Multiple PCR assay based on the cigR gene for detection of *Salmonella* spp. and *Salmonella* Pullorum/Gallinarum identification

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ABSTRACT  *Salmonella* spp. are important zoonotic pathogens that are responsible for severe diseases in both animals and humans. *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (*S. Gallinarum*) and biovar Pullorum (*S. Pullorum*) are typical infectious pathogens detected in the chicken industry that have caused great economic losses. To facilitate their detection and prevent contamination, we developed a rapid multiple PCR method, which can simultaneously detect *Salmonella* spp. and further identify the biovars *S. Pullorum* and *Gallinarum*. This PCR detection method is based on the *cigR* gene, which is conserved among *Salmonella* spp. but has a 42-bp deletion in *S. Pullorum/Gallinarum*. The specificity and sensitivity of the PCR assay was evaluated with 41 different strains: 34 *Salmonella* strains, including 5 *S. Pullorum/Gallinarum* strains, and 7 non-*Salmonella* strains. The lower limit of detection was 8.15 pg of *S. Pullorum* (S06004) genomic DNA and 20 cfu in PCR, which shows a great sensitivity. In addition, this method was applied to detect or identify *Salmonella* from processing chicken liver and egg samples, and the results corresponded to those obtained from serotype analysis using the conventional slide agglutination test. Overall, the new *cigR*-based PCR assay is efficient and practical for *Salmonella* detection and *S. Pullorum/Gallinarum* identification and will greatly reduce the workload of epidemiologic investigation.

Key words: *Salmonella*, *S. Pullorum/Gallinarum*, PCR assay, *cigR*, detection

INTRODUCTION

As foodborne pathogenic bacteria, *Salmonella* spp. represent an important public health issue that has become increasingly recognized by consumers worldwide. Although more than 2,600 *Salmonella* serovars have been identified to date (Issenhuth-Jeanjean et al., 2014), not all are harmful to human health because some *Salmonella* serovars are only pathogenic to their specific hosts. For example, the *Salmonella Gallinarum* biovars Pullorum and Gallinarum are restricted to poultry and can be transmitted both vertically and horizontally to cause pullorum disease or fowl typhoid in chickens (Barrow and Freitas Neto, 2011). Recently, the major *Salmonella enterica* serovars reported to cause infections in chickens include *S. enterica* serovar Enteritidis (*S. Enteritidis*) and *S. enterica* serovar Gallinarum (*S. Pullorum/Gallinarum*) (Gong et al., 2014; Fei et al., 2017; Li et al., 2018).

Traditional serotype analysis of *Salmonella* is mainly based on the White-Kauffmann-Le Minor scheme, which identifies the somatic (*O*) and flagellar (*H*) antigens using the slide agglutination test (Grimont and Weill, 2007). However, testing the H antigen of *S. Enteritidis* is a grueling and time-consuming process, which requires induction of flagellum growth, adding extensive workload to researchers especially when a range of samples need to be determined. For convenience, the specific and sensitive PCR method has been widely developed and applied to detect different
pathogens (Hoorfar, 2011; Majchrzak et al., 2014). For example, a two-step PCR assay using genes encoding the O, H, and Vi antigens (rfb, fltC, fltB, and viaB) was used to identify Salmonella serotypes (Muñoz et al., 2010). Multiplex PCR assays have also been applied for detecting specific O and H antigen gene alleles to identify the S. enterica serovars Enteritidis, Hadar, Heidelberg, and Typhimurium (Hong et al., 2008). The single gene SPUL-2693 or ipaJ was used as targets in identifying S. Pullorum/Gallinarum or S. Pullorum, respectively (Xu et al., 2018a,b). In epidemiologic investigation of Salmonella, simple and rapid PCR detection of Salmonella and its serotype are on demand, and more and more genes that are specific in all Salmonella or a certain serotype of Salmonella are explored.

