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***Salmonella Enteritidis* in Layer Farms of Different Sizes Located in Northern China: On-Farm Sampling and Detection by the PCR Method**

ABSTRACT

Salmonella enterica subspecies *enterica* serotype Enteritidis (SE) has caused foodborne infections over decades. It is transmitted mainly from contaminated eggs to humans. SE is commonly present in layer houses, and closely interacts with environmental factors. The objective of the present study was to develop a viable PCR method to identify SE in environmental samples collected in layer farms of different sizes, and to evaluate SE contamination status in four main egg-production provinces of northern China. After specificity retrieval using Primer-BLAST against the NCBI database, three SE specific oligonucleotide primers were selected as candidate primers. The primers targeting *Prot6e* gene were adopted and primers targeting *Sdf 1* were also selected to validate the results, after testing eight different types of pooled poultry environmental samples (overshoe, air, drinking nipple, feed, egg collection belt, eggshell, air inlet, and air outlet) by PCR. A PCR detection limit of 1 CFU/mL was determined using cell lysates from pure cultures. Testing time was less than 48 h. On-farm samples were collected from two layer farm sizes (one housing more than 50,000 layers, and the other, less than 50,000 layers) in each province. The applied PCR method was shown to be simple, inexpensive and effective for screening SE in a large amount of farm samples. The study identified only one SE-positive farm, which a large farm and where nine samples were found to be contaminated with SE: drinking nipples (3), egg collection belt (1), air inlet (1), air (1), overshoe (1) and eggshell (2).

INTRODUCTION

The prevalence in humans of the most frequently isolated *Salmonella* serotype (Gantois *et al.*, 2009), *Salmonella enterica* serovar Enteritidis (SE), has increased since the late 1960s (Bäumler *et al.*, 2000). Several outbreaks of human SE infection have been reported, and were associated with the consumption of contaminated eggs (Castanheira *et al.*, 1995; Crespo *et al.*, 2005; Much *et al.*, 2009; Vaz *et al.*, 2010; Guo *et al.*, 2015; Zhou *et al.*, 2015). Around 80% of 371 known SE outbreaks in the United States between 1985 and 1999 were linked to contaminated eggs (Patrick *et al.*, 2004). This pathogen spreads mainly through food production chain, and particularly increases with egg consumption (CDC, 2000; Osimani *et al.*, 2016, Li *et al.*, 2016). Poultry eggs are initially infected with SE inside the layer houses (Guard-Petter, 2001; Dewaele *et al.*, 2012; Ricke *et al.*, 2013). Research indicated that contamination persisted even after effective terminal cleaning and disinfection (C&D) processes (Carrique-Mas *et al.*, 2009). In addition, asymptomatic infection increases the difficulties in detecting SE infection in poultry flocks (Gast & Holt, 1998; Guard-Petter, 2001). Although some strategies for reducing SE in the production chain were studied (Ricke *et al.*, 2013; Upadhyaya *et al.*, 2013), particular importance has to be



given to monitoring and controlling SE contamination in the layer house environment. According to the latest statistics of the Food and Agriculture Organization of the United Nations (FAO), egg production in China has exceeded 24.78 million tons, accounting for about 36.33% of world's total production. However, few investigations on egg contamination with SE have been carried out in China.

Several methods of SE identification have been evaluated (Oliveira *et al.*, 2002; Moongkarndi *et al.*, 2011; Dewaele *et al.*, 2012). The polymerase chain reaction (PCR) is a well-established technique and has proven to be practical in terms of cost, time, and sensitivity (Almeida *et al.*, 2013), particularly for the detection of a large number of field samples. Although some multiplex PCR systems have been proposed (Trafny *et al.*, 2006; O'Regan *et al.*, 2008; Park *et al.*, 2009; de Freitas *et al.*, 2010), a study reported that they have relatively poor sensitivity (Chen *et al.*, 2012). Multiple primer pairs were later applied, but this significantly increased the probability of producing non-specific bands and even false positive results, especially when the poultry house environment contained countless unknown bacteria communities (Dumas *et al.*, 2011). Real-time PCR method also showed high specificity in SE detection, but the high cost of the detection instruments and test reagents hinders its use for large-scale screening (Espy *et al.*, 2006).

