A new multiplex PCR for the accurate identification and differentiation of *Salmonella enterica* serovar Gallinarum biovars Pullorum and Gallinarum

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Salmonella enterica* serovar Gallinarum* biovars Gallinarum* and *Pullorum* cause severe chicken salmonellosis, a disease associated with high mortality and morbidity among chickens worldwide. The conventional serotyping and biochemical reactions have been used to identify *Salmonella* serovars. However, the conventional methods are complicated, time-consuming, laborious, and expensive. Furthermore, it is challenging to distinguish *S. Gallinarum* and *S. Pullorum* via biochemical assays and serotyping because of their antigenic similarity. Although various PCR methods were established, a PCR protocol to detect and discriminate *S. Gallinarum* and *S. Pullorum* simultaneously is lacking. Herein, a one-step multiplex PCR method was established for the accurate identification and discrimination of *S. Pullorum* and *S. Gallinarum*. Three specific genes were used for the multiplex PCR method, with the *I137_14445* and *ybgL* genes being the key targets to identify and differentiate *S. Gallinarum* and *S. Pullorum*, and *stn* being included as a reference gene for the *Salmonella* genus. In silico analysis showed that the *I137_14445* gene is present in all *Salmonella* serovars, except for *S. Gallinarum*, and could therefore be used for the identification of *S. Gallinarum*. A 68-bp sequence deficiency in *ybgL* was found only in *S. Pullorum* compared to other *Salmonella* serovars, and this could therefore be used for the specific identification of *S. Pullorum*. The developed PCR assay was able to distinguish *S. Gallinarum* and *S. Pullorum* among 75 various *Salmonella* strains and 43 various non-*Salmonella* pathogens with excellent specificity. The detection limit for the genomic DNA of *S. Gallinarum* and *S. Pullorum* was 21.4 pg. /μL, and the detectable limit for bacterial cells was 100 CFU. The developed PCR method was used for the analysis of *Salmonella* isolates in a chicken farm. This PCR system successfully discriminated *S. Gallinarum* and *S. Pullorum* from other different *Salmonella* serovars. The PCR results were confirmed by the conventional serotyping method. The newly established multiplex PCR is a
simple, accurate, and cost-effective method for the timely identification and differentiation of S. Pullorum and S. Gallinarum.

KEYWORDS
Salmonella pullorum, Salmonella gallinarum, multiplex PCR, I137_14445, ybgL, accurate differentiation

Introduction

Salmonella enterica can cause severe enteric fever, gastroenteritis, and septicaemia, leading to serious public health problems globally (Foley and Lynne, 2008; Odoch et al., 2017; Williams et al., 2022). Salmonella spp. contains over 2,650 serovars by the diverse combinations of lipopolysaccharide (O antigens) and flagellar structure (H antigens; Guibourdenche et al., 2010; Cheng et al., 2019). However, some Salmonella serovars are only infectious to the specific hosts (Saeki et al., 2013; Zhu et al., 2015).

Fowl typhoid (FT) and Pullorum disease (PD) are caused by Salmonella enterica serovar Gallinarum biovars Gallinarum (S. gallinarum) and Pullorum (S. pullorum), respectively. The two types of septicemic diseases have significant effects on poultry industry, resulting in serious economic losses in many countries (Foley et al., 2011). PD, caused by S. pullorum, is associated with high mortality and morbidity rates among the young chicks, especially those less than 3 weeks of age. The characteristics of the infected chicks are the acute septicemia and white viscous diarrhea (Rettger, 1909). S. Pullorum can also be transmitted to the eggs through the ovary in the infected hens, resulting in both horizontal and vertical transmission and causing serious economic burden for the poultry industry (Geng et al., 2017; Xu et al., 2018). S. Gallinarum, a close relative of S. Pullorum, can lead to chronic and acute septicemia in different ages of poultry, and is a host-specific pathogen causing FT (Barrow and Freitas Neto, 2011).