The cigR gene is located on Salmonella pathogenicity island 3 (Niemann et al., 2011), which encodes CigR that acts as a T3SS2 effector and a putative inner membrane protein (Kingsley et al., 2013). Through sequence alignment, we found that although CigR is generally conserved in Salmonella, there is a slight sequence difference between S. Pullorum/Gallinarum and other Salmonella serotypes. Thus, we firstly hypothesized that the cigR gene could serve as a potential classification marker for S. Pullorum/Gallinarum to facilitate detection of these serovars.

Accordingly, in this study, we developed a rapid one-step PCR system using 2 pairs of primers to detect Salmonella and to specifically identify S. Pullorum/Gallinarum. The specificity and sensitivity of the PCR assay were evaluated, and the method was applied to processed chicken and egg samples for validation.

**Bacterial Culture and Genomic DNA Extraction**

All verified Salmonella and non-Salmonella strains were recovered in Luria–Bertani nutrient agar (Oxoid, Basingstoke, UK) or Brain Heart Infusion agar (Becton, Dickinson and Company, Sparks, MD) for 18 h at 37°C. The colonies were transferred to the relevant broth and cultured overnight at 37°C with shaking at 180 rpm for DNA purification. One to 5 mL of bacterial culture medium was centrifuged to isolate genomic DNA as per the manufacturer’s instructions of TIANamp Bacterial DNA extraction kit (TianGen, Beijing, China). The concentration and purity of the isolated genomic DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and DNA was stored at −20°C until use.

**Bioinformatics Analysis**

To detect Salmonella and differentiate S. Pullorum/Gallinarum based on a PCR assay, we exploited the Basic Local Alignment Search Tool from the National Center for Biotechnology Information. The cigR (GenBank accession no. CP022963.1 region 74,576-75,013) gene was used in searches of the nonredundant nucleotide collection (nr/nt) database. The number of nucleotide sequences was set to the maximum value of 20,000 to ensure that all aligned target sequences in the database were displayed. Two pairs of primers specific for cigR gene were designed using the online software Primer-BLAST in the National Center for Biotechnology Information.

**PCR Procedure**

All PCR assays were performed in a 25-μL reaction mixture, containing 12.5 μL 2 × Taq Master Mix (Vazyme Biotech Co., Nanjing, China), 9.5 μL of double-distilled water, 0.4 μmol L⁻¹ cigR-F primer, 0.2 μmol L⁻¹ cigR-R1 and cigR-R2 primers, and 1.0 μL of DNA template. The reaction mixture was incubated in a programmable DNA thermal cycler (Bio-Rad, Hercules, CA). PCR amplifications started with an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 95°C for 15 s, 50°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose and visualized using a GelDoc XR Gel Documentation System (Bio-Rad, Hercules, CA).

**Specificity and Sensitivity of the PCR Assay**

The specificity of the PCR assay based on the designed cigR primers was checked using DNA from 28 serovars of Salmonella and 7 non-Salmonella strains.

The sensitivity of the PCR assay was assessed to determine the lower limit of detection of the method. In brief, an overnight culture of S. Pullorum strain

**MATERIALS AND METHODS**

**Bacterial Strains**

A total of 41 strains were used to establish and verify the PCR method, including 28 serovars of Salmonella from various serogroups: S. Pullorum, S. Gallinarum, Salmonella Anatum, Salmonella Agona, Salmonella Chester, Salmonella Derby, Salmonella Dublin, S. Enteritidis, Salmonella Indiana, Salmonella Infantis, Salmonella London, Salmonella Newport, Salmonella Pakistan, Salmonella Potsdam, Salmonella Paratyphoid A, Salmonella Paratyphoid B, Salmonella Rissen, Salmonella Typhimurium, Salmonella Kentucky, Salmonella Typhi, Salmonella Mbandaka, Salmonella Montevideo, Salmonella Thompson, Salmonella Tennessee, Salmonella Abortusequi, Salmonella Ughelli, Salmonella Choleraesuis, and Salmonella Yoruba. The other 7 strains were non-Salmonella strains, including Mycobacterium tuberculosis, Listeria monocytogenes, Campylobacter jejuni, Campylobacter coli, Escherichia coli, Shigella flexneri, and Staphylococcus aureus, which were used to evaluate the specificity of the method.
S06004 was harvested by centrifugation and then washed with phosphate-buffered saline 3 times. The optical density at 600 nm of S06004 was adjusted to 1, and then, the bacterial suspension was diluted 10 times. The cfu count was determined by the plate count method. In addition, diluted S06004 of different densities was boiled at 100°C for 10 min and centrifuged at 10,000 g for 2 min to obtain the supernatant DNA. After plate counting, the supernatants were adjusted to a final concentration of 400, 200, 80, 40, 20, 10, and 5 cfu mL⁻¹. The genomic DNA of S06004 was obtained from a 1-mL bacterial suspension of optical density at 600 nm = 1 and then serially diluted to the following concentrations: 163 ng mL⁻¹, 16.3 ng mL⁻¹, 1.63 ng mL⁻¹, 163 pg mL⁻¹, 16.3 pg mL⁻¹, 8.15 pg mL⁻¹, and 4.075 pg mL⁻¹. Finally, 1 μL of each dilution was used for the PCR.

