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Rapid detection of flagellated and non-flagellated *Salmonella* by targeting the common flagellar hook gene *flgE*

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Abstract

Salmonella spp. can cause animal and human salmonellosis. In this study, we established a simple method to detect all *Salmonella* species by amplifying a specific region within the *flgE* gene encoding the flagellar hook protein. Our preliminary sequence analysis among flagella-associated genes of *Salmonella* revealed that although *Salmonella* Gallinarum and *Salmonella* Pullorum are lacking flagella, they did have flagella-associated genes, including *flgE*. To investigate in detail, a comparative *flgE* sequence analysis was conducted using different bacterial strains including flagellated and non-flagellated Salmonella as well as non-*Salmonella* strains. Two unique regions (481–529 bp and 721–775 bp of the reference sequence) within the *flgE* open reading frame were found to be highly conserved and specific to all *Salmonella* species. Next, we designed a pair of PCR primers (*flgE*-UP and *flgE*-LO) targeting the above two regions, and performed a *flgE*-tailored PCR using as template DNA prepared from a total of 76 bacterial strains (31 flagellated *Salmonella* strains, 26 non-flagellated *Salmonella* strains, and 19 other non-*Salmonella* bacteria strains). Results showed that specific positive bands with expected size were obtained from all *Salmonella* (including flagellated and non-flagellated *Salmonella*) strains, while no specific product was generated from non-*Salmonella* bacterial strains. PCR products from the positive bands were confirmed by DNA sequencing. The minimum detection amount for genomic DNA and bacteria cells reached 18.3 pg/μL and 100 colony-forming unit (CFU) per PCR reaction, respectively. Using the *flgE*-PCR method to detect *Salmonella* in artificially contaminated milk samples, as low as 1 CFU/mL *Salmonella* was detectable after an 8-h pre-culture. Meanwhile, the *flgE*-tailored PCR method was applied to evaluate 247 clinical samples infected with *Salmonella* from different chicken breeding farms. The detection results indicated that *flgE*-PCR could be used to specifically detect *Salmonella* in concordance with the traditional bacterial culture-based detection method. It is worthwhile noticed that identification results using *flgE*-tailored PCR should be completed within less than 1 day, expanding the result of much faster than the standard method, which took more than 5 days. Overall, the *flgE*-tailored PCR method can specifically detect flagellated and non-flagellated *Salmonella* and can serve as a powerful tool for rapid, simple, and sensitive detection of *Salmonella* species.

Yi Yang and Pengzhi Wang contributed equally to this work and shared the first authorship.

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Key points

- *Targeting flgE gene for all Salmonella spp. found.*
- *The established PCR assay is used to specifically detect all Salmonella spp.*
- *The PCR method is applied to detect clinical Salmonella spp. samples within less than 1 day.*

Keywords *Salmonella* · Flagellar hook · *flgE* · PCR · Detection

Introduction

As a member of *Enterobacteriaceae, Salmonella* is a group of Gram-negative bacterial pathogens and more than 2 600 different serovars have been reported until now (Issenhuth-Jeanjean et al. 2014). Some *Salmonella* species can cause gastroenteritis or systemic typhoid fever in several animal hosts including human. These pathogenic *Salmonella* are potential risk factors for public health safety (Foley and Lynne 2008). Accurate identification of this pathogen is one of the key processes to control salmonellosis. Therefore, it is imperative to develop a rapid, accurate, and convenient method for *Salmonella* detection in order to improve the security of human and animal health.

There have been numerous reports on *Salmonella* detection methods. Conventional methods based on bacterial culture, isolation, biochemical tests, and serotyping have been regarded as a "gold standard" (Cho and Ku 2017). However, the traditional *Salmonella* detection process is time-consuming and labor-intensive. According to White-Kauffmann-Le Minor (WKLM) scheme (Issenhuth-Jeanjean et al. 2014) and based on determination somatic (O) and flagellar (H) antigens, *Salmonella* can be serotyped by slide agglutination test (SAT). Although the SAT is simple, rapid, and the most important diagnostic tool under on-the-spot inspection conditions, it has the drawbacks of low detection sensitivity, easy to produce false positive results, and not economical because of the expensive commercial specific antisera. These shortcomings limit its usefulness as an ideal detection method.

Currently, the polymerase chain reaction (PCR) has been proved as a sensitive, rapid, and specific method, which is widely used for the detection of *Salmonella* (Cho and Ku 2017). Many PCR detection methods employed various genes as a target for the detection of *Salmonella*. Some of them use housekeeping genes such as 16S rDNA and *gyrB* as detection targets (Lin et al. 2004; Ye et al. 2011), while other methods are using virulence-associated genes as a target, such as *invA* (Rahn et al. 1992), *hilA* (Pathmanathan et al. 2003), *stn* (Makino et al. 1999), and *ompC* (Kwang et al. 1996).

In addition, some genes coding for bacterial surface antigens, such as flagellum and fimbriae, have been introduced as genetic targets for the identification of *Salmonella* (Doran et al. 1993; Hirose et al. 2002; Munir et al. 2015; Zhang et al. 2014). Among them, flagellum-associated genes have been proved to be potential targets suitable for *Salmonella* identification, for example, flagellin protein coding genes (*fliC*) have been reported as a target for the detection of *Salmonella* (Chiu et al. 2005; Khan et al. 2012). These methods can identify one or several particular serotype serovars of *Salmonella*. For instance, Chiu et al. (2005) developed a PCR method based on the *fliC* gene for detection of *Salmonella enterica* serovar Choleraesuis; Khan et al. (2012) reported a nested PCR targeting the flagellin gene for the detection of *Salmonella* Typhi. However, to our knowledge, there are no reports about using only single pair of primers targeting the flagellar gene, to identify all *Salmonella* species, including flagellated and non-flagellated *Salmonella* strains. We believe that this is due to the non-flagellated phenotype of *Salmonella* Pullorum and *Salmonella* Gallinarum, which limits the idea of using flagellum-associated genes to identify non-flagellum bacteria. Since the infections of *Salmonella* Pullorum and *Salmonella* Gallinarum, both of which are non-flagellated, remain an important problem for the poultry industry in many countries and regions (Barrow and Freitas Neto 2011), it is necessary to conduct improvement for the existing detection methods.

Salmonella Pullorum and *Salmonella* Gallinarum serovars are known as non-flagellated bacteria (Barrow and Freitas Neto 2011). However, when we performed bioinformatic and subsequent experimental sequencing analysis, we found that there are flagella coding genes in the genome of the two serotypes of *Salmonella*, including *flgE* gene, which encodes the flagellate hook protein hook. Besides, we found that *flgE* gene sequences from *Salmonella* Pullorum and *Salmonella* Gallinarum showed a very high sequence identity (100% sequence identity) when comparing with the *flgE* gene sequence from flagellated *Salmonella* Enteritidis. These unexpected discoveries were encouraging to investigate the possibility of using the flagellum-associated gene *flgE* as target to detect flagellated and non-flagellated *Salmonella* species.

