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

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 cause infectious diseases that are complex and difficult to control and manage (Park et al., 2014). Among these, *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*), *Salmonella enterica* 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). These 2 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 pathogens in China, which is the first step of formulating effective control strategies. Therefore, a rapid and accurate method is urgently needed to identify *S. Pullorum* and
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 DNA Kit (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 A260/280 obtained by the Biophotometer (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'-TGCACATCGCCACCTCCAG-3'; citE2-R, 5'-CGCAATCACCTCATACAT-3'; SPS4_00301–SPS4_00311-F, 5'-GCACGGACGTTCATCTAC-3'; and SPS4_00301–SPS4_00311-R, 5'-GACGGTCACACCAATAAGC-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) |
|-----|------------------|--------------------|-----------|-----------------------------------------------|
|     |                  |                    |           | *citE2* | *citE2* | ROD | SPS4_00301-SPS4_00311 |
| 1   | S. Pullorum      | CVCC 530<sup>a</sup> | D         | −      | +      | +   | +                      |
| 2   | S. Pullorum      | CVCC 1791          | D         | +      | +      | +   | +                      |
| 3   | S. Pullorum      | CVCC 1799          | D         | −      | +      | +   | +                      |
| 4   | S. Pullorum      | CVCC 535           | D         | −      | +      | +   | +                      |
| 5   | S. Pullorum      | ATCC 9120<sup>b</sup> | D     | −      | +      | +   | +                      |
| 6   | S. Pullorum      | ATCC 9120          | D         | −      | +      | +   | +                      |
| 7   | S. Enteritidis   | ATCC 4931          | D         | −      | +      | +   | +                      |
| 8   | S. Typhimurium   | ATCC 13311         | B         | −      | +      | +   | +                      |
| 9   | S. Typhimurium   | CMCC20115<sup>c</sup> | 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 10708         | D         | −      | +      | +   | +                      |
| 16  | S. Choleræausis  | ATCC 10708         | D         | −      | +      | +   | +                      |
| 17  | S. Enteritidis   | ATCC 4931          | D         | −      | +      | +   | +                      |
| 18  | S. Enteritidis   | Laboratory stock   | D         | −      | +      | +   | +                      |
| 19  | S. Choleræausis  | ATCC 10708         | C1        | +      | +      | +   | +                      |
| 20  | S. Dulbin        | Laboratory stock   | D1        | +      | +      | +   | +                      |
| 21  | S. Indiana       | ATCC 51959         | /         | +      | +      | +   | +                      |
| 22  | S. Oranenbourg   | 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. Dulbin        | Laboratory stock   | /         | +      | +      | +   | +                      |
| 30  | S. Enteritidis   | Laboratory stock   | /         | +      | +      | +   | +                      |
| 31  | S. Westhampton   | ATCC 9712          | /         | +      | +      | +   | +                      |
| 32  | S. Saintpaul     | ATCC 19430         | +         | −      | +      | +   | +                      |
| 33  | S. Typhi         | Laboratory stock   | D         | +      | +      | +   | +                      |

<sup>a</sup>CVCC, China Veterinary Culture Collection Center, China.
<sup>b</sup>ATCC, American Type Culture Collection, USA.
<sup>c</sup>CMCC, National Center for Medical Culture Collections, China.
<sup>d</sup>+, 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 conditions in 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 subs. 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 OD600 value of S. Pullorum culture was adjusted to 1, its serial 10-fold dilutions with sterile DDW were from 10^6 CFU/mL to 10^0 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 Polyclonal 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 of the 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 subs. 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 subs. 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 subs. 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 Effect of 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
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 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, tetra-thionate broth base; XLD, Xylose Lysine Desoxycholate Medium.

**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).
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.
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 x 10⁶ CFU/mL to 5 x 10³ CFU/mL. Results showed that the purpose fragment (as low as 10 CFU per reaction) was still detected (Figure 5B).

**Multiplex PCR Assay Evaluation in Artificially Contaminated Egg Samples**

The different concentrations of S. Pullorum and S. Enteritidis from 10⁶ CFU/mL to 10⁵ 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 10⁶ and 10⁵ CFU/mL per reaction, positive signals could be identified without enrichment. The limits of detection of the multiplex PCR assay in egg were 10⁵ CFU/mL after 2 h enrichment, 10⁴ CFU/mL after 6 h enrichment, and 10³ 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 10⁴ 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 Assay in Fecal Samples**

A total of 69 typical anal swab samples from chicken farms were simultaneously subjected to the 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_00311 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
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. *enterica* 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 *flyB* 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.* Pullorum individually, 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 10^6 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...
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.