Application of the PCR Method to Clinical Samples

The PCR assay was applied to evaluate the contamination of Salmonella collected from 87 sick or dead chickens that came from chicken farms in Shanghai and Jiangsu Province, China. All animal experimental protocols were approved by the Committee on the Ethics of Animal Experiments of Yangzhou University, Yangzhou, China. Concrete performance was determined as described in our previous report (Fei et al., 2017). In brief, the liver of each chick was aseptically obtained, and approximately 10 g of the tissue was suspended in 100 mL buffered peptone water for incubation at 37°C for 16–18 h. Next, 100 μL of this pre-enriched culture was transferred into 9.9 mL of Rappaport-Vassiliadis enrichment broth (Difco, BD, Sparks, MD) and then subcultured at 42°C for 24 h. Then, 100 μL of the bacterial suspension from the enrichment broth was added to double-distilled water and washed 3 times, and then, dissolved in 50 μL double-distilled water. After boiling, the supernatants containing the DNA were prepared by centrifugation and used as template in PCR. In the meantime, one loopful of each selective enriched culture was inoculated onto Xylose Lysine Tergitol-4 (XLT-4) agar (Difco, BD, Sparks, MD) plates, which were incubated at 37°C for 24–48 h. A presumptive Salmonella colony growing on XLT-4 was inoculated into liquid Luria–Bertani and then biochemically confirmed using an API-20E test kit (bioMérieux, Marcy l’Etoile, France). Salmonella serotyping was then performed in accordance with the White-Kauffmann-Le Minor scheme by slide agglutination with O and H antigen-specific sera (SSI Diagnostica, Hiller, Denmark).

In addition, 40 clinical samples from eggs that had experienced pre-enrichment and selective enrichment were chosen for practical evaluation of the method. The PCR templates were prepared as described previously. The results from the PCR assay were compared with those from traditional serotyping.

RESULTS AND DISCUSSION

Bioinformatics Analysis and Primer Design

Bioinformatics analysis revealed that cigR existed in all Salmonella, and a 42-bp fragment was found to be absent in S. Pullorum/Gallinarum compared with other serotypes of Salmonella (Supplementary Figure 1). Because this 42-bp difference is not sufficiently obvious to clearly distinguish S. Pullorum/Gallinarum from other Salmonella, as shown in Figure 1, 2 pairs of primers were designed, including a reverse primer that is specific to the 42-bp fragment, which allowed for the specific identification of these serovars from other Salmonella present in a sample.

The primers used in this study were designed based on the nucleotide sequence of the cigR gene in S. Typhimurium (Accession no. NC-003197.2 region 3960760-3961239). The sequences of the primers were cigR-F, 5’-ATGAATAATCGTGCTGTTTT-3’, cigR-R1, 5’-TAATAATCGCCGTGAACCACC-3’, and cigR-R2, 5’-GTAGCGTCTAGGGAAAACG-3’.

