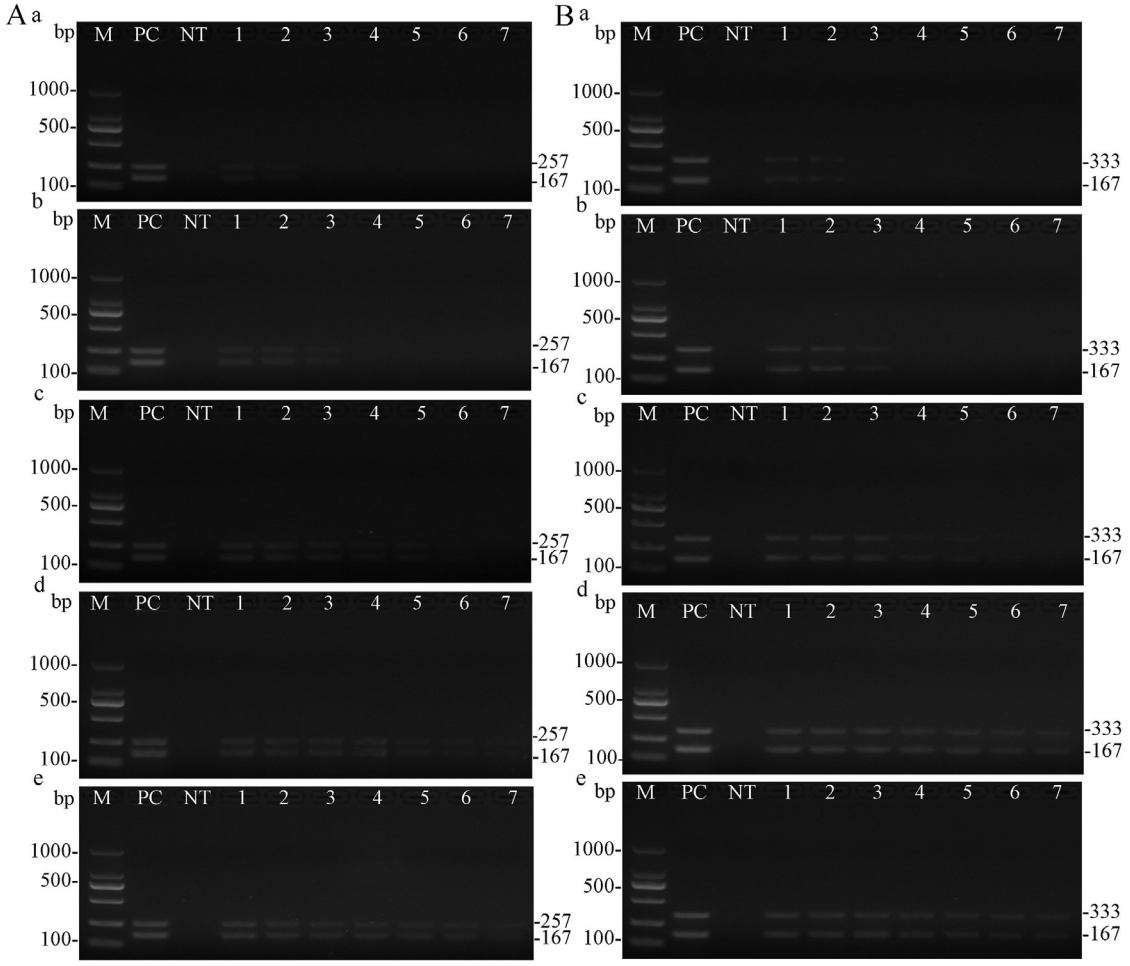

Figure 6. Evaluation of multiplex PCR assay by using artificially contaminated chicken egg samples. (A) *S.* Pullorum and (B) *S.* Enteritidis from (Lane 1) 10^6 CFU/mL to (Lane 7) 10° CFU/mL. (a) 0, (b) 2, (c) 6, (d) 10, and (e) 12 h enrichment. Lane M: Takara DL2000 DNA marker, PC: positive control (*S.* Pullorum CVCC 530 or *S.* Enteritidis ATCC 4931), NT: negative template (sterile DDW).