In the present study, we first aligned collected primer pairs from previous PCR methodology studies

on SE detection with Primer-BLAST against the NCBI database. Based on the results of specificity retrieval, selected candidate primers were tested in pooled poultry environment samples using a general PCR procedure. After adjusting the DNA extraction process and PCR program parameters, the final candidate primer located on *Prot6e* gene was applied to PCR protocols, and primers targeting *Sdf I* were designed to verify on-farm results. The detection limit was finally determined using 10-fold serially diluted SE bacterium solution. Finally, environmental SE contamination was investigated in four main egg production areas in northern China. In summary, the aim of the present study was to establish a simple, inexpensive, and effective method for SE screening for the surveillance of SE contamination status of laying hen farms.

MATERIALS AND METHODS

Screening of specific SE primers with Primer-BLAST

We reviewed 19 papers on SE detection using PCR published between 1994 and 2012. A total of eight primer pairs expected to be SE-specific were chosen from seven representative papers, and the details are shown in Table 1.

Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) was used to check the specificity of those eight primers. Default checking parameters remained unchanged, except that Genome (chromosomes from

Table 1 – Oligonucleotide primers used for verifying *Salmonella* Enteritidis detection

Target gene	Primers ¹⁾	Sequence (5'-3')	Location	Amplicon size (bp)	Accession number (GenBank)	References
<i>sefA</i>	A058 (F)	GATACTGCTGAACGTAGAAGG	NG ²⁾	488	-	(Doran <i>et al.</i> , 1996)
	A01 (R)	GCGTAAATCAGCATCTGCAGTAGC				
	Forward (F)	GGCTTCGGTATCTGGTGGTGTA	330–351	97	L03833	(Seo <i>et al.</i> , 2004)
	Reverse (R)	GTCATTAATATTGGCTCCCTGAATA	402–426			
<i>Sdf I</i>	Forward (F)	TGTGTTTTATCTGATGCAAGAGG	23-45	293	AF370707	(Agron <i>et al.</i> , 2001) ³⁾
	Reverse (R)	CGTCTTCTGGTACTTACGATGAC	295-351			
<i>insertion element</i>	IE1L (F)	AGTGCCATACTTTAATGAC	397–416	316		
	IE1R (R)	ACTATGTCGATACGGTGGG	694–712		Z83734	(Wang and Yeh, 2002)
	IE2L (F)	GGATAAGGGATCGATAATTGC	610–630	560		
	IE3R (R)	GGACTTCCAGTTATAGTAGG	1150–1169			
<i>Prot6e</i>	Prot6e-5 (F)	ATATGTCGTTGCTGCTTCC	387–406	206		(Malorny <i>et al.</i> , 2007) ³⁾
	Prot6e-6 (R)	CATTGTTCCACCGTCACTTTG	572–592		U66901	
	438 (F)	GGCACCGCAGCAATGGTTGG	438-457	135		(Hadjinicolaou <i>et al.</i> , 2009)
	572 (R)	GGTCGAGCTACAGAGAGTCACAC	550-572			
<i>invA</i>	invA-F (F)	TCCCTTTGCGAATAACATCC	NG	725	-	(Chen <i>et al.</i> , 2012)
	invA-B (R)	ATTACTTGTGCCGAAGAGCC				

¹⁾ Oligonucleotide primer names were quoted as shown the original papers; Primer orientation is indicated in parenthesis: F, forward; R, reverse.

²⁾ NG means the location of primer-targeted gene was not given in the reference.

³⁾ Primer pairs of *Salmonella* Enteritidis finally adopted in this study.



all organisms) was selected as active-search database. Based on the Primer-BLAST results, three pairs of the SE candidate primer were further tested by PCR using farm samples.

Experimental farm samples and SE culture procedures

Experimental farm samples were collected from over shoes, house air, drinking nipples, feed, egg collection belt, eggshell, air inlet and air outlet from a layer house of the Experimental Unit for Poultry Genetic Resource and Breeding at China Agricultural University (CAU), and were pooled per sample type.

Samples were firstly placed on a culture plate and homogenized with Buffered Peptone Water (BPW) at 1:9 dilution, and incubated at 37°C for 18 h with shaking at 170 rpm. Then, 1 mL of the bacterial solution was transferred to 9 mL of Selenite Cystine Broth (SC) and incubated at 37°C for 18 h with shaking at 170 rpm.