In this work, based on the bioinformatics and sequencing analysis, we established a rapid PCR method to identify both flagellated and non-flagellated *Salmonella* simultaneously. The upstream and downstream primers were designed to specifically target unique sequences regions of *Salmonella flgE* gene. The specificity and sensitivity of the PCR method were determined in this study. Result showed that the *flgE*-PCR method

Table 1 Bacterial strains used in this study and specificity of the *flgE* gene-PCR method

Serovar/Species	Strain	Results of *flgE*-PCR	Serovar/Species	Strain	Results of *flgE*-PCR
Salmonella Enteritidis	CMCC(B)50336	+	*Salmonella* Pullorum	CVCC523	+
	994	+		CVCC526	+
	CVCC3377	+		CVCC535	+
	S0014	+		CVCC540	+
	SE43	+		S07	+
	S. 09	+		S08	+
	DUCK MY	+		S10	+
	T48	+		S11	+
	T64	+		S78	+
Salmonella Typhimurium	W32	+		S12-1	+
	T14	+		S12-2	+
	Stm	+		45SP13	+
	U27	+		SP68	+
	W2	+		SP73	+
	A12	+		SP79	+
	STM isolate 1	+		SP80	+
	STM isolate 2	+		SP82	+
	STM isolate 3	+		SP90	+
	STM isolate 4	+		SP95	+
Salmonella Choleraesuis	29	+	*Escherichia coli*	CE7	-
	U80	+		O161	-
	U81	+		1521F18ac	-
	U82	+		O157:H7	-
Salmonella Agona	36	+		Nissle 1917	-
	Agona	+		F4ad	-
Salmonella Typhi	W33	+		F4ac	-
Salmonella Paratyphi A	50115	+		F18ac	-
Salmonella Dublin	C79-84	+		Swine 1522	-
Salmonella Tennessee	Tennessee	+		Cow *E. coli*	-
Salmonella Saintpaul	Saintpaul	+		HB101	-
Salmonella Newport	31	+		BL21	-
Salmonella Gallinarum	SG9R	+		DH5α	-
	SG01	+	*Edwardsiella tarda*	Et-13	-
	U20	+	*Enterobacter cloacae*	Egg-23	-
	T63	+		Brood-2	-
	T88	+		Brood-3	-
	T89	+		Brood-4	-
	T90	+	*Staphylococcus*	*Staphylococcus*	-

"■" stands for positive reaction and " ■" stands for negative reaction

was specific and sensitive enough to detect *Salmonella* spp. In addition, by detecting 247 samples of chicken embryos from breeding farms, the *flgE*-PCR method was proved to be capable for *Salmonella* detection in clinical samples. In summary, the PCR method was suitable for an accurate, rapid, and convenient detection of all *Salmonella* spp.

Materials and methods

Bacterial strains

A total of 76 bacteria strains including 31 flagellated *Salmonella* strains, 26 non-flagellated *Salmonella* strains, and 19 other non-*Salmonella* bacteria strains (Table 1) were used to characterize the specificity of *flgE*-PCR method. The *Salmonella* Enteritidis strain CMCC (B) 50336 (abbreviated as SE50336 in subsequent text) was kindly offered by Professor Xinan Jiao in Yangzhou University. *Salmonella* Enteritidis strains T48 and T64; *Salmonella* Typhimurium strains W32, T14, U27, W2, A12; *Salmonella* Typhi W33; *Salmonella* Choleraesuis U80, U81, U82; and *Salmonella* Gallinarum U20 were kindly offered by Professor Shulin Liu, Harbin Medical University. *Salmonella* Pullorum strains CVCC523, CVCC526, CVCC535, and CVCC540 were from China Veterinary Culture Collection Center (CVCC). *Escherichia coli* strains CE7 was provided by Professor Chengping Lu in Nanjing Agricultural University. The *Staphylococcus* strain was a gift from the Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Sciences. The other strains were preserved in our laboratory. The SE50336 strain was used as a reference strain to perform the optimization of PCR conditions and sensitivity of the PCR assay. All the strains were grown in Luria-Bertani (LB) broth (NaCl [Sinopharm Chemical Reagent Co., Ltd, Beijing, China] 10 g/L, Tryptone [Oxoid, Hampshire, UK] 10.0 g/L, Yeast extract [Oxoid, Hampshire, UK] 5.0 g/L) or on LB agar plates at 37 °C.

Genomic DNA extraction

Genomic DNA was extracted from cultured bacterial cells by boiling method as previously described (Zhang et al. 2014). Briefly, 1.5 mL of overnight bacteria culture was centrifuged at 10000 rpm for 5 min, and the pellet was washed with sterilized double-distilled water (DDW) twice. Then, 200 μL of DDW was added and the mixture was suspended by pipetting then boiled at 100 °C for 10 min. After centrifuging at 10000 rpm for 5 min, the supernatants were transferred into a clean Eppendorf tube. The concentration of genomic DNA was measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). These DNA were stored at − 20 °C and then served as templates for PCR amplification.

Bioinformatics analysis

Multiple sequence alignments were performed using *flgE* gene sequences from different flagellated and non-flagellated *Salmonella* strains and non-*Salmonella* strains. The *flgE* sequences were downloaded from the GenBank database. The *flgE* nucleotide sequence from *Salmonella* Enteritidis strain P125109 (AM933172.1), a serovar harbor flagella-associated genes and has the nearest genetic relationships with *Salmonella* Pullorum and *Salmonella* Gallinarum, was selected as a reference sequence. The presence of the *flgE* gene in non-flagellated *Salmonella* strains, including *Salmonella* Gallinarum strains (T63, T88, T89, T90, U20, SG9R, and SG01) and *Salmonella* Pullorum strains (CVCC523, CVCC526, CVCC535, CVCC540, S07, S08, S10, S11, S78, S12-1, S12-2, 45SP13, SP68, SP73, SP79, SP80, SP82, SP90, and SP95) (Table 1), was confirmed by PCR amplification. Primers for PCR-amplifying the complete sequence of *flgE* were described in Table 2. Then, the PCR products were purified and cloned into pMD19-T vector (Takara Biotechnology Co., Dalian, China) and sequenced by GENEWIZ (Suzhou, China). The sequenced data were analyzed and aligned by ClustalW method using the MEGA 7 software (Version 7.0.26) and Jalview (Version 2.11.0) (Waterhouse et al. 2009).