been demonstrated as a robust drug target for the physiology and virulence of *Mycobacterium tuberculosis* (Arora et al., 2018). The sequence alignment of the *citE2* gene was present in all *S. enterica* subsp. *enterica* strains, but *S.* Pullorum strains had a 76 bp deletion in *citE2*. The intergenic sequence between SPS4_00301 and SPS4_00311 existed in all *S. enterica* subsp. *enteric* stains and was the first to be reported as a diagnostic marker of *Salmonella*. Zhou et al. (2020) developed a PCR-based assay by targeting the cigR ROD, and this assay has excellent effectivity and suitability. The conserved ROD represents a potential marker for the specific identification of *S.* Pullorum/Gallinarum (Xiong et al., 2016, 2018). Similarly, the intergenic sequence between 2 genes in *Salmonella* is conservative in the process of evolution and has important biological functions (Tang et al., 2017). In epidemiologic investigation and transmission of *S.* Pullorum/Gallinarum, increased specific genes are explored to determine *Salmonella* serovars for PCRs (Batista et al., 2018). For example, the *fliC* and *fljB* genes present in *S.* Typhimurium (Khaltabadi et al., 2019) are not highly conservative to discriminate clinical mutants. The multiplex real-time PCR assay reduces the problems of atypical strains or false-negative results (Naberhaus et al., 2019).

The conservation status of *citE2* ROD and the intergenic sequence of SPS4_00301−SPS4_00311 were exploited as target sequences to design *S.* Pullorum primers. In this study, the multiplex PCR assay produced the 2 expected bands from 6 strains of *S.* Pullorum and 27 strains of non-*S.* Pullorumin dividually, but 13 strains of non-*Salmonella* did not have a band (Figure 4). Detection limits for the multiplex PCR could be analyzed at 6.25 pg/μL for the genomic DNA of *S.* Pullorum and 10^4 CFU/mL for pure *S.* Pullorum cells in a single tube. As shown in Table 2, our method exhibited a sensitivity that was comparable with other earlier reported data for the detection of *S.* Pullorum (Sahu et al., 2019). From the results of the spiked eggs with two of the individual target bacteria, a 10-fold enrichment could be observed to detect 10^0 CFU/mL viable *S.* Pullorum and *S.* Enteritidis. Wan et al. (2021) applied a real-time fluorescent quantitative PCR to detect *Salmonella* spp and *S.* Enteritidis in food samples, and 10 CFU in BPW could be detected by applying this quantitative PCR. A total of 69 chicken anal swab samples from chicken farms were detected using the multiplex PCR system, which was also identical with the culture method, to verify the clinical application of this method. Cumulatively, the specificity and sensitivity of two special primer pairs