**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).

**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*  | $4 \times 10^7$–$2 \times 10^8$ CFU/mL | $2 \times 10^4$ CFU/mL | Zhou et al. (2020)         |
| One-step PCR| *S. Pullorum*/Gallinarum | *fbB*   | $2 \times 10^7$–$2 \times 10^8$ CFU/mL | $2 \times 10^4$ CFU/mL | Xiong et al. (2016)        |
| *fbE*-PCR   | *S. Pullorum*/Gallinarum | *fbE*   | $10^6$–$10^7$ CFU/mL | $10^5$ CFU/mL | Yang et al. (2020)         |
| LFNAA       | *S. Pullorum*          | *SEEP*  | $10^{-5}$–$10^{-3}$ ng/μL | $5 \times 10^{-3}$ ng/μL | Liu et al. 2020            |
| LP-LAMP     | *S. Pullorum*          | *fbS*   | $49.2$ pg/μL–$4.92$ pg/μL | $4.92$ pg/μL | Shen et al. (2020)         |
| EA-probe    | *S. Pullorum*          | *fbS*   | $4.53$ pg/μL–$4.53$ pg/μL | $4.53$ pg/μL | Wen et al. (2021a)         |
| Multiplex qPCR| *S. Pullorum*/Gallinarum | *pSG*/pSG/pSP | $10^8$–$10^9$ CFU/mL | $10^5$ CFU/mL | Rubio et al. (2017)        |
| Multiplex PCR| *S. Pullorum*/Gallinarum | *tcpS*  | $10^7$–$2 \times 10^8$ CFU/mL | $2 \times 10^4$ CFU/mL | Xiong et al. (2017)        |
| PCR         | *S. Pullorum*/Gallinarum | *ipaJ*  | $10^{-10}$–$10^{-6}$ 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 | Xu et al. (2018b)          |
| PCR         | *S. Pullorum*          | *SPUL 2693* | $6.24 \times 10^{-6}$–$6 \times 10^{-7}$ CFU/mL | $6 \times 10^{-3}$ CFU/mL | Xu et al. (2018b)          |
| LAMP        | *S. Gallinarum*        | *sefA*  | $2 \times 10^6$–$2 \times 10^7$ CFU/mL | $2 \times 10^4$ CFU/mL | Gong et al. (2016)         |
| Multiplex PCR| *S. Pullorum*          | *citE2* | $4 \times 10^6$–$10^7$ CFU/mL | $10^6$ 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.

REFERENCES

Arora, G., D. Chaudhary, S. Kidwai, D. Sharma, and R. Singh. 2018. CitE enzymes are essential for Mycobacterium tuberculosis to establish infection in macrophages and guinea pigs. Front. Cell. Infect. Microbiol. 8:385.

Babu, U. S., L. M. Harrison, I. R. Patel, M. K. Mammel, E. Bigley III, and K. V. Balan. 2021. Development and validation of an improved method for the detection of Salmonella in cinnamon bark and oregano leaves using the adsorbent beta zeolite in the pre-enrichment media. Food Microbiol 100:103852.

Barrow, P. A., and O. C. F. de Freitas Neto. 2011. Pulmonary disease and fowl typhoid—new thoughts on old diseases: a review. Avian Pathol 40:1–13.

Batista, D. F. A., O. C. de Freitas Neto, A. M. de Almeida, P. A. Barrow, F. de Oliveira Barbosa, and A. B. Junior. 2016. Molecular identification of Salmonella enterica subsp. Enteric serovar Gallinarum biogors Gallinarum and Pullorum by a duplex PCR assay. J. Vet. Diagn. Invest. 28:419–422.

Batista, D. F. A., O. C. de Freitas Neto, A. M. de Almeida, G. Maboni, T. F. de Carvalho, T. P. de Carvalho, P. A. Barrow, and A. B. Junior. 2018. Evaluation of pathogenicity of Salmonella Gallinarum strains harbouring deletions in genes whose orthologues are conserved pseudogenes in S. Pullorum. PLoS One 13: e020555.

Blanco, G., and J. A. D. de Tuesta. 2018. Culture-and molecular-based detection of swine-adapted Salmonella shed by avian scavengers. Sci. Total Environ. 634:1513–1518.

Celia-Estúpitan, A. L. D. P., D. F. A. Batista, M. V. Cardozo, A. I. S. de Souza, L. B. R. Alves, A. M. de Almeida, P. A. Barrow, A. B. Junior, and O. C. de Freitas Neto. 2017. Further investigations on the epidemiology of fowl typhoid in Brazil. Avian Pathol 46:416–425.

China National Food Safety Standard. 2016. Detection of Food Microorganisms -Salmonella spp. Ministry of Health of the People’s Republic of China. National Food Safety Standard, China GB4789.4–2016.

Dobrindt, U., and J. Hacker. 2001. Whole genome plasticity in pathogenic bacteria. Curr. Opin. Microbiol. 4:550–557.