Specificity of the PCR Assay

To ensure the specificity of the PCR assay, 34 Salmonella strains from 28 different serotypes included

![Figure 1](image-url). Diagram of the primer design of cigR to distinguish Salmonella Pullorum/Gallinarum from other serovars. cigR gene of Salmonella Pullorum/Gallinarum has a 42-bp-deficient region compared with that of other serovars, which was exploited to design the primers. The arrows indicate the positions of the designed primers. The PCR amplifies a 421-bp product of S. Pullorum/Gallinarum and 2 products of 463 and 65 bp of non-S. Pullorum/Gallinarum.
in various serogroups and 7 non-Salmonella strains (Table 1) were examined. As shown in Figure 2, five different strains of S. Pullorum/Gallinarum could be differentiated from 29 other Salmonella strains: only 1 band (421 bp) was observed in the PCR products for S. Pullorum/Gallinarum, whereas 2 bands (463 bp and 65 bp) were detected for the other Salmonella strains. No band was detected for the 7 non-Salmonella strains. These results suggested that the PCR method can broadly detect various types of Salmonella and is specific to identify S. Pullorum/Gallinarum.

Although the cigR gene also exists in Pseudomonas putida (Nelson et al., 2001), it has no significant sequence similarity with Salmonella cigR. Compared with the multiplex PCR method targeting invA, sdf, and STM4492 for detection of Salmonella spp. and differentiation of S. Typhimurium and S. Enteritidis (Saeki et al., 2013), the cigR-based PCR assay is simpler and yet also allows for both Salmonella detection and S. Pullorum/Gallinarum identification. Because PCR detection of ipaJ or rfbS has been suggested for the detection of S. Pullorum or S. Enteritidis.

Table 1. Salmonella and non-Salmonella strains used in this study.

| Serotype/species | Strain | Source | Serogroup | PCR result (band number) |
|------------------|--------|--------|-----------|--------------------------|
| Salmonella | Paratyphoid A | 50,093 | Laboratory stock | A | 2 |
| Salmonella | Abortus equi | A | Laboratory stock | B | 2 |
| Salmonella | Typhimurium | C7 | Laboratory stock | B | 2 |
| Salmonella | Paratyphoid B | 1 | Laboratory stock | B | 2 |
| Salmonella | Chester | B1 | Laboratory stock | B | 2 |
| Salmonella | Agona | T7N1 | Laboratory stock | B | 2 |
| Salmonella | Derby | F10 | Laboratory stock | B | 2 |
| Salmonella | Indiana | T4 | Laboratory stock | B | 2 |
| Salmonella | Tennessee | SH78 | Laboratory stock | C1 | 2 |
| Salmonella | Thompson | J093 | Laboratory stock | C1 | 2 |
| Salmonella | Montevideo | S10 | Laboratory stock | C1 | 2 |
| Salmonella | Mbandaka | SH138 | Laboratory stock | C1 | 2 |
| Salmonella | Rissen | C10 | Laboratory stock | C1 | 2 |
| Salmonella | Infantis | T6N1 | Laboratory stock | C1 | 2 |
| Salmonella | Choleraeusis | C500 | Laboratory stock | C1 | 2 |
| Salmonella | Newport | GS3-1 | Laboratory stock | C2 | 2 |
| Salmonella | Pakistan | A6 | Laboratory stock | C3 | 2 |
| Salmonella | Kentucky | S190 | Laboratory stock | C3 | 2 |
| Salmonella | Typhi | 50,071 | Laboratory stock | D | 2 |
| Salmonella | Enteritidis | C50041 | Laboratory stock | D | 2 |
| Salmonella | Enteritidis | C50336 | Laboratory stock | D | 2 |
| Salmonella | Enteritidis | Z11 | Laboratory stock | D | 2 |
| Salmonella | Enteritidis | P125109 | Tang et al., 2018 | D | 2 |
| Salmonella | Pullorum | S06004 | Laboratory stock | D | 1 |
| Salmonella | Pullorum | C79-13 | Laboratory stock | D | 1 |
| Salmonella | Pullorum | RK55078 | Laboratory stock | D | 1 |
| Salmonella | Pullorum | 449/87 | Tang et al., 2018 | D | 1 |
| Salmonella | Gallinarum | SG9 | Wigley et al., 2005 | D | 1 |
| Salmonella | Dublin | SL5928 | Laboratory stock | D1 | 2 |
| Salmonella | London | G11 | Laboratory stock | E | 2 |
| Salmonella | Anatum | S21 | Laboratory stock | E1 | 2 |
| Salmonella | Ughelli | C14 | Laboratory stock | E1 | 2 |
| Salmonella | Yoruba | H2-G14 | Laboratory stock | I | 2 |
| Non-Salmonella | Mycobacterium tuberculosis | H37Rv | ATCC 27294 | 0 |
| | Listeria monocytogenes | EGD | ATCC | 0 |
| | Campylobacter jejuni | 11,168 | ATCC 700819 | - | 0 |
| | Campylobacter coli | 115-1 | Isolate from chicken | - | 0 |
| | Escherichia coli | 1,314 | Isolate from chicken | - | 0 |
| | Shigella flexneri | 301 | Jin et al., 2002 | - | 0 |
| | Staphylococcus aureus | 502A | ATCC 27217 | - | 0 |