The White–Kauffmann–Le Minor scheme has been used as the conventional method for Salmonella serotyping. The method is based on specific O (somatic) and H (flagellar) antisera by using slide agglutination tests (Majchrzak et al., 2014). However, this method is time-consuming, taking 5–6 days for the whole procedure. Besides, the expensive typing antisera are required for the assay (Bell et al., 2016). Furthermore, the differentiation of S. Pullorum and S. Gallinarum is difficult as the same O antigens 1, 9, and 12 between them (Christensen et al., 1993). Most Salmonella species possess flagella and exhibit motility. However, S. Pullorum and S. Gallinarum are two notable exceptions, having been shown lack of motility and flagella (H antigen; Holt and Chaulal, 1997). The biochemical tests, based on the fermentation types of ornithine, spironolactone, dulcitol, and maltose, were conducted previously to identify the two Salmonella biovars (Van Immerseel et al., 2013). However, the characteristics of typical and atypical colonies by the naked eyes increased the workload because the possibility of contamination must be excluded (Bai et al., 2019).

Molecular methods have shown high ability for the sensitive and specific discrimination of different pathogens and closely related variants. The increasing development of DNA-based detection methods has been applied to identify Salmonella spp., such as amplified fragment length polymorphism, pulsed-field gel electrophoresis, and DNA–DNA microarray hybridization (Swaminathan et al., 2001; Liebana, 2002; Morales et al., 2005). More recently, whole-genome sequencing has further enhanced the genetic analysis of Salmonella. The whole bacterial genome of each strain can be determined and the results can be compared to the large genetic databases (Carroll et al., 2021; Atlaw et al., 2022). However, this method is expensive and professional technicians are required for the data analysis.

Therefore, accurate and cost-effective methods for the fast and sensitive detection of specific Salmonella serovars in poultry products are urgently needed. A number of PCR methods have been developed, and the assays demonstrated high specificity and sensitivity in detecting Salmonella serotypes using such methods (Massi et al., 2003; Kim et al., 2017). The combination of PCR and restriction fragment length polymorphism was used for the differentiation of S. Pullorum and S. Gallinarum (Ribeiro et al., 2009; Soler-Garcia et al., 2014). However, these complex analyses have limited the diagnostic applications of assays for the differential diagnosis of PD and FT (Ren et al., 2017). A rapid and cost-effective diagnostic method is therefore in urgent need to detect and differentiate S. Gallinarum and S. Pullorum.

In this study, a new one-step multiplex PCR method was developed for the specific detection and differentiation of S. Pullorum and S. Gallinarum simultaneously. The assay involved three pairs of primers based on the I137_14445, ybgL, and stn genes. The sensitivity and specificity of the multiplex PCR method were determined, and the assay was used for the specific identification of S. Gallinarum and S. Pullorum among clinical Salmonella isolates from a chicken farm.

Materials and methods

Bacterial strains

The bacterial strains including Salmonella spp. and non-Salmonella pathogens for the development and verification
of the multiplex PCR method are listed in Supplementary Table S1. A total of 75 Salmonella strains and 43 various non-Salmonella strains were used in the present study, which were isolated previously as part of our routine monitoring and were stored at the Jiangsu Key Laboratory of Zoonosis, Yangzhou University. The biochemical identification of all Salmonella strains was conducted with the API identification kits (BioMérieux, Marcy, France). The Salmonella serovars were verified with the diagnostic antisera (Tianrun Bio-Pharmaceutical, Ningbo, China) in accordance with the White–Kaufmann–Le Minor scheme. For the discrimination of S. Pullorum and S. Gallinarum, the ornithine decarboxylation and dulcitol fermentation were conducted.

Bacterial growth and genomic DNA extraction

Frozen stocks of the isolates were recovered on brain heart infusion (BHI) agar (Becton, Dickinson and Company, Sparks, MD, United States) or Luria–Bertani (LB) agar (Oxoid, Basingstoke, United Kingdom) overnight at 37°C. The bacterial strains were cultured in LB or BHI broth at 37°C for 16 h with 180 rpm for the genomic DNA extraction.

The extraction of bacterial genomic DNA was conducted with the TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) in accordance with the manufacturer’s procedures. The DNA purity and concentration were determined with the NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, United States). The DNA was subsequently stored at −20°C prior to use.