Gand, M., W. Mattheus, N. H. C. Roosens, K. Dierick, K. Marchal, S. C. J. de Keersmaecker, and S. Bertrand. 2020. A multiplex oligonucleotide ligation-PCR method for the genosotyping of commonSalmonellae using a liquid bead suspension assay. Food Microbiol 87:103394.

Geng, S., Y. Wang, Y. Xue, H. Wang, Y. Cai, J. Zhang, P. Barrow, Z. Pan, and X. Jiao. 2019. The SseL protein inhibits the intracellular NF-κB pathway to enhance the virulence of Salmonella Pullorum in a chicken model. Microb. Pathog. 129:1–6.

Gogoi, P. P., B. Borah, I. Husein, L. Das, G. Hazarika, S. Tamuly, and L. M. Barkalita. 2018. Efficacy of pulsed-field gel electrophoresis and repetitive element sequence-based PCR in typing of Salmonella isolates from Assam. India. J.Clin. Microbiol. 56:e02043–17.

Gong, J., L. Zhuang, C. Zhu, S. Shi, D. Zhang, L. Zhang, Y. Yu, X. Dou, B. Xu, and C. Wang. 2016. Loop-mediated isothermal amplification of the sefA gene for rapid detection of Salmonella Enteritidis and Salmonella Gallinarum in chickens. Foodborne Pathog Dis 13:177–181.

Guo, R., Z. Li, Y. Jiao, S. Geng, Z. Pan, X. Chen, Q. Li, and X. Jiao. 2017. O-polyasaccharide is important for Salmonella Pullorum survival in egg albumen, and virulence and colonization in chicken embryos. Avian Pathol 46:535–540.

Issenhuth-Jeanjean, S., P. Roggentin, M. L. Mikolét, M. Guibourdenche, E. de Pinna, S. Nair, P. I. Fields, and F. X. Weill. 2014. Supplement 2008-2010 (no. 48) to the white-knaufmann-le minor scheme. Res. Microbiol. 165:526–530.

Khalatabi, R. F., N. Shahrokhi, M. Ebrahimi-Rad, and P. Ehsani. 2019. Salmonella Typhimurium in Iran: contribution of molecular and IS200 PCR methods in variants detection. PLoS One 14:e0213726.

Kirk, M. D., S. M. Pires, R. E. Black, M. Caipo, J. A. Crump, B. Devleeschauwer, D. Döpfer, A. Fazil, C. L. Fischer-Walker, T. Hald, A. J. Hall, K. H. Keddy, R. J. Lake, C. F. Lanata, P. R. Torgerson, A. H. Havelaar, and F. J. Angulo. 2015. World-Health organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. PLoS Med 12:e1001921.

Kubo, I., M. Kajiyia, N. Aramaki, and S. Furutani. 2020. Detection of Salmonella Enterica in egg yolk by PCR on a microfluidic disc device using immunomagnetic beads. Sensors 20:1060.

Liu, R., Z. Wang, X. Liu, A. Chen, and S. Yang. 2020. Rapid on-site detection of Salmonella Pullorum based on lateral flow nucleic acid assay combined with recombinase polymerase amplification reaction. Poult. Sci. 99:7225–7232.

Liu, Z., Q. Zhang, N. N. Yang, M. G. Xu, J. F. Xu, M. L. Jing, W. X. Wu, Y. D. Lu, F. Shi, and C. F. Chen. 2019. Rapid and sensitive detection of Salmonella in chickens using loop-mediated isothermal amplification combined with a lateral flow dipstick. J. Microbiol. Biotechnol. 29:454–464.

Naberhaus, S. A., A. C. Krull, L. K. Bradner, K. M. Harmon, P. Arruda, B. L. Arruda, O. Sahin, E. R. Burrough, K. J. Schwartz, and A. J. Kreuder. 2019. Emergence of Salmonella enteric serovar 4,[5],12:i:-as the primary serovar identified from swine clinical samples and development of a multiplex real-time PCR for improved Salmonella serovar-level identification. J. Vet. Diagn. Invest. 31:818–827.

Park, S. H., M. Aydin, A. Khaiwara, M. C. Dolan, D. F. Gilmore, J. L. Bouldin, S. Ahn, and S. C. Bickie. 2014. Current and emerging technologies for rapid detection and characterization of Salmonella in poultry and poultry products. Food Microbiol 38:250–262.

Pradhan, D., and V. D. Negi. 2019. Stress-induced adaptations in Salmonella: a ground for shaping its pathogenesis. Microbiol. Res. 129:6311.

Priya, B. G., R. K. Agrawal, A. A. P. Milton, M. Mishra, S. K. Mendiratta, A. Luke, S. Inbaraj, B. R. Singh, D. Kumar, C. A. Sharman, and S. K. Agrawal. 2020. Rapid and visual detection of Salmonella in meat using invasion A (invA) gene-based loop-mediated isothermal amplification assay. LWT-Food Sci. Technol. 126:109262.