PCR procedure

The PCR assay was performed in a 25 μL reaction volume containing: 2.5 μL of 10 × PCR reaction buffer (Mg^{2+} free) (Takara Biotechnology Co., Dalian, China), 2 μL of dNTPs (2.5 mM each), 1 μL of upstream and downstream primer (10

Table 2 List of primers used in this study

Primer	Sequence	Use of the primers
flgE-N	5'-ATGTCTTTTTCTCAAGCGGT TAG-3'	*flgE* gene sequencing
flgE-C	5'-TTAGCGCAGGTTAACCAGCGT-3'	
flgE-UP	5'-ACGGACCCTGTACCGTCTAAA-3'	*Salmonella* spp. detection
flgE-LO	5'-TGATGTTCACCGTACCGCC-3'	

μM), 0 mM to 2.0 mM of final concentrations of $MgCl_2$, 1.5 U of rTaq DNA polymerase (Takara Biotechnology Co., Dalian, China), and 1 μL(183 ng/μL) of extracted genomic DNA template. Positive and negative controls were the DNA of reference strain SE50336 and DDW, respectively. Amplifications were carried out using a DNA thermocycler (Applied Biosystems, Foster City, CA, USA) and the following amplification program: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at gradient temperature (from 50 °C to 60 °C) for 45 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min.

Amplification products were analyzed by electrophoreses on 2% agarose gel with a DL2000 ladder (Takara Biotechnology Co., Dalian, China) as molecular weight marker. The gels were imaged by a gel image system (BIO-RAD, Hercules, CA, USA).

Specificity analysis of the *flgE*-PCR

The specificity of the *flgE*-PCR was assessed using genomic DNA from 57 *Salmonella* strains (including 31 flagellated *Salmonella* strains and 26 non-flagellated *Salmonella* strains) and 19 non-*Salmonella* bacterial strains (Table 1).

Sensitivity of the *flgE*-PCR

To determine the minimum amount of DNA that can be detected by *flgE*-PCR method, genomic DNA extracted from *Salmonella* Enteritidis strain SE50336 was 10-fold serially diluted with DDW from 183 ng/μL to 183 fg/μL and then served as template for the PCR reaction. To determine the minimum amount of bacteria cells that can be detected by the *flgE*-PCR method, 30 μL of overnight culture for *Salmonella* Enteritidis strain SE50336 was subcultured into 3 mL of LB at 37 °C with shaking at 200 revolutions per minute (RPM) for about 1.5 h. The culture was first adjusted to an optical density of 1.0 at 600 nm (OD_{600nm} = 1.0), and then prepared for serial 10-fold dilutions in PBS. An aliquot (1 μL) of each dilution was directly used as templates to perform the PCR test. In parallel, an aliquot (100 μL) of each dilution was plated onto a LB agar plate, then incubated overnight at 37 °C to determine the colony-forming unit (CFU) of SE50336.

Milk sample was obtained from the local market and confirmed negative for *Salmonella*. Then, 0.5 ml of milk was mixed with 4.5 ml of selenite cystine (SC) broth (Qingdao Hope Bio-Technology Co., Ltd, Qingdao, China). To these mixtures, 0 to 1×10^3 CFU/mL of *Salmonella* SE50336 cells was inoculated, and the mixtures were incubated at 37 °C for 6–10 h. DNA was then extracted from 1 mL of each culture and used as template for PCR assays following DNA amplification conditions described above.

Salmonella detection of the samples from the chicken embryos in the breeding farms

A total of 247 samples were collected from healthy and dead chicken embryos in breeding farms located in Guangxi province during 2019. Samples were pre-enriched in Buffered Peptone Water (BPW) for 18 hours at 37 °C, then selectively enriched in SC broth for 18–24 h at 37 °C. After pre-enrichment, the presence of *Salmonella* in samples was determined using both the described *flgE*-PCR method and a modified method from standard microbiological analysis procedure of China (GB 4789. 4-2010). Positive samples were confirmed on Xylose Lysine Deoxycholate (XLD) agar and MacConkey (MAC) agar (Qingdao Hope Bio-Technology Co., Ltd, Qingdao, China), and serotyped by SAT using a commercial kit of *Salmonella*-specific O and H antisera (Tianjin biochip corporation, Tianjin, China) based on WKLM scheme.

Results

Bioinformatics analysis of the *flgE* gene of the flagellated *Salmonella*

We began our study by nucleotide sequences alignment of *flgE* gene from bacterial strains belonging to *Salmonella* (24 serovars of flagellated *Salmonella*) and non-*Salmonella* (13 strains) genera (Supplementary Fig. S1). Results showed that the *flgE* gene is distributed in all flagellated *Salmonella* strains (100 %) used in this study. In order to find a unique region for identification of *Salmonella*, the difference of the *flgE* gene between *Salmonella* and non-*Salmonella* was analyzed. The result showed that, two regions of the *flgE* gene, corresponding to nucleotides 481–529 and 721–775 of the *flgE* open reading frame, were found to have the least identity with non-*Salmonella* sequences (Supplementary Fig. S1). Since these two regions of the *flgE* gene were both specific and unique for *Salmonella* strains, they were considered suitable regions to design a specific pair of primer for the identification of *Salmonella* strains.

Bioinformatics analysis of the *flgE* gene of non-flagellated *Salmonella* (i.e., *Salmonella* Gallinarum/Pullorum)

Although *Salmonella* Gallinarum and *Salmonella* Pullorum did not contain flagella, complete genome sequence analysis of these two serovars showed that the genomes of both *Salmonella* species harbor flagella-associated genes, including the *flgE* gene (Table 3). We downloaded the *flgE* gene sequences from the complete genome data (listed in Table 3) and compared them with the reference sequences from a confirmed flagellated *Salmonella* Enteritidis strain P125109

Table 3 Available complete genome sequences of *Salmonella* Gallinarum/Pullorum and the distribution of flagella-associated genes

Serovar	Strains	Accession no.	Flagella	Flagella-associated genes
Salmonella Enteritidis	Strain P125109[a]	AM933172.1	+	+
Salmonella Gallinarum	Strain 287/91	AM933173.1	–	+
	Strain 9184	CP019035.1,	–	+
	Strain 9	CM001153.1	–	+
Salmonella Pullorum	Strain CDC1983-67	CP003786.1	–	+
	Strain RKS5078	CP003047.1	–	+
	Strain S06004	CP006575.1	–	+
	Strain ATCC 9120	CP012347.1	–	+
	Strain S44987_1	NZ_ LK931482.1	–	+

[a] *Salmonella* Enteritidis P125109 serves as a known flagella and flagella-associated genes positive reference strain

(AM933172.1) (Supplementary Fig. S2). Furthermore, in order to evaluate whether the *flgE* gene was present in all the non-flagellated *Salmonella*, PCR assays were carried out using the genome of *Salmonella* Gallinarum and Pullorum strains as template which were collected strains in our laboratory (Table 1), and *flgE*-N and *flgE*-C primer pairs were used (Table 2). Then, the sequences of *flgE* gene from the above strains were sequenced and compared with the reference sequence (AM933172.1). Results suggested that the *flgE* gene is present in all the tested 7 strains of *Salmonella* Gallinarum and 19 strains of *Salmonella* Pullorum (Fig. 1). In addition, the *flgE* sequence from *Salmonella* Pullorum/Gallinarum showed