Table 2. Clinical samples used to evaluate the application of the PCR method.

| Source of samples | No. of enriched samples | No. of Salmonella positive samples by PCR | PCR result (band number) | Serovar\(^a\) (no. of isolates) | Coincidence rate between PCR and traditional method of bacteria separation |
|-------------------|-------------------------|----------------------------------------|--------------------------|-----------------------------|---------------------------------------------------------------|
| Chickens          | 87                      | 24                                     | 2\(^1\)                  | Enteritidis (15)            | 100%                                                          |
| Eggs              | 40                      | 23                                     | 1\(^2\)                  | Pullorum (9)                |                                                              |

\(^1\)PCR result with 2 bands suggested that this sample contained Salmonella except Salmonella Pullorum/Gallinarum.

\(^2\)PCR result with 1 band suggested that this sample contained Salmonella Pullorum/Gallinarum.

\(^3\)Serovar was determined by agglutination tests using specific H and O antisera (SSI Diagnostika, Hiller, Denmark).
Gallinarum identification (Shah et al., 2005; Xu et al., 2018a), 1 of these 2 genes can be combined with \textit{cigR} for the detection of \textit{Salmonella} spp. and differentiation of \textit{S. Pullorum} and \textit{S. Gallinarum}.

**Figure 2.** Specificity of the PCR assay for \textit{Salmonella} detection and \textit{Salmonella} Pullorum/Gallinarum identification. The \textit{cigR} gene was PCR-amplified using genomic DNA from various \textit{Salmonella} and non-\textit{Salmonella} strains. Lane M: DL2000 DNA marker. Detailed strain information is provided in Table 1.

**Sensitivity of the PCR Assay**

The genomic DNA of \textit{S. Pullorum} S06004 serially diluted from 163 ng \(\mu\text{L}^{-1}\) to 4.075 pg \(\mu\text{L}^{-1}\) was used

**Figure 3.** Sensitivity of the PCR assay for detection of genomic DNA and cells from \textit{Salmonella} Pullorum S06004. (A) PCR for the detection of genomic DNA. Lanes 1-7, \textit{S. Pullorum} genomic DNA used as a template in the following amounts: 163 ng, 16.3 ng, 1.63 ng, 163 pg, 16.3 pg, 8.15 pg, and 4.075 pg. (B) PCR for the detection of S06004 cells. Lanes 1-7, cfu per PCR assay: 400, 200, 80, 40, 20, 10 and 5. Lane M: DL2000 DNA marker.
Figure 4. One-step PCR for detection of *Salmonella* and identification of *Salmonella* Pullorum/Gallinarum from processing chicken and egg samples. The enrichment broths of chicken (A) and egg (B) samples were used as templates in PCR. The PCR assay produced 1 product of 421 bp for *S.* Pullorum/Gallinarum and 2 products for other *Salmonella*. Lane M: DL2000 DNA marker. See Table 2 for detailed information of the chicken and egg samples.
to determine the limit of detection of the PCR assay. A specific band was still visible when the DNA content was as low as 8.15 pg (Figure 3A). This limit of detection is similar to that previously determined in the PCR detection of S06004 using JhB (Xiong et al., 2016). In addition, 20 cfu was the lowest amount of strain S06004 that could be detected with the PCR assay (Figure 3B). This minimum detectable cell number is lower than that reported previously in the PCR identification of S. Pullorum (Xu et al., 2018b).

