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/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

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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

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pathogens (Hoorfar, 2011; Majchrzak et al., 2014). For example, a two-step PCR assay using genes encoding the O, H, and Vi antigens (*rfb*, *fliC*, *fliB*, 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.

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.

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

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 μL^{-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 μL^{-1}, 16.3 ng μL^{-1}, 1.63 ng μL^{-1}, 163 pg μL^{-1}, 16.3 pg μL^{-1}, 8.15 pg μL^{-1}, and 4.075 pg μL^{-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 show 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′-ATGAATAATCGTCGTGGTTT-3′, *cigR*-R1, 5′-TAATAATCGCCGTGACCACC-3′, and *cigR*-R2, 5′-GTAGCGCTCAGGGAAAACG-3′.

Specificity of the PCR Assay

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

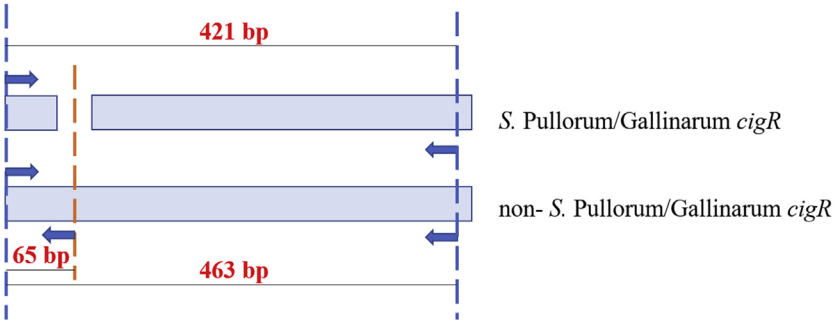

Figure 1. Diagram of the primer design of *cigR* to distinguish *Salmonella* Pullorum/Gallinarum from other serovars. *cigR* gene of *Salmonella* Pullorum/Gallinarum has a 42-bp–deficient region compared with that of other serovars, which was exploited to design the primers. The arrows indicate the positions 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.

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

	Serotype/species	Strain	Source	Serogroup	PCR result (band number)
Salmonella	*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 Potsdam	GE2	Laboratory stock	C1	2
	Salmonella Infantis	T6N1	Laboratory stock	C1	2
	Salmonella Choleraesuis	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	RKS5078	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	EGDe	ATCC BAA-679	-	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

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.*

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[3] (no.of isolates)	Coincidence rate between PCR and traditional method of bacteria separation
Chickens	87	24	2[1]	Enteritidis (15)	100%
			1[2]	Pullorum (9)	
Eggs	40	23	2[1]	Enteritidis (23)	100%

[1]PCR result with 2 bands suggested that this sample contained *Salmonella* except *Salmonella* Pullorum/Gallinarum.
[2]PCR result with 1 bands 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).