In silico analysis and primer design

To establish a useful PCR method to identify and differentiate the biovars S. Gallinarum and S. Pullorum, we analyzed differences in the nucleotide sequences of the I137_14445 and ybgL genes. The genes I137_14445 (GenBank acc. no. CP006575.1, region 3,087,481–3,088,224) and ybgL (GenBank acc. no. AM933173.1, region 770825–771559) were searched in the non-redundant nucleotide collection (nr/nt) database. The displayed number of nucleotide sequences was set to the maximum value of 5,000 to ensure that all aligned sequences in the database were included. DNA sequence alignment of I137_14445 and ybgL genes from S. Pullorum, S. Gallinarum and other Salmonella serotypes was performed using ClustalW. Three pairs of primers were optimized for the development of the multiplex PCR. The positions of the designed primers were based on the gap in the ybgL gene, the unique sequence in the I137_14445 gene, and a conserved sequence to all Salmonella serotypes in the stn gene. The primers were designed by length so that they could be distinguished on an agarose gel. The first primers of I137_14445-F/R amplified a 525-bp product to differentiate S. Gallinarum from other serovars. The second primer set, ybgL-F/R, amplified a 307-bp fragment to allow for the discrimination of S. Pullorum. The third primer set, stn-F/R, amplified a 731-bp fragment to identify the Salmonella genus from other microorganisms (Table 1). The three primer sets were designed and checked using the software Primer Premier 5.0. The primer specificity was determined with the basic local alignment search tool (BLASTn; NCBI, Bethesda, MD, United States). The primers were commercially synthesized by GenScript (Nanjing, China).

Development and optimization of the multiplex PCR assay

The multiplex PCR system was conducted in a final volume of 25 μl, including 2× Taq Master mix (12.5 μl; Vazyme, Nanjing, China), the I137_14445-F/R primers (80 nM), the ybgL-F/R primers (80 nM), the stn-F/R primers (40 nM), and the bacterial genomic DNA (100 ng). The PCR reaction was conducted in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, United States), and programmed as an initial denaturation at 94°C for 3 min; 30 sequential cycles of 94°C for 40 s, 53°C for 30 s, and 72°C for 60 s; and a final step of 72°C for 10 min. The PCR products were stained with GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, United States) following 1% agarose gel electrophoresis. The visualization of the amplified PCR fragments was obtained by using the Gel Doc XR Gel Documentation System (Bio-Rad).

### Table 1. Primer sequences for the specific detection and differentiation of S. gallinarum and S. pullorum with the multiplex PCR system.

| Primers | Primer sequence (5′ → 3′) | Size (bp) | GenBank acc. no./Nt segments | Salmonella serovars |
|---------|--------------------------|-----------|-------------------------------|--------------------|
| stn F   | TCCTGTGTGCTCGCTATCAGTG   | 731       | L16014.1                      | +                  |
| stn R   | TTTTGGCATACGGTTATCAGC    | 353–1,083 | 308675.103086769–3,088,224    | +                  |
| I137_14445 F | AGCGAAGAGGCGGTTGTGAT     | 525       | CP006575.13086769–3,088,225   | +                  |
| I137_14445 R | CCGCCCGACTGATGTGATGAC    |           |                                | −                  |
| ybgL F  | AAGCCGCCGATCGCTTCTAAC    | 307       | AM933173.1771135–771,441      | +                  |
| ybgL R  | ACGCCGATACGGCTTCTAC      |           |                                | +                  |

SP: S. pullorum; SG: S. gallinarum.
Specificity of the multiplex PCR assay

The specificity of the multiplex PCR method was determined by using the genomic DNA extracted from 12 S. Pullorum strains, 4 S. Gallinarum strains, 59 strains from other Salmonella serovars, and 43 non-Salmonella pathogens. Detailed information for these strains is presented in Supplementary Table S1.