Quick, J., N. D. Grubau, S. T. Pullan, I. M. Claro, A. D. Smith, K. Gangavarapu, G. Oliveira, R. Robles-Sikisaka, T. F. Rogers, N. A. Butlcer, D. R. Burton, L. L. Lewis-Ximenez, J. G. De Jesus, M. Giovanetti, S. C. Hill, A. Black, T. Bedford, M. W. Carroll, M. Nunes, L. C. Acaentara Jr, E. C. Sabino, S. A. Baylis, J. L. Bouldin, S. Ahn, and S. C. Ricke. 2014. Current and emerging technologies for rapid detection and characterization of Salmonella enterica in poultry and poultry products. Food Microbiol 38:250–262.

Ren, X., Y. Fu, C. Xu, Z. Peng, M. Li, L. Zhang, J. Zhang, and M. Liao. 2017. High resolution melting (HRM) analysis as a new tool for rapid identification of Salmonella enteric serovar Gallinarum biovars Pullorum and Gallinarum. Poult. Sci. 96:1088–1093.
Wilson, A., E. M. Fox, N. Fegan, and D. I. Kurthbøke. 2019. Comparative genomics and phenotypic investigations into antibiotic, heavy metal, and disinfectant susceptibilities of *Salmonella enterica* strains isolated in Australia. Front. Microbiol. 10:1620.

Wiltew, L., R. Jeyashakila, B. Sivaraman, B. B. Nayak, H. S. Kumar, A. K. Jaiswar, V. P. Ratrey, and G. Jeyasekaran. 2021. In-house and on-field validation of the multiplex PCR assay developed for authentication of three commercially important shrimp species. LWT-Food Sci. Technol. 148:111701.

Xiong, D., L. Song, S. Geng, J. Tao, S. An, Z. Pan, and X. Jiao. 2016. One-step PCR detection of *Salmonella* Pullorum/Gallinarum using a novel target: the flagellar biosynthesis gene flfB. Front. Microbiol 7:3863.

Xiong, D., L. Song, Z. Pan, and X. Jiao. 2018. Identification and discrimination of *Salmonella enterica* serovar Gallinarum biovars Pullorum and Gallinarum based on a one-step multiplex PCR assay. Front. Microbiol 9:1718.

Xiong, D., L. Song, J. Tao, H. Zheng, Z. Zhou, S. Geng, Z. Pan, and X. Jiao. 2017. An efficient multiplex PCR-based assay as a novel tool for accurate inter-serovar discrimination of *Salmonella* Enteritidis, S. Pullorum/Gallinarum and S. Dublin. Front Microbiol 8:420.

Xu, Y., Y. Hu, Y. Guo, Z. Zhou, D. Xiong, C. Meng, Q. Li, S. Geng, Z. Pan, and X. Jiao. 2018. A new PCR assay based on the new gene-SPUL_2693 for rapid detection of *Salmonella enterica* subsp. Enteric serovar Gallinarum biovars Pullorum and Pullorum. Poult. Sci. 97:4000–4007.

Xu, Z., M. Wang, C. Zhou, G. Gu, J. Liang, X. Hou, M. Wang, and P. Wei. 2020. Prevalence and antimicrobial resistance of salmonella enterica serovar Gallinarum biovars Pullorum and Gallinarum. Poult. Sci. 97:3619–3625.

Yang, Y., P. Wang, P. Xia, B. Yang, P. Dai, T. Hong, J. Li, X. Meng, S. El Qadil, and G. Zhu. 2020. Rapid detection of flagellated and non-flagellated *Salmonella* by targeting the common flagellar hook gene flgE. Appl. Microbiol. Biot. 104: 9719-9732.

Yang, Y., F. Xu, H. Xu, Z. P. Aguilar, R. Niu, Y. Yuan, J. Sun, X. You, W. Lai, Y. Xiong, C. Wan, and H. Wei. 2013. Magnetic nano-beads based separation combined with propidium monoazide treatment and multiplex PCR assay for simultaneous detection of viable *Salmonella* Typhimurium, *Escherichia coli* O157:H7 and *Listeria monocytogenes* in food products. Food Microbiol 34:418–424.

Zhang, J. F., B. Wei, S. Y. Cha, K. Shang, H. K. Jang, and M. Kang. 2020. The use of embryonic chicken eggs as an alternative model to evaluate the virulence of *Salmonella enterica* serovar Gallinarum. PLoS One 15:e0238630.

Zhou, Y., X. Kang, C. Meng, D. Xiong, Y. Xu, S. Geng, Z. Pan, and X. Jiao. 2020. Multiple PCR assay based on the ciga gene for detection of *Salmonella* spp. and *Salmonella* Pullorum/Gallinarum identification. Poult. Sci. 99:5991–5998.