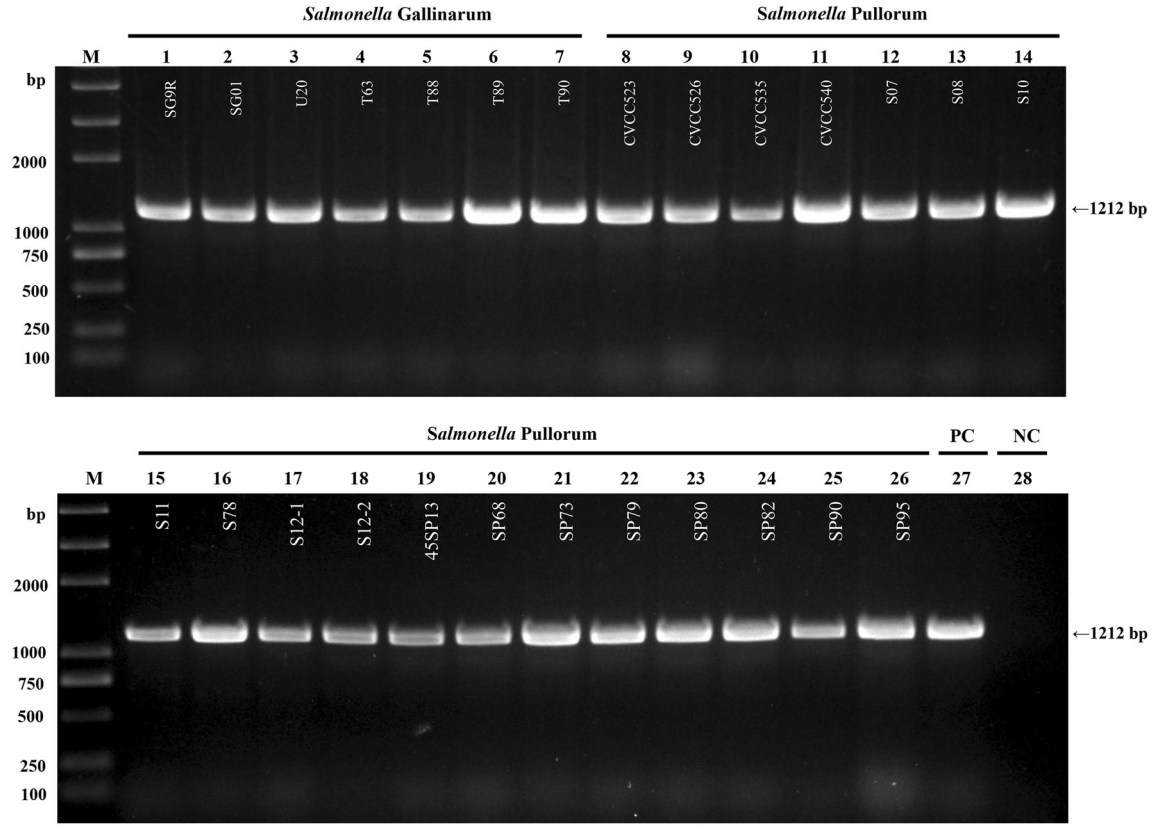

Fig. 1 The presence and identification of the flagella hook gene *flgE* in non-flagellated *Salmonella* (*Salmonella* Pullorum and *Salmonella* Gallinarum) strains. Lane M, 2 K plus DNA ladder; lanes 1–26, genomic DNA samples from different *Salmonella* Pullorum and *Salmonella* Gallinarum strains which were listed in Table 1. Lane 27, positive control (PC) use genomic DNA from a known positive for *flgE* gene strain (*Salmonella* Enteritidis str. SE50336) as template, Lane 28, negative control (NC), the genomic DNA was replaced with equal-volume of DDW. The *flgE* gene presents in all strains of non-flagellated *Salmonella* tested

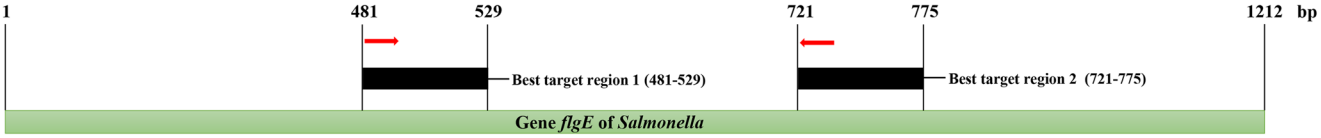

Fig. 2 Primer design for the *flgE*-PCR method for *Salmonella* spp. detection. Black box indicates the best target region determined by bioinformatics analysis for the identification of *Salmonella* spp. The red arrows represented the positions of the designed primers

a very high sequence identity (100%) with the *flgE* sequence from reference strain P125109 (Supplementary Fig. S2). These results suggested that the flagellated *Salmonella*-specific regions in the *flgE* gene also exist in non-flagellated strains and were conserved among all the analyzed *Salmonella* strains.

Primer designs for detecting *Salmonella* spp.

The bioinformatic analysis showed that the flagellar hook gene *flgE* was distributed in all *Salmonella* species including flagellated and non-flagellated *Salmonella* strains. In addition, results suggested that the above two regions of the *flgE* gene (481–529 bp and 721–775 bp of the reference sequence) were conserved, and present in all *Salmonella* strains. These evidences supported the potential idea of using these two unique regions of the *flgE* gene to design primers for *Salmonella* spp. detection. By taking advantage of this feature, we designed upstream primer (*flgE*-UP) within the region one and downstream primer (*flgE*-LO) within the region two (Fig. 2, Table 2). This pair of primer was then utilized to exploit a PCR method to rapidly and efficiently detect flagellated and non-flagellated *Salmonella.*

PCR procedure

The PCR amplification system was developed and the reaction conditions were optimized, including annealing temperature and Mg^{2+} concentration. The results showed that under the conditions of annealing temperature 50–61 °C (Fig. 3a), and concentration of 0.4–2.0 mM Mg^{2+} (Fig. 3b), target fragments can be amplified very well, which means that the established reaction system allows for PCR at a wide range of annealing temperature and Mg^{2+} concentrations. The primers yield to the expected 262-bp amplicon (Fig. 3). Based on the conditions described in the "Materials and methods" section, the optimal conditions for the two other parameters were determined as follows: an annealing temperature of 56 °C and a final concentration of 2.0 mM $MgCl_2$. The determined parameters were used in subsequent experiments.

The *flgE*-PCR method proved to be both unique and specific to all the *Salmonella* spp.