Sensitivity of the multiplex PCR assay

The PCR sensitivity was conducted to evaluate the detection limit of the method. The bacterial genomic DNA was from the two Salmonella biovars S. Gallinarum strain SG9 and S. Pullorum strain S06004. A serial dilution (10-fold) was obtained from 21.4 to 2.14 pg/μl to serve as the templates in the developed multiplex PCR method. Overnight cultured bacteria of S. Pullorum and S. Gallinarum were washed twice with PBS and the optical densities at 600 nm (OD_{600}) of the two strains were adjusted to 1. The CFU concentrations of the two strains when the OD_{600} = 1 were determined by plate counts. Freshly cultured bacteria were adjusted to the OD_{600} = 1 and diluted to the same CFU concentrations with PBS. The bacteria were serially diluted (10-fold) from 2 × 10^{10} to 2 × 10^{7} CFU/μl, and the bacterial concentrations were verified by plate counts. The genomic DNA was extracted from these diluted bacterial suspensions, and 5 μl of the extracted DNA was served as a template in the multiplex PCR.

Analysis of chicken egg samples using the PCR assay

Naturally contaminated samples were collected from clinically dead eggs from a chicken farm in Yangzhou, China. The isolation of Salmonella pathogens from the chicken egg samples were conducted as previously described (Cai et al., 2016; Zhou et al., 2017). In brief, each sample was pre-enriched at 37°C for 24 h in 50 ml of buffered peptone water (Difco, BD, Sparks, MD, United States). The bacterial culture was streaked onto xylose lysine tergitol 4 (Difco, BD) agar, and incubated at 37°C for 16 h. The presumptive Salmonella colonies were individually picked as templates for the multiplex PCR. Meanwhile, each sample was also subjected to a standard traditional serum agglutination assay.

Results and discussion

Sequence alignment analysis and primer design

Due to the advantages of rapidity, specificity and sensitivity, PCR has been used widely to detect and identify the particular bacterial pathogens. The choice of target is key to the development of a specific and sensitive PCR assay. In silico analysis confirmed the presence of the i137_14445 gene in all Salmonella serovars except for S. Gallinarum, indicating that this discrepancy could be used for the identification of S. Gallinarum. A 68-bp region in the ybgL gene was present in S. Gallinarum and other Salmonella serovars but deleted in S. Pullorum, and this region could therefore be targeted for the specific identification of S. Pullorum (Figure 1). Thus, the i137_14445 and ybgL genes could be used as targets for the accurate identification and differentiation of S. Gallinarum and S. Pullorum, and the xtn gene was used as a reference gene for the Salmonella genus. Specific primer sets were designed to target these three DNA fragments (Table 1).

Other new genes have been reported for the identification of Salmonella. The ipaF gene of S. Pullorum was used for the development of a PCR method to detect S. Pullorum (Xu et al., 2018). Another PCR method was developed based on the cigR gene for the efficient detection of Salmonella and identification of S. Gallinarum/Pullorum (Zhou et al., 2020). However, these PCR methods could not identify and differentiate S. Pullorum and S. Gallinarum simultaneously. In this study, the i137_14445 and ybgL genes were used as targets to differentiate S. Gallinarum and S. Pullorum for the first time.

Specificity of the primers for S. Gallinarum and S. Pullorum identification and differentiation

The timely identification, genotyping, and serotyping of Salmonella pathogens could provide important information about the strain identification and source of infection during an outbreak (Gebreyes et al., 2006). Even so, most genotyping assays such as plasmid profile analysis, ribotyping, amplified fragment length polymorphism, and pulsed-field gel electrophoresis could not
produce results consistent with Salmonella serotypes and genotypes (Bailey et al., 2002; Wang et al., 2011; Ozdemir and Acar, 2014).