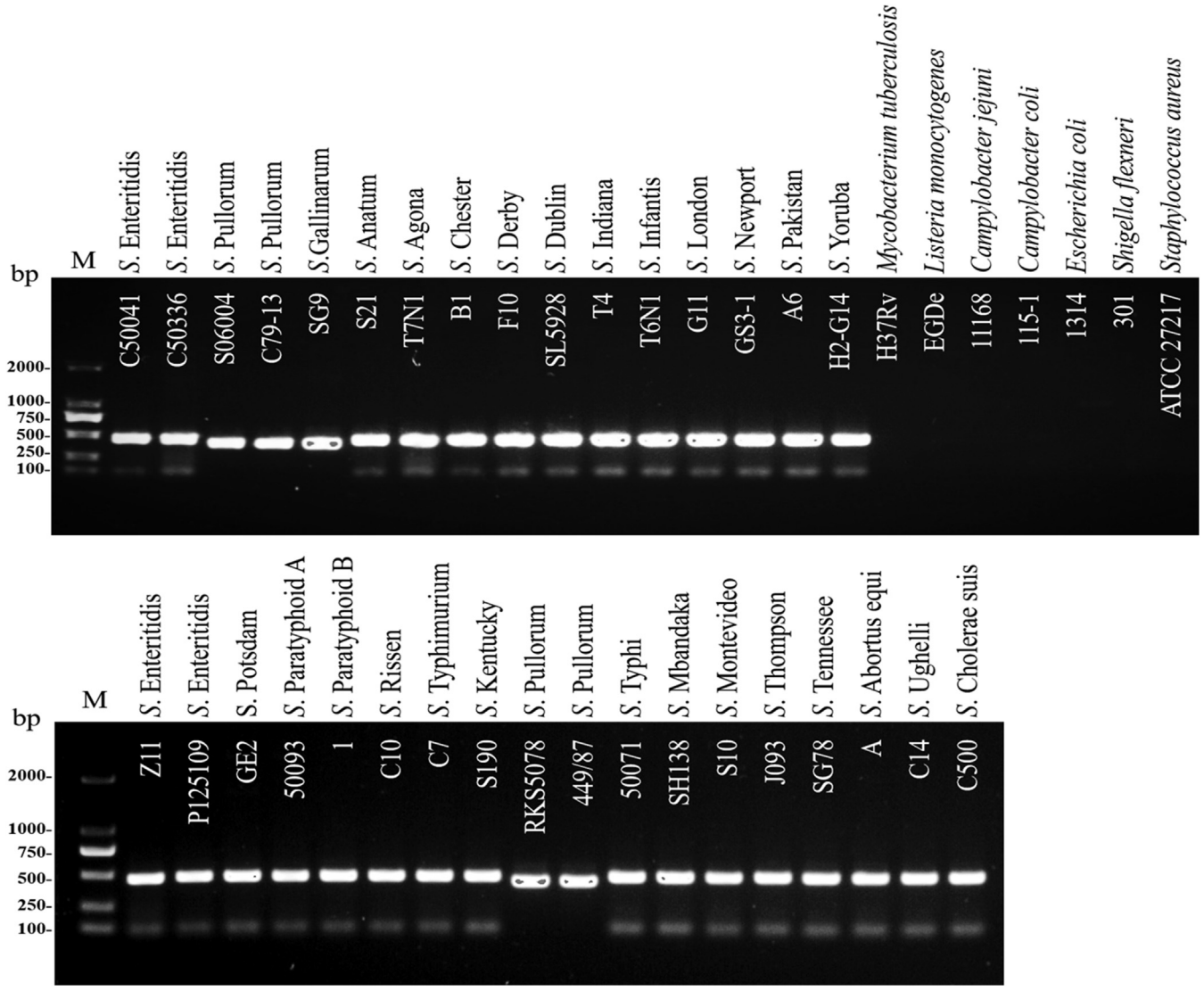

Figure 2. Specificity of the PCR assay for *Salmonella* detection and *Salmonella* Pullorum/Gallinarum identification. The *cigR* gene was PCR-amplified using genomic DNA from various *Salmonella* and non-*Salmonella* strains. Lane M: DL2000 DNA marker. Detailed strain information is provided in Table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

Gallinarum identification (Shah et al., 2005; Xu et al., 2018a), 1 of these 2 genes can be combined with *cigR* for the detection of *Salmonella* spp. and differentiation of *S.* Pullorum and *S.* Gallinarum.

Sensitivity of the PCR Assay

The genomic DNA of *S.* Pullorum S06004 serially diluted from 163 ng μL^{-1} to 4.075 pg μL^{-1} was used

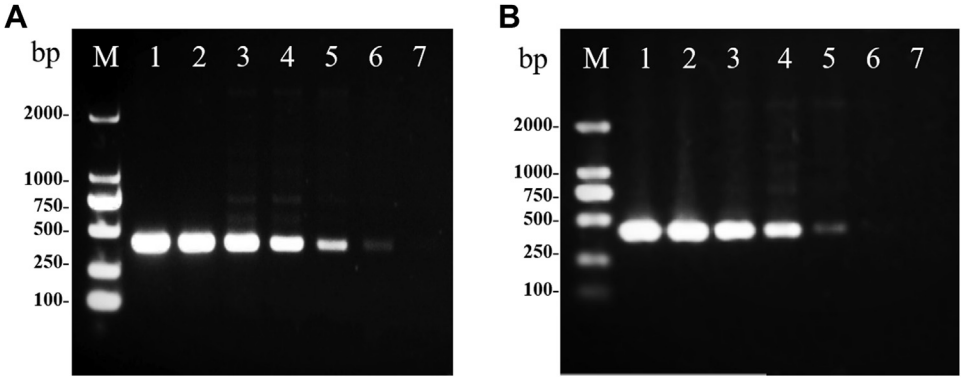

Figure 3. Sensitivity of the PCR assay for detection of genomic DNA and cells from *Salmonella* Pullorum S06004. (A) PCR for the detection of genomic DNA. Lanes 1-7, *S.* Pullorum genomic DNA used as a template in the following amounts: 163 ng, 16.3 ng, 1.63 ng, 163 pg, 16.3 pg, 8.15 pg, and 4.075 pg. (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.