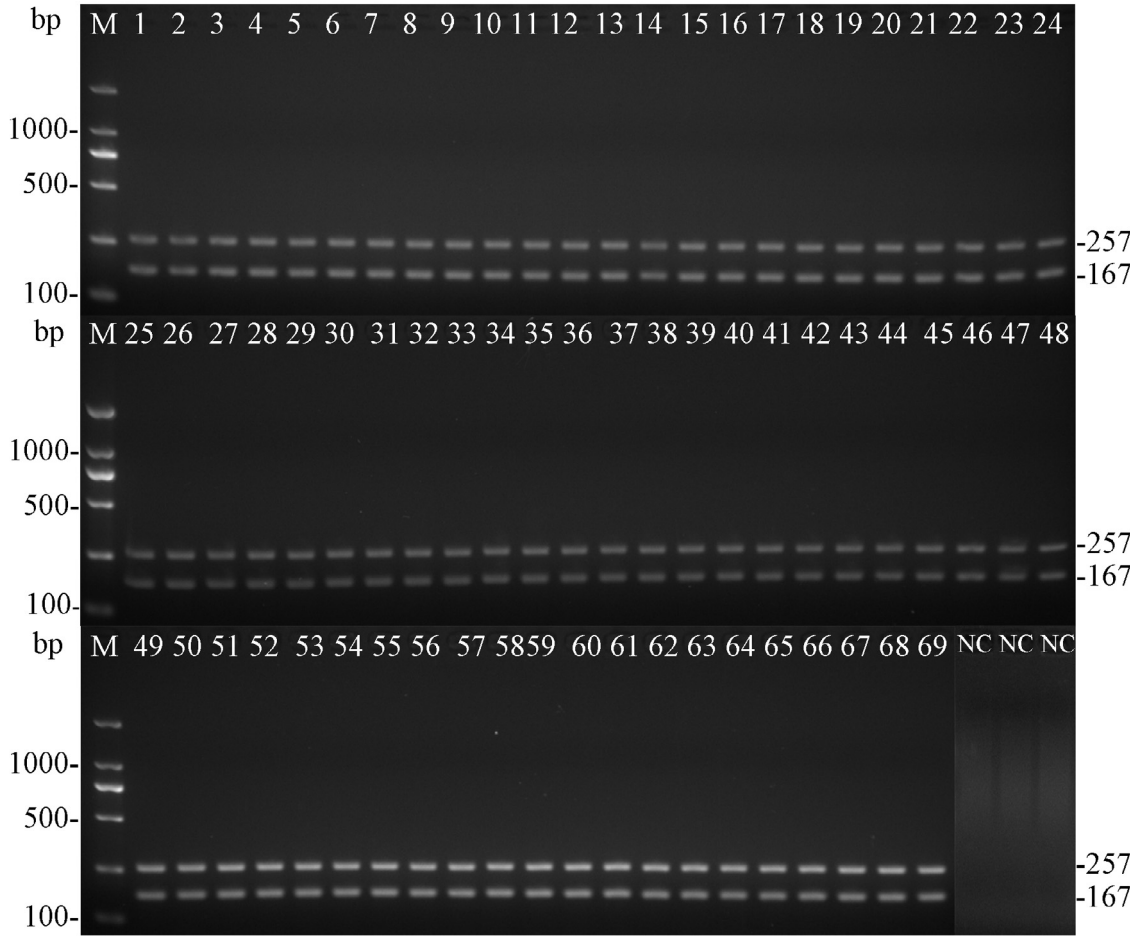

Figure 7. Multiplex PCR assay for the detection of *S.* Pullorum from chicken anal swab samples. Lane M: Takara DL2000 DNA marker, Lanes 1−69: 69 wild strains of *S.* Pullorum from chicken farms, NC: negative control (*Salmonella*-free).

were effectively sufficient to construct a multiplex PCR system for the identification and diagnosis of *S.* Pullorum. Our results indicated that the proposed PCR method provided a useful information to identify *S.* Pullorum from *S. enterica* subsp. *enterica* in laboratory and real samples efficiently.

In summary, a rapid, accurate, and economical multiplex PCR for detecting of *S.* Pullorum was successfully established by the *citE2* gene and the intergenic sequence of SPS4_00301−SPS4_00311. Our result revealed that the multiplex PCR was a highly efficient and practical method to distinguish *S.* Pullorum of natural chicken anal swab and egg samples artificially contaminated with *S.* Pullorum and *S.* Enteritidis. Presumably, the present approach might be a valuable strategy for the early diagnosis and epidemiological investigation of *S. enterica* subsp. *enterica* in microbiology laboratories.

Table 2. List of nucleic acid-based assays for the determination of *S.* Pullorum/Gallinarum.

Methods	Target	Comment	Linear range	Sensitivity	References
Multiple PCR	*S.* Pullorum/Gallinarum	*cigR*	4×10^5- 2×10^4 CFU/mL	2×10^4 CFU/mL	Zhou et al. (2020)
One-step PCR	*S.* Pullorum/Gallinarum	*flhB*	2×10^6- 2×10^3 CFU/mL	2×10^3 CFU/mL	Xiong et al. (2016)
flgE-PCR	*S.* Pullorum/Gallinarum	*flgE*	10^9 -10^5 CFU/mL	10^5 CFU/mL	Yang et al. (2020)
LFNAA	*S.* Pullorum	*SEEP*	10-5 $\times 10^{-3}$ ng/uL	5×10^{-3} ng/ uL	Liu et al. 2020
LP-LAMP	*S.* Pullorum	*rfbS*	49.2ng/μL-4.92 pg/μL	4.92 pg/μL	Shen et al. (2020)
EA-probe	*S.* Pullorum	*rfbS*	4.53pg/mL-45.3 ng/μL	4.53 pg/μL	Wen et al. (2021a)
Multiplex qPCR	*S.* Pullorum/Gallinarum	*pSGP/pSG/pSP*	10^8-10^1 CFU/mL	10^1 CFU/mL	Rubio et al. (2017)
Multiplex PCR	*S.* Pullorum/Gallinarum	*tcpS*	2×10^7- 2×10^4 CFU/mL	2×10^4 CFU/mL	Xiong et al. (2017)
PCR	*S.* Pullorum/Gallinarum	*ipaJ*	10^{10}-10^5 CFU/mL	10^5 CFU/mL	Xu et al. (2018a)
CACA	*S.* Pullorum	*rfbS*	3.98 $\times 10^3$-3.98 pg/μL	3.98 pg/μL	Wen et al. (2021b)
PCR	*S.* Pullorum	*SPUL 2693*	6.24 $\times 10^8$-6 $\times 10^3$ CFU/mL	6×10^3 CFU/mL	Xu et al. (2018b)
LAMP	*S.* Gallinarum	*sefA*	2×10^8- 2×10^5CFU/mL	2×10^5 CFU/mL	Gong et al. (2016)
Multiple× PCR	*S.* Pullorum	*citE2*	4×10^6-10^4 CFU/mL	10^4 CFU/mL	in this study

Multiplex PCR assay based on the *citE2* gene and intergenic sequence for the rapid detection of *Salmonella* Pullorum in chickens.

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DISCLOSURES

All authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101981.

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