Application of the PCR Method to Clinical Samples

To validate the PCR method based on the cigR gene, the liver samples of 87 sick or dead chickens were tested. DNA samples were prepared from bacterial suspensions in selective enrichment broth and then added to the PCR system to amplify the cigR gene. Figure 4A demonstrates that 9 strains were identified as S. Pullorum/Gallinarum. 15 strains were non-S. Pullorum/Gallinarum, and others had no Salmonella. All 87 bacterial samples collected from selective enrichment broths were also spread onto XLT-4 plates, and a single suspected colony was analyzed by serotyping. Traditional serotyping confirmed that in 24 colonies obtained from XLT-4, 9 strains were S. Pullorum and the other 15 strains were S. Enteritidis, which were in accordance with the PCR results.

To further extend the application of the PCR assay, the method was also tested with the processing samples from eggs. As shown in Figure 4B, 23 samples produced 2 bands, representing the presence of Salmonella but not S. Pullorum/Gallinarum. No PCR product was detected in eight DNA samples, indicating no Salmonella contamination. The results from traditional bacterial isolation and serotype analysis were identical to those of the cigR gene-based PCR assay. Furthermore, the use of PCR with bacteria in selective enrichment broth helps reduce the time required for Salmonella growth on XLT-4 by 24–48 h when compared with PCR identification of a single colony. This advantage may be particularly helpful when conducting a large epidemiologic study and high-throughput screen because the entire PCR assay could be completed in less than 2 h.

The proposed PCR method could discriminate S. Pullorum/Gallinarum from other serotypes of Salmonella. For the chicken samples, 9 S. Pullorum strains were identified and the other 15 strains were S. Enteritidis. All 23 Salmonella isolates obtained from egg samples were identified as S. Enteritidis. This finding corresponds with a report from the United States indicating that S. Enteritidis is one of the leading bacterial causes of foodborne illness and that shell eggs are a primary source of human S. Enteritidis infections (FDA, 2009; CDC, 2015). S. Enteritidis differs from other serovars in its capacity for transovarian transmission, that is, to infect the egg’s internal contents (Moffatt and Musto, 2013). Thus, a rapid and efficient PCR method for S. Enteritidis detection is also immediately needed. Toward this end, the specific genes prof6c or sdf that target S. Enteritidis can be integrated into the PCR system established in this study for further identification of S. Enteritidis in chicken or egg samples (Agron et al., 2001; Malorny et al., 2007).

In epidemiologic surveys, the samples from the selective enrichment broth proven to be negative by this PCR assay could be ruled out, which will help to save human and material resources. Moreover, because animals are needed to prepare antisera used for traditional serotyping, this PCR assay could also contribute toward reducing the use of antisera with the benefit of animal protection. Because the existing method for Salmonella detection in clinical samples is not sufficiently rapid for practical purposes, our PCR method will help save the time spent in single colony formation and serotype identification. However, because the pre-enrichment and selective enrichment steps are also time consuming, these steps should be optimized and shortened, and the PCR should be validated for effective application of this method in testing clinical samples.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program Special Project (2017YFC1601203 and 2016YFD0501607), China, the Special Fund for Agroscientific Research in the Public Interest (201403054), the National Natural Science Foundation of China (nos. 31730094), China, the Project for Agricultural Products Quality and Safety Supervision (Risk Assessment) (181721301092362264), the Yangzhou University Science and Technology Innovation Team, China, and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), China.

Conflict of Interest Statement: The authors declare that they have no conflict of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.07.026.

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