In this study, the specificity of the I137_14445 and ybgL primer sets was evaluated with genomic DNA extracted from 75 Salmonella strains and 43 various non-Salmonella pathogens. The
Salmonella strains included 29 various serovars (Supplementary Table S1). The results showed that for S. Pullorum, only two amplified products, 731-bp stn and 525-bp I137_14445, were generated. For S. Gallinarum, two products, 731-bp stn and 307-bp ybgL, were generated. For other Salmonella serovars, three products, stn, I137_14445, and ybgL, were amplified. By contrast, no bands were observed for the 43 non-Salmonella pathogens (Figure 2). No false negative or positive fragments were produced, suggesting that the developed multiplex PCR had high specificity. Both biovars could be identified and distinguished based on the newly developed multiplex PCR. Previous methods were developed based on a single-nucleotide polymorphism or the variable regions of a certain gene to distinguish S. Gallinarum and S. Pullorum, such as PCR restriction fragment length polymorphism and single-stranded conformational polymorphism (Kisiela et al., 2005; Soler-García et al., 2014). However, in the present study, S. Gallinarum and S. Pullorum could be directly differentiated simultaneously based on three specific targets in a multiplex PCR. The multiplex PCR assay generated products of the I137_14445 and ybgL genes, which enabled clear discrimination between S. Gallinarum, S. Pullorum, and other serovars. Importantly, as the primers I137_14445 F/R are S. Gallinarum-specific, and the ybgL gene is S. Pullorum-specific, the two candidate genes could be used separately, to identify the two biovars, respectively.

Sensitivity of the multiplex PCR assay for S. Gallinarum and S. Pullorum identification

The genomic DNA of S. Pullorum S06004 and S. Gallinarum SG9 was diluted serially from 21.4 ng/μl to 2.14 pg./μl to evaluate the detectable concentration of the PCR method. The minimum detection limit of the three sets of primers for the identification of S. Gallinarum and S. Pullorum was 21.4 pg./μl of genomic DNA (Figure 3A). The result was comparable to the HRM-PCR method established previously (Ren et al., 2017). In addition, for different concentrations of bacterial cells, the PCR products were detected successfully at concentrations ranging from 10⁵ to 10² CFU per reaction (Figure 3B). The minimum number of detectable cells of S. Gallinarum or S. Pullorum in the developed PCR method was much lower than that of the sefA-based PCR assay (400 CFU; Gong et al., 2016), and it was comparable to the ipaJ-based PCR assay (100 CFU; Xu et al., 2018). The results showed that the multiplex PCR method has low detection limit, and thereby low concentrations of S. Gallinarum and S. Pullorum could
be detected. The lowest detection limit of each single PCR reaction was also determined. The results showed that the lowest number of cells of *S. Pullorum* and *S. Gallinarum* was 10 CFU for *ybgL* and *stn*, and 100 CFU for *I137_14445* (Supplementary Figure S1). As the detectable limit for bacterial cells of the developed multiplex PCR method was 100 CFU (Figure 3), the detectable limit of 100 CFU would be recommended for the accurate identification and differentiation of *S. Pullorum* and *S. Gallinarum*.

### TABLE 2 The developed multiplex PCR method was applied for the identification of *Salmonella* isolates from one chicken farm.

| Serovar (no. of isolates) | Isolate no. | PCR results (stn/I137_14445/ybgL) | Dulcitol fermentation | Ornithine decarboxylase |
|---------------------------|-------------|-----------------------------------|-----------------------|-------------------------|
| *Pullorum* (10)           | Ch4         | +/+/+                             | −                     | +                       |
|                           | Ch5         | +/+/+                             | −                     | +                       |
|                           | Ch9         | +/+/+                             | −                     | +                       |
|                           | Ch10        | +/+/+                             | −                     | +                       |
|                           | Ch11        | +/+/+                             | −                     | +                       |
|                           | Ch12        | +/+/+                             | −                     | +                       |
|                           | Ch16        | +/+/+                             | −                     | +                       |
|                           | Ch17        | +/+/+                             | −                     | +                       |
|                           | Ch18        | +/+/+                             | −                     | +                       |
|                           | Ch24        | +/+/+                             | −                     | +                       |
| *Gallinarum* (2)          | Ch22        | +/+/+                             | +                     | −                       |
|                           | Ch23        | +/+/+                             | +                     | −                       |
| *Enteritidis* (9)         | Ch1         | +/+/+                             | /                     | /                       |
|                           | Ch2         | +/+/+                             | /                     | /                       |
|                           | Ch3         | +/+/+                             | /                     | /                       |
|                           | Ch6         | +/+/+                             | /                     | /                       |
|                           | Ch7         | +/+/+                             | /                     | /                       |
|                           | Ch8         | +/+/+                             | /                     | /                       |
|                           | Ch13        | +/+/+                             | /                     | /                       |
|                           | Ch14        | +/+/+                             | /                     | /                       |
|                           | Ch15        | +/+/+                             | /                     | /                       |
| *Indiana* (3)             | Ch19        | +/+/+                             | /                     | /                       |
|                           | Ch20        | +/+/+                             | /                     | /                       |
|                           | Ch21        | +/+/+                             | /                     | /                       |