Genomic DNA extraction

Genomic DNA extraction process was carried out by transferring 1 mL of selective enriched sample to a microcentrifuge tube. Cell suspension was centrifuged at 12,000 rpm for 5 min at 4°C in a centrifuge (5424R, Eppendorf®, Hamburg, Germany), then the supernatant was discarded. The pellet was washed twice with double-distilled water (ddH₂O) taken from a Millipore Milli-Q® Academic Water Purification System (Merck KGaA, Darmstadt, Germany), and washed again in 1-mL anhydrous ethanol suspension. The precipitate was suspended in 200 µL ddH₂O after air drying. The microcentrifuge tube was then boiled for 15 min and chilled immediately on ice for 2 min. The tube was centrifuged at 12,000 rpm for 15 min at 4°C. Afterwards, the supernatant was transferred to a new microcentrifuge tube and used as DNA template for the PCR.

Selection of candidate primers using Polymerase Chain Reaction

Amplification of the farm samples was carried out in a total volume of 20 µL as follows: 10 µL 2 × Power Taq PCR MasterMix (Biotek Corporation, Beijing, China) with Taq DNA polymerase, PCR buffer, dNTP mixture containing Mg²⁺, 1 µL 10µM of each primers, 2 µL of extracted DNA template and 6 µL of ddH₂O. PCR amplification was performed in an Applied Biosystems® Veriti™ 96-Well Thermal Cycler (Life Technologies, CA, USA) with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30

s, 72°C for 20 s and finished with a final extension at 72°C for 10 min.

Each PCR assay systematically included positive and negative controls with genomic DNA of SE strains and ddH₂O, respectively. Aliquots (5 µL) of the PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels (BIOWEST®, Nuaille, France) using PowerPac™ Basic Power Supply (Bio-Rad Laboratories, CA, USA), stained with GelStain (Transgen Biotech, Beijing, China), and visualized under UV light in a ImageQuant™ 300 Imager (GE Healthcare, Buckinghamshire, UK).

Determination of SE detection sensitivity

Optimal PCR annealing temperature was firstly determined. Temperatures of 56°C, 58°C, 60°C, 62°C, 64°C, and 66°C were set as annealing temperatures along with forward & reverse *Prot6e* primers. PCR amplification was performed in triplicate. Amplification efficiency was estimated by calculating the standardized grayscale for each expected band. Grayscale analysis was performed using ImageJ software version 1.50a (The University of Queensland, unknown). Amplification efficiency was calculated as:

$$AE = \text{Gra (AP)} / \text{Gra (Mk)}$$

Where AE is the amplification efficiency at different annealing temperatures, Gra (Ap) is the grayscale of amplicons targeting the *Prot6e* gene, and Gra (Mk) the 400 bp band derived from Marker 1 (Dongsheng Biotech, Guangdong, China).

Both cell lysate and SE liquid were used as templates to perform PCR amplification for the comparative evaluation of sensitivity. The strains of *Salmonella enterica* serovar Enteritidis were obtained from the China Institute of Veterinary Drugs Control (IVDC). Detection limit was evaluated using 10-fold dilutions of the reference SE strains. At each dilution step, sufficient amount was saved for PCR analyses. Each dilution was enriched as mentioned above, and the extracted genome or bacterial liquid was used as DNA template for the PCR assay. The plate count method was used to determine CFU. Viable SE amounts were obtained by plating 10-fold serial dilutions of inoculated broth cultures onto nutrient agar thrice and incubating the plates at 37°C for 24 h.

Cell lysate amplification was carried out as described in the section of candidate primers selection. For amplification with bacterial liquid, pure SE suspensions were firstly homogenized with an adequate amount of BPW, and incubated overnight at 37°C with shaking at 170 rpm for the next step. PCR assay was carried out



in a total volume of 20 µL as follows: 10 µL One Taq® 2 × Master Mix with Standard Buffer (NEW ENGLAND Biolabs®, MA, USA), 0.4 µL 10 µM of each primer, 2 µL of bacterial liquid, and 7.2 µL ddH₂O. PCR amplification was performed with initial denaturation at 94°C for 30 s, followed by 30 cycles of 94°C for 30 s, 58°C for 1 min, 68°C for 20 s, and final extension at 68°C for 5 min. Agarose gel electrophoresis was proceeded as described in this study.