The specificity of the *flgE*-PCR system was determined by testing 31 flagellated *Salmonella* strains, 26 non-flagellated *Salmonella* strains, and 19 non-*Salmonella* bacterial strains (Table 1). The results revealed that all the flagellated *Salmonella* strains generated the 262-bp of specific target band. In addition, all the non-flagellated *Salmonella* (*Salmonella* Pullorum/Gallinarum) strains also generated the specific target band (Fig. 4, Table 1). In contrast, no amplification products were observed in 19 strains of different non-*Salmonella* pathogens (Fig. 4, Table 1). This result shows that the *flgE*-PCR method has the potential to specifically detect flagellated *Salmonella* (lanes 1–31) and the non-flagellated *Salmonella* (lanes 32–57), without any detectable cross-reactivity with non-*Salmonella* bacteria strains.

The sensitivity of the *flgE* gene-PCR method for detecting all *Salmonella* spp.

To evaluate the sensitivity of the PCR method, genomic DNA of *Salmonella* Enteritidis strain SE50336 was consecutively diluted and used as templates for PCR reaction. The target DNA fragment was amplified using template DNA concentrations ranging from of 183 ng/μL to 183 fg/μL. The results

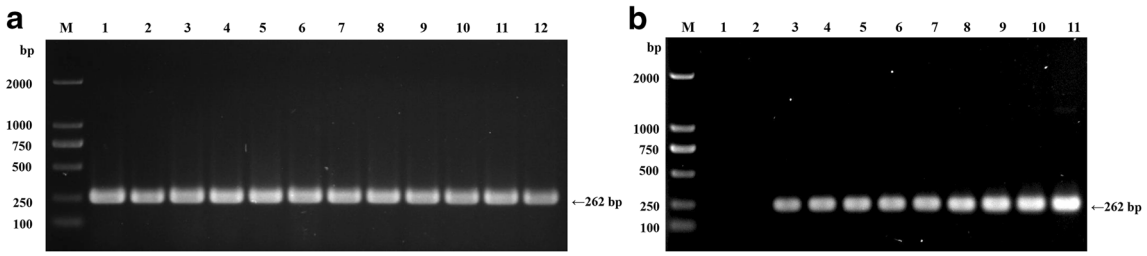

Fig. 3 The effects of the annealing temperature and $MgCl_2$ concentration on *flgE*-PCR reaction. Lane M, DL2000 DNA ladder. **a** The effects of the annealing temperature on *flgE*-PCR reaction. Lane 1–12, the PCR reaction at the different annealing temperature (50 to 61 °C). **b** The effects of the $MgCl_2$ concentrations on *flgE*-PCR reaction. Lanes 1–11, PCR amplification system with different final concentrations of $MgCl_2$ (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 mM)

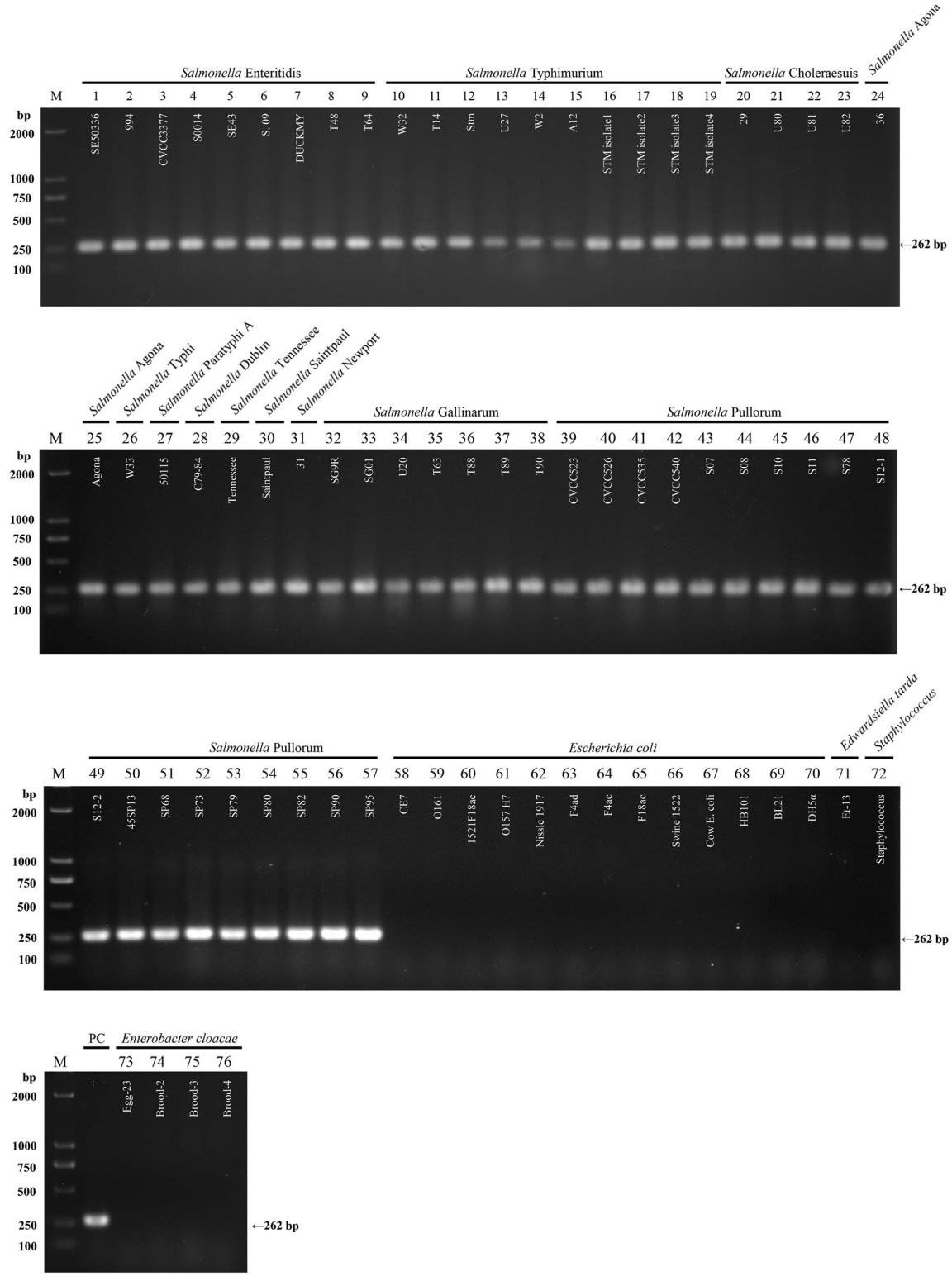

Fig. 4 Specificity of the *flgE*-PCR for detecting all *Salmonella* spp. The PCR assays were conducted using genomic DNA as template from different flagellated, non-flagellated *Salmonella* strains, and non-*Salmonella* bacterial strains. Lane M, DL2000 DNA ladder; lane 1–31, PCR products amplified from flagellated *Salmonella* strains. Lane 32–57, PCR products amplified from non-flagellated *Salmonella* strains. Lane 58–76, PCR products amplified from non-*Salmonella* strains. Positive control (PC), using genomic DNA from a known positive strain (SE50336) as template. Detailed strain information were listed in Table 1