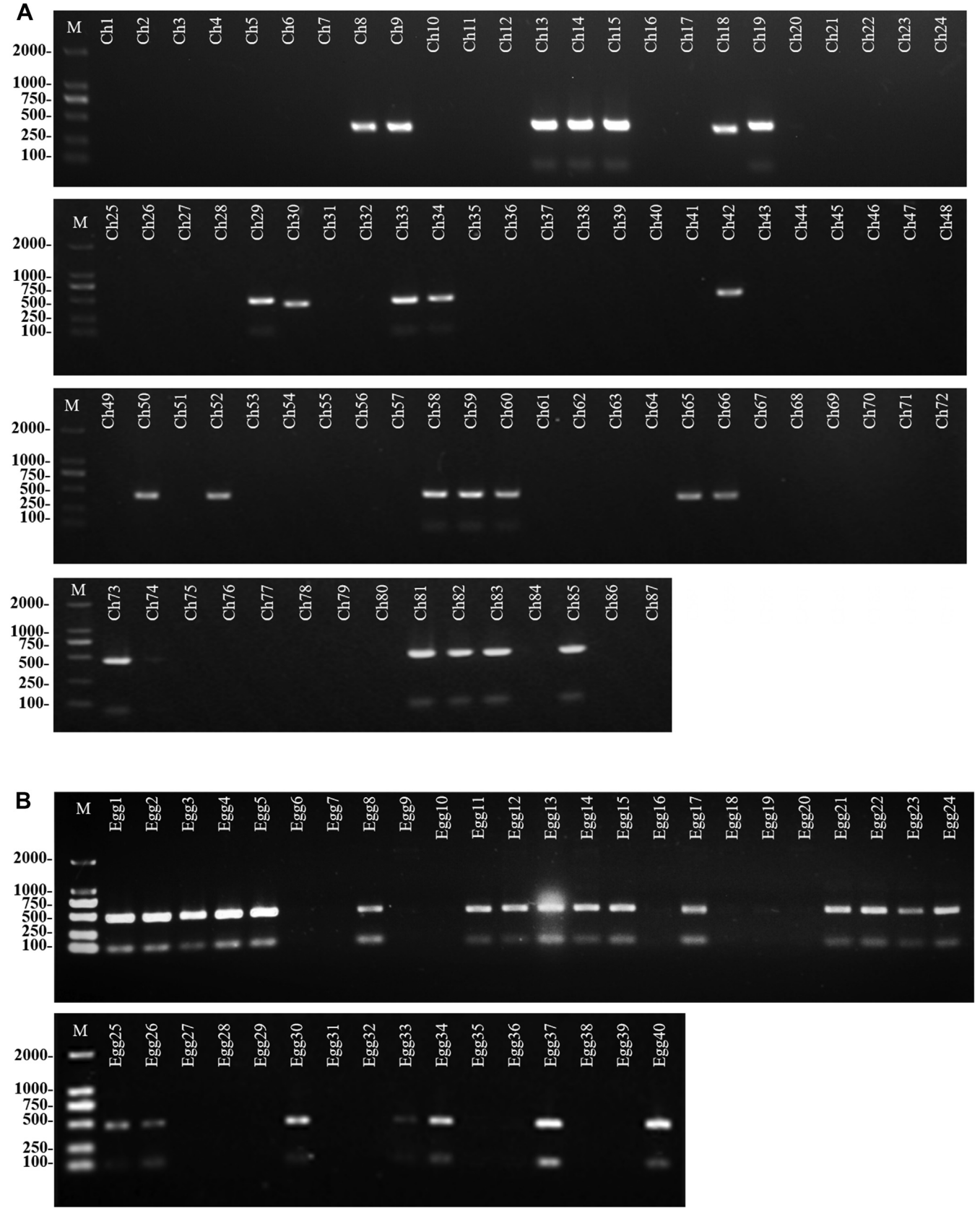

Figure 4. One-step PCR for detection of *Salmonella* and identification of *Salmonella* Pullorum/Gallinarum from processing chicken and egg samples. The enrichment broths of chicken (A) and egg (B) samples were used as templates in PCR. The PCR assay produced 1 product of 421 bp for *S.* Pullorum/Gallinarum 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 *flhB* (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.* Enteritis, which was 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.* Enteritis. All 23 *Salmonella* isolates obtained from egg samples were identified as *S.* Enteritis. This finding corresponds with a report from the United States indicating that *S.* Enteritis is one of the leading bacterial causes of foodborne illness and that shell eggs are a primary source of human *S.* Enteritis 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 *prot6e* 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.

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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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