Environmental contamination of commercial farms with SE

In total, 1512 samples were collected on eight battery-cage commercial farms in the four main egg-production provinces in a voluntary SE surveillance study carried out in northern China between August and December 2014. Detailed information is shown in Table 2. Two commercial egg-production farm sizes were selected in each province: one housing more than 50,000 layers, and the other, less than 50,000 layers. Layer house and its corresponding egg collection area were randomly chosen as sampling sites in each farm. Feed, drinking nipple, egg collection belt, air inlet, air outlet, air, overshoe, and eggshell were sampled inside each house, and air, overshoes, and eggshells in the egg collecting area. Sampling was carried by scraping or wetting with sterile cotton swab according to the sample type.

Table 2 – Sampled farms from four main egg production areas in the northern China

Province	Farm ¹⁾	Scale	Layer strain ²⁾	Housing system	Structure
Shandong	A	30,000	Hy-Line Brown	Battery cage	Semi open
	B	50,000	Hy-Line Brown		Semi open
Liaoning	C	19,000	Hy-Line Brown		Semi open
	D	60,000	Hy-Line Brown		Semi open
Henan	E	40,000	Nongda No.3		Semi open
	F	250,000	Nongda No.3		Closed
Hebei	G	4,000	Hy-Line Sonia		Open
	H	100,000	Hy-Line Brown		Closed

¹⁾ Farms were named alphabetically.

²⁾ Same strain was kept in each sampled layer house.

The sterile cotton swab covered with targeted samples were put in a culture plate with BPW at 1:9 dilution, stored in a cooler box at about 4°C, and transported to the laboratory, where they were submitted to the detection procedures described above.

Statistical analysis and plotting

Statistical analyses of annealing temperature determination were executed using SAS version 9.3

(SAS Institute Inc., North Carolina, US). One-way analysis of variance process was applied to examine differences in amplification efficiency at different annealing temperatures. Plotting was accessed using R software with ggplot2 package, parameters were set according to practical requirements.

RESULTS

Primer screening via BLAST

SE primers used in the present study were checked for their specificity by Primer-BLAST. Results showed that the primers shown in Table 1 were capable of detecting *Salmonella enterica* subspecies *enterica* serovar Enteritidis, but not all primers excluded other serotypes. The serotypes corresponding to the target genes detected with each primer are shown in Table 3. We listed target genes rather than its original primer pairs, since primers located on the same gene yielded identical specificity results. In summary, primer pairs located on *sefA*, *insertion element* and *invA* genes were able to detect other *Salmonella* serotypes belonging to *Salmonella* serogroup A, B, C₁ and D serovars, in addition to Enteritidis, while oligonucleotide primers targeting *Sdf I* and *Prot6e* genes identified only Enteritidis. Therefore, three primer pairs located on these two genes were nominated as candidate primers.

Table 3 – Target gene and its specific *Salmonella* serotypes matched by Primer-BLAST

Gene target/ Serotype	<i>SefA</i>	<i>Sdf I</i>	<i>Insertion element</i>	<i>Prot6e</i>	<i>invA</i>
Enteritidis	√ ²⁾	√	√	√	√
Dublin					
Pullorum	√		√		√
Gallinarum					
Typhi					
Paratyphi A	√				√
Paratyphi C					
Typhimurium					√
...					

¹⁾ The symbol "√" indicates certain *Salmonella* serotype was detectable by targeted primer pairs.

Specific examination by PCR assay

After Primer-BLAST alignment, two primer pairs targeting *Prot6e* gene and an oligonucleotide primer targeting *Sdf I* gene were shown to be SE-specific. PCR analysis was performed to check the identifiability of these three primers with farm samples. Results showed that non-specific bands between 100 and 600 bp were captured using primer pairs of *Prot6e* 438 (F) & 572 (R), while environmental sample lanes were much cleaner, with primers targeting *Sdf I* gene and primer



pairs of *Prot6e* 5 (F) & 6 (R). Eventually, *Prot6e* 5 (F) & 6 (R) were chosen for further SE screening, considering experimental results by Malorny *et al.* (2007), and another SE-specific marker targeting *Sdf I* was selected to screen all the environmental samples.

Detection sensitivity of the PCR assay

Relative to the detection sensitivity of the primers *Prot6e* 5 (F) & 6 (R), band intensity of the target amplicons was reduced as annealing temperature was gradually increased. Standardized band intensities of the target amplicons were not considerably different at 56°C and 58°C (Figure 1). Band intensities sharply decrease at temperatures higher than 58°C, and were significantly lower compared with those obtained at 56°C and 58°C ($p < 0.0001$). Therefore, the optimal annealing temperature suggested was 58°C.