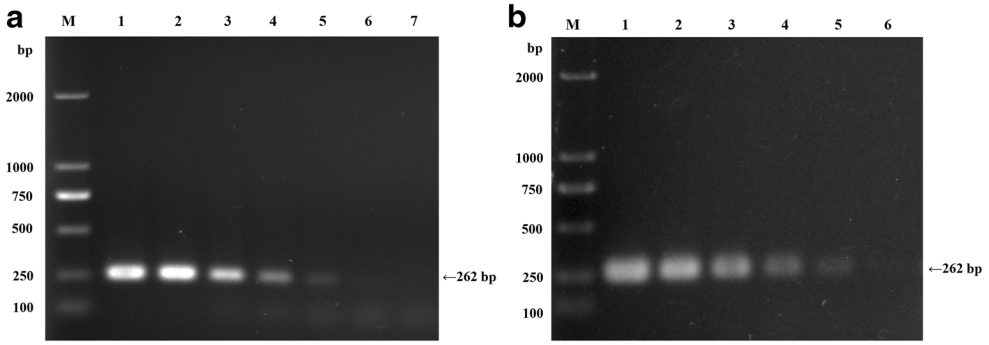

Fig 5 Sensitivity of the *flgE*-PCR assay for detection of bacterial genomic DNA and bacterial cells from *Salmonella* Enteritidis (str. SE50336). The PCR amplified a product of 262 bp. Lane M, DL2000 DNA ladder. **a** The PCR detection for the bacterial genomic DNA, lanes 1–7, 183 ng/μL, 18.3 ng/μL, 1.83 ng/μL, 183 pg/μL, 18.3 pg/μL, 1.83 pg/μL, 183 fg/μL of bacterial genomic DNA from SE50336 were used as template. **b** The PCR detection for the *Salmonella* Enteritidis cells, lanes 1–6, the number of bacteria cells per PCR reaction was 10^6 CFU, 10^5 CFU, 10^4 CFU, 10^3 CFU, 10^2 CFU, and 10 CFU

showed that the minimum amount of DNA that could be detected by this method was 18.3 pg/μL of genomic DNA (Fig. 5a). Furthermore, the minimum of bacterial cells of *Salmonella* Enteritidis that could be detected using this *flgE*-PCR method was determined using different dilutions of SE50336 cells. After analyzed by the *flgE*-PCR method, the least bacterial cells that could be detected were validated as 100 CFU per reaction (Fig. 5b). Milk samples artificially contaminated with different cell numbers of *Salmonella* SE50336 were analyzed through *flgE*-PCR after 6, 8, and 10-h incubation. Our data showed that it was possible to detect an inoculation concentration of 1 CFU/mL of *Salmonella* after 8 h of enrichment with the SC mixture (Fig. 6).

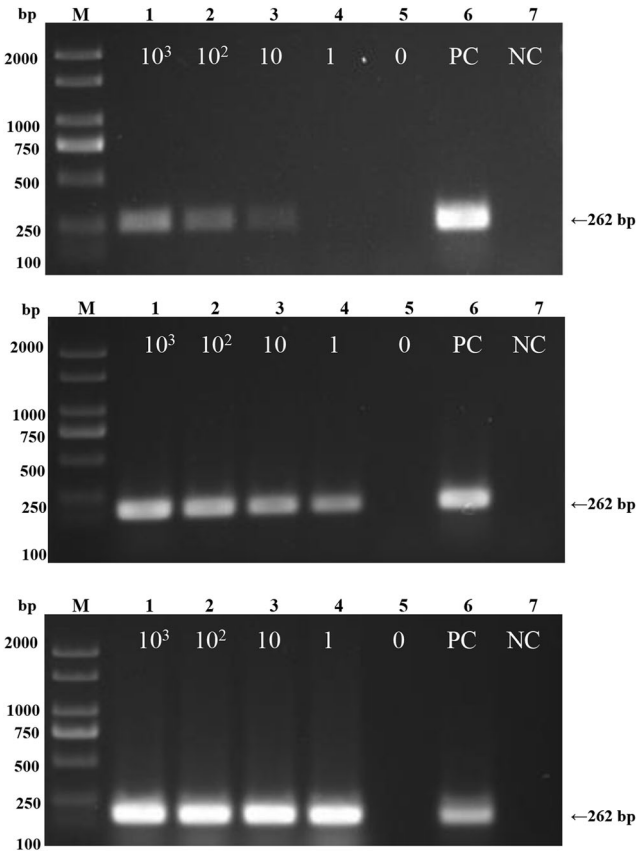

Fig. 6 Sensitivity of the *flgE*-PCR for detection of *Salmonella* Enteritidis CMCC (B) 50336 in milk samples with pre-enrichment for 6 h (A), 8 h (B), or 10 h (C). Lane M, DL2000 DNA ladder, lane 1–4, milk samples spiked with bacterial culture serially diluted 10-fold from 10^3 (lane 1) to 10^0 (lane 4) CFU/mL, lane 5, unspiked (0 CFU/mL) milk sample as PCR template, lane 6, positive control (PC) use genomic DNA from CMCC (B) 50336 as template, lane 7, negative control (NC), equal-volume of DDW as PCR template

The *flgE*-PCR method is adequate for the *Salmonella* detection in clinical samples

A total number of 247 chicken embryos samples were tested for the presence of *Salmonella* using both the *flgE*-PCR method and the traditional standard method. The results were listed in Table 4. After pre-enrichment and selective enrichment (less than 1 day), 46 of the 247 samples were tested positive for the presence of *Salmonella* using *flgE*-PCR method (less than 3 h). An identical number of *Salmonella* positive samples (46 out of 247) was obtained when performing the traditional microbiological identification process (about 5 days, Fig. 7). Taken together our designed PCR method, which is highly sensitive and in complete agreement with the traditional identification method, could have potential application as molecular microbiology tool for rapid *Salmonella* detection in aviculture.

Discussion

Salmonella induces infections in humans and a broad range of animals, which remains a serious health problem in human and veterinary medicine (Foley and Lynne 2008; Kirk et al. 2015; Rukambile et al. 2019; Threlfall 2002). Traditional culture-based detection procedure is still the most commonly used routine method for the identification of *Salmonella*. This diagnosis method for *Salmonella* detection is based on the use

Table 4 Detection consistency of the *flgE*-PCR method compared with traditional culture-based method

Methods	Time cost	Positive	Negative	Coincidence rate
The *flgE*-PCR method	1 days	46	201	100%
Traditional culture-based method	5 days	46	201	

of selective culture media such as SC, XLD, MAC, Tetrathionate broth (TTB), Bismuth Sulfite (BS), Deoxycholate Hydrogen Sulfide Lactose (DHL), and serological tests according to the White-Kauffmann-Le Minor scheme and biochemical features analysis. The culture-based detection is time-consuming and laborious because it always takes up to about 5–7 days to complete the detection process (Cho and Ku 2017). Nucleic acid-based analytical methods have undergone considerable improvements in sensitivity, specificity, and speed during the past decades. Among these methods, PCR serves as a powerful tool to detect *Salmonella* in a simple, rapid, and convenient manner (Ricke et al. 2018).