The traditional serotyping of the *Salmonella* isolates was determined following the White–Kauffmann–Le Minor scheme. The traditional differentiation of *Salmonella* biovars *Pullorum* and *gallinarum* was conducted with the ornithine decarboxylation and dulcitol fermentation.

### Application of the *S. Pullorum*– and *S. Gallinarum*-specific multiplex PCR assay

*S. Gallinarum* and *S. Pullorum* can cause FT and PD respectively, and thus resulted in substantial economic losses of livestock (Ren et al., 2017). Some prevalent poultry bacterial diseases are caused by *S. Gallinarum* and *S. Pullorum* (Gong et al., 2014). Traditional assays for the identification of *Salmonella* serovars relied on diagnostic serological agglutination. However, the cross-reactivity could occur with *S. Enteritidis* or other serogroup D serovars (Ren et al., 2017). The phenotypic characterization of *Salmonella* serovars has been mainly based on the serotyping. However, incorporation of serotyping analysis with other identification and molecular typing methods is often necessary for the rapid determination of the epidemiological linkage (Liu et al., 2011). Although various PCR methods have been developed to identify different serovars, including a multiplex qPCR assay (Rubio et al., 2017) and a duplex PCR assay (Batista et al., 2016) for detecting *S. Gallinarum* and *S. Pullorum*, few molecular methods are available to detect and differentiate *S. Pullorum* and *S. Gallinarum* simultaneously.

The PCR assay developed in this study satisfies this requirement, and could contribute to the purification of the two *Salmonella* biovars in the poultry farms.

A total of 24 unknown serovars of *Salmonella* isolates were tested using our newly developed multiplex PCR method. The results showed that ten isolates produced the specific 731-bp target band of the *stn* gene and the 525-bp target band of the *I137_14445* gene, suggesting that these isolates were *S. Pullorum*. Two isolates produced the specific 731-bp target band of *stn* and the 307-bp target band of *ybgL*, indicating that these isolates were *S. Gallinarum* (Table 2). The multiplex PCR results were completely concordant with the conventional serotyping assay. Traditional serotyping method is complicated including non-selective and selective enrichment, biochemical identification, and serological characterization (Ren et al., 2017), which are time- and labor-intensive processes. The
developed PCR assay presented in this study has the ability to detect and differentiate S. Pullorum and S. Gallinarum within 2 h, thereby greatly shortening the time required for serotype identification. However, it would not be able to identify the serovars if it is a mixture of S. Gallinarum/Pullorum and other Salmonella serotypes. A mixture of different Salmonella serovars will produce all three bands, and S. Pullorum and S. Gallinarum could not be identified and distinguished. Thus, the developed multiplex PCR method is suitable to identify the purified colonies or cultures.

Conclusion

In summary, we established a multiplex PCR method that could detect and differentiate S. Pullorum and S. Gallinarum simultaneously based on three specific gene targets for the first time. Our assay exhibited efficient identification of both cultured bacteria and clinical Salmonella isolates. The results indicate that this multiplex PCR assay represents a one-step, economical, and accurate procedure for the sensitive, specific, and rapid identification of S. Gallinarum and S. Pullorum, respectively. The newly established PCR method could timely detect the presence of serovars Pullorum and Gallinarum accurately, especially when large quantities of samples to be tested, potentially facilitating the implementation of more timely and efficient control measures for PD or FT.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

XJ, ZP, and DX designed the experiments. DX, LY, and LS performed the PCR assays and analyzed the results. XJ, ZP, and DX wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.983942/full#supplementary-material

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