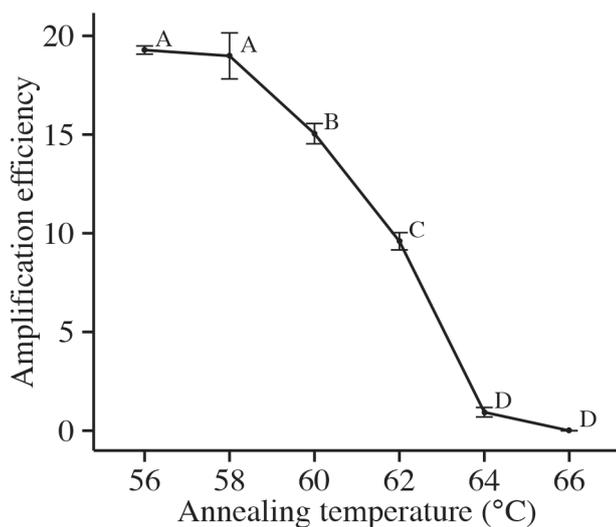


Figure 1 – Amplification efficiency of the adopted *Prot6e* primers at different annealing temperatures. Amplification efficiency was estimated based on the standardized production of the amplicon, which was calculated as the grayscale value of the targeted band divided by that of indicating marker. Higher values indicate superior amplification efficiency. Common letters beside each solid point indicate no significant differences ($p < 0.05$). Vertical bars denote standard deviation (SD) within each group.

Simultaneously to PCR sensitivity calculations, a comparative evaluation of sensitivity was also made using amplifications of bacterial cell suspensions. A detection limit of 1 CFU/mL was obtained after the general SE identification procedure with dilutions of the reference SE strains (Figure 2A), which was more sensitive than the direct amplification of the SE suspensions. The PCR sensitivity using bacterial liquid was about 800 CFU/mL (approximately as low as 8 CFU per PCR reaction), as shown in Figure 2B. In addition, lane 1 in Figure 2B was inverted and enhanced for better identification resolution (Figure 2C).

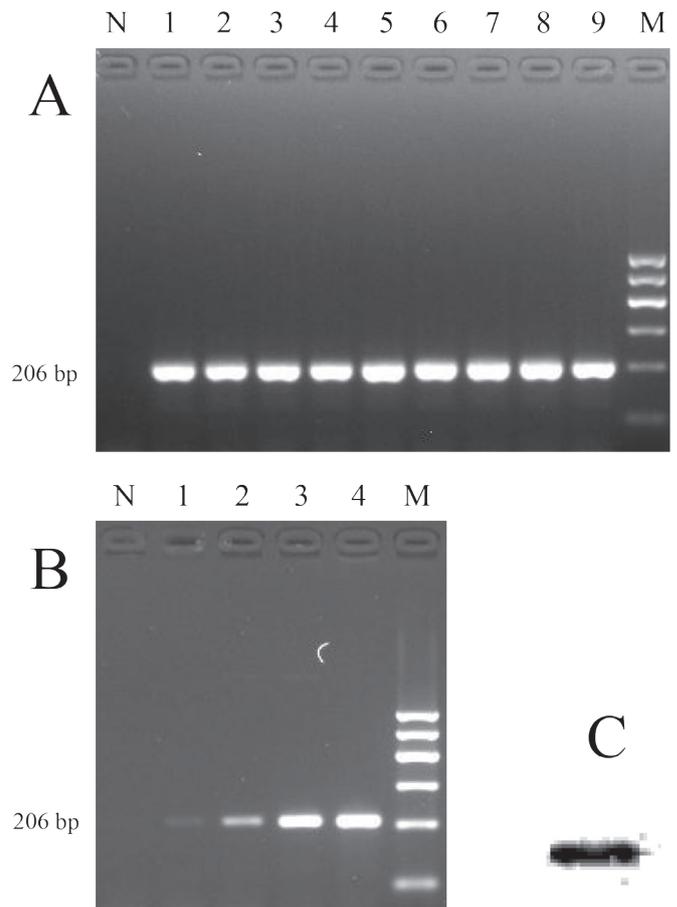


Figure 