Bacterial flagellum is a motile organelle, which provide bacteria swimming or swarming motilities and has multiple biological functions on bacterial pathogenicity, such as cell adhesion, biofilm formation, and host cell invasion (Chaban et al. 2015; Duan et al. 2013; Zhou et al. 2015). A typical bacterial flagellum consists of three parts: a basal body, a filament, and a hook (Chaban et al. 2015). The basal body is located in the cytoplasmic membrane and function as a motor. The flagellar filament is a long tubular structure and functions as a helical propeller that enables bacterial motility. Flagellin, which is encoded by the *fliC* gene, is a subunit protein of flagellar filament. The bacterial flagellin is a well-studied and typical pathogen-associated molecular pattern (PAMP), which can be recognized by pattern recognition receptors (PRRs), such as Toll-like receptor (TLR) and NOD-like receptors (NLRs), and subsequently trigger the innate immunity (Cui et al. 2018; Hayashi et al. 2001; Hajam et al. 2017). In addition, as a strong immunogen, flagellin can also activate the adaptive immune response (Honko and Mizel 2005). Therefore, it has been widely recognized that flagellin can serve as an immune adjuvant to enhance antigenic immunogenicity of the vaccines (Gupta et al. 2014; Kim et al. 2018).

The flagellar hook is a short, highly curved tubular structure and serves as a universal joint that connects the basal body to the flagellar filament. The gene *flgE* codes a 403 amino acid (AA) hook protein FlgE, which forms the hook of the *Salmonella* flagellum. Previous studies are mainly focused on the structural feature of hook subunit protein FlgE and hook assembling mechanisms (Matsunami et al. 2016; Moriya et al. 2011; Saijo-Hamano et al. 2019; Samatey et al. 2004). However, recent research has revealed that the flagellar hook and hook monomer protein FlgE of *Pseudomonas aeruginosa* have the potency of pro-inflammation or immune-stimulation (Shen et al. 2017; Li et al. 2019). This means that the flagellar hook and its monomer protein FlgE are novel key factors, which contribute to host-bacterial interactions. Thus, just like flagellin, the flagellar hook and protein FlgE have the potential to serve as a vaccine adjuvant (Shen et al. 2017; Li et al. 2019).

In this study, we developed a PCR method for detecting all *Salmonella* spp. based on targeting the flagellar hook protein coding gene *flgE*. The primers were designed within two unique regions (481–529 bp and 721–775 bp of the reference sequence) in *flgE* gene, which were conserved and unique to *Salmonella* species. Interestingly, although the two non-flagellated *Salmonella* serovars (*Salmonella* Gallinarum and

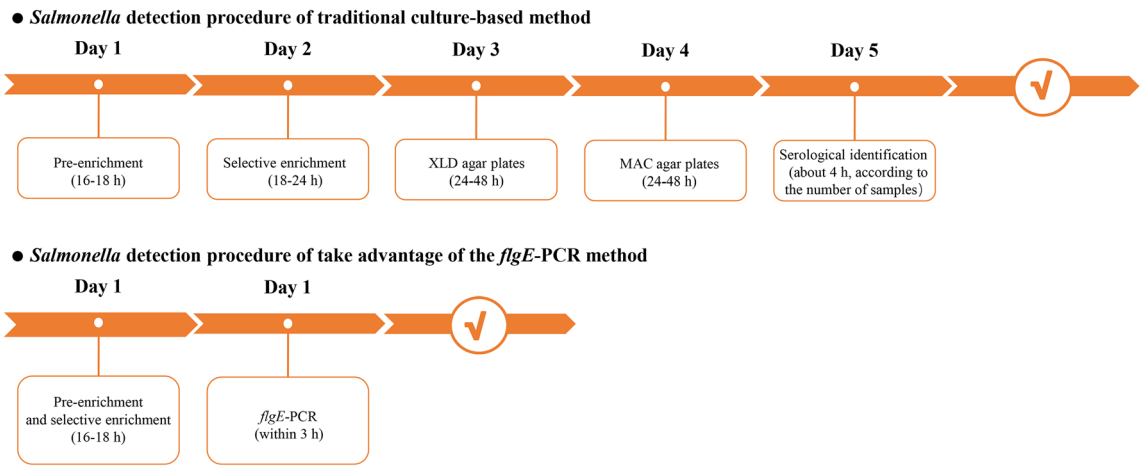

Fig. 7 Comparison of the *Salmonella* spp. detection procedures

Table 5 Applications of nucleic acid-based methods for the detection of *Salmonella*

Method/ technology	Target species	Target gene	Pre-enrichment broth and time	$MDA_{directly}$|$MDA_{pre\text{-}culture}$	References
qPCR	*Salmonella* spp.	*invA*	BPW/overnight	2 (CFU/PCR)|3 (CFU/25 g)	Chen et al. 1997
qPCR	*Salmonella* spp.	*invA*	UPB/18 h	10^3-10^4 (CFU/ml)| < 10 (CFU/ml)	Nam et al. 2005
qPCR	*Salmonella* spp.	*ttrRSBCA*	BPW/overnight	10^4 (CFU/mL)|50 (CFU/PCR)	Malorny et al. 2004
qPCR	*Salmonella* spp.	*ssaN*	BPW/6 h	130 (CFU/mL)|1 (CFU/10 g)	Chen et al. 2010
qPCR	*Salmonella* spp.	*bipA*	BPW/16 h-20 h	10^2(Genomes/PCR)|3-6 (CFU/25 g)	Calvo et al. 2008
qPCR	*Salmonella* spp.	*himA*	NM	2 (CFU/PCR)|NM	Chen et al. 2000
Multiplex qPCR	*S.* Heidelberg *S.* Kentucky *S.* Hadar *S.* Enteritidis *S.* Dublin	proteins type II restriction enzyme gene *prot6e* hypothetical protein gene ICESe4 gene CAAX protease *gene*	BPW/20 h RVSB/12 h	12, 9, 40, 13, and 5,280 (CFU/mL)|1-10 (CFU/25 mL)	Afroj et al. 2017
Multiplex qPCR	*S.* Enteritidis *S.* Typhimurium	*invA* *prot6E* *fliC*	NM	10 (copies of DNA/PCR)|NM	Hadjinicolaou et al. 2009
Multiplex qPCR	*Salmonella* spp. *E. coli* O157:H7	*invA* *fliC*	NM	10^2 (CFU/mL)|NM	Zhou et al. 2017
Multiplex qPCR	*S. aureus* *L. monocytogenes* *Salmonella* spp.	*nuc* *hlyA* *orgC*	BHIB/4 h	10^2 (CFU/mL)|12, 14, and 10 (CFU/25 mL)	Ding et al. 2017
PCR	*Salmonella* spp.	*invA*	NM	300 (CFU/PCR)|NM	Rahn et al. 1992
PCR	*Salmonella* spp.	*stn*	TSB/16 h HTTB/16 h	NM|1 (CFU/g)	Makino et al. 1999
PCR	*Salmonella* spp.	*sifB*	BPW/18 h	NM|10 (CFU/25 g)	de Almeida et al. 2014
PCR	*Salmonella* spp.	16S rDNA	SCB/8 h	1-9 (CFU/PCR)|1-9 (CFU/g)	Lin et al. 2004
PCR	*Salmonella* spp.	*gyrB*	NM	3.2 (CFU/PCR)|NM	Ye et al. 2011
PCR	*Salmonella* spp.	*hilA*	BHIB/6 h	120 (CFU/PCR)|1.2 (CFU/PCR)	Pathmanathan et al. 2003
PCR	*Salmonella* spp.	*ompC*	TSB/4-6 h	400 (CFU/PCR)|100 (CFU/PCR)	Kwang et al. 1996
PCR	*Salmonella* spp.	*flgE*	SCB/8 h	100 (CFU/PCR)|1 (CFU/mL)	This study

NM not mentioned

$MDA_{directly}$ | $MDA_{pre\text{-}culture}$

$MDA_{directly}$: the minimum detectable amount of bacteria cells in sample that can be detected directly (without pre-culture steps), $MDA_{pre\text{-}culture}$: the minimum detectable amount of bacteria cells in sample that can be detected after a certain period of pre-culture step

BPW buffered peptone water, *UPB* universal pre-enrichment broth, *RVSB* Rappaport vassiliadis *Salmonella* enrichment broth, *TSB* Tryptone soy broth, *SCB* selenite cystine broth, *HTTB* Hajna tetrathionate broth, *BHIB* brain heart infusion broth

Salmonella Pullorum) did not contain flagella, we noticed that their genome harbors flagella-associated genes, including *flgE* gene. Subsequently, we confirmed that *flgE* gene was present in all the *Salmonella* Gallinarum/Pullorum strains tested in this study. In addition, the *flgE* sequences from *Salmonella* Pullorum/Gallinarum are 100% identical to the *flgE* sequence from the reference strain. This means that the new clue described in this study is competent for the detection of non-flagellated *Salmonella*. Subsequently, to validate the proof of concept of our designed method, we tested various *Salmonella* Gallinarum and *Salmonella* Pullorum strains available in our laboratory using this *flgE*-PCR method. In addition, the *flgE*-PCR method was also carried out in clinical chicken embryo sample detection. The result of PCR has a high accordance (100%) with traditional identification methods. Besides, the *flgE*-PCR method showed low time consumption compared with the standard method.

Several molecular targets have been applied for *Salmonella* detection (Table 5). *Salmonella* have been detected using conventional PCR, quantitative real-time PCR (qPCR), multiplex real-time PCR (Multiplex qPCR), etc. (Malorny et al. 2009). The sensitivity of the different methods is varied. Generally,

the qPCR assay was more sensitive than the conventional PCR assay. However, the higher cost of the equipment and reagent of the qPCR method somewhat limited its wide application, despite high sensitivity of identification. Conventional PCR shows advantages in simple operation and cost-effectiveness; therefore, it is more suitable for low cost routine analysis. Our *flgE*-PCR method was capable to detect as few as 100 CFU/PCR of the pure culture of *Salmonella* SE50336 directly. This result showed that our method is significantly more sensitive than the previous PCR assays established by Kwang et al. (1996) and Pathmanathan et al. (2003), in which a minimum of 400 CFU and 120 CFU was respectively required for detecting *Salmonella ompC* and *hilA* genes. However, using our *flgE*-PCR method, the minimum amount for bacterial cell required for *Salmonella* detection was 10- and 100-fold behind the methods reported by Lin et al. (2004) and Ye et al. (2011), respectively. In their reports, 1–9 CFU (by targeting 16S rDNA gene) and 3.2 CFU (by targeting *gyrB* gene) of bacteria cells could be detected. A pre-culture step of a few hours is always necessary before performing PCR to fulfil the detection requirements of national legislations (GB 4789. 4-2010). The minimum detection amount of bacterial cells in our *flgE*-PCR method was improved to 1 CFU/mL in contaminated milk after 8 h of pre-culture. The results are consistent with the early research that the PCR demonstrated increased sensitivity when pre-enrichment was performed prior to the PCR detection (Nam et al. 2005). In short, the *flgE*-PCR method can be used for routine detection of *Salmonella* with a reasonable sensitivity.

Overall, through bioinformatics analysis of the *flgE* gene and subsequent experimental verification, a practical PCR method for identification of *Salmonella* was provided in this study. Furthermore, the detailed sequence analysis of the *flgE* gene in this work will provide the basis and the necessary information for further study of the function of the *Salmonella* FlgE protein. Future research can focus on, but not limited to, the following aspects: designing experiments to identify the major domains of *Salmonella* FlgE protein that are responsible for stimulating the inflammatory response of host cells, and formulating strategies to evaluate the potential of the FlgE protein to serve as an immune adjuvant, and so on.

In conclusion, we found two unique regions within the flagellar hook protein encoding gene *flgE*, which are both conserved and unique to the *Salmonella* genus. By taking advantage of this characteristic, one pair of PCR primers was designed within these two regions. Based on this pair of primers, a direct PCR assay specific for all *Salmonella* spp. was developed. The specificity and sensitivity of the *flgE*-PCR method were proved by detecting different *Salmonella* and non-*Salmonella* strains preserved in our laboratory. Furthermore, *flgE*-PCR method was also proved to be competent for the *Salmonella* detection in clinical samples collecting from chicken farms. Compared with the traditional methods, this *flgE*-PCR method was rapid, accurate, and easy to operate since the entire *Salmonella* detection process can be rapidly completed without intensive labor force. Due to the potential of our *flgE*-PCR method, it is worth further investigations to optimize and apply this PCR assay in the clinical detection and surveillance of *Salmonella* in the near future.

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Authors' contributions YY and PW performed the experiments, analyzed the data, and wrote the manuscript. PX, BY, PD, TH, and JL participated in the data analysis and wrote the paper. QS contributed to the experiments designing, manuscript writing, and language polishing. GZ and XM conceived and designed the study, participated in experimental work, and wrote the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethics approval and consent to participate This article does not contain any studies with human participants performed by any of the authors. The current study was approved by the by the Institutional Animal Care and Use Committee of the Yangzhou University College of Veterinary Medicine of China.

Consent for publication All authors listed on this manuscript have read and agreed to the publication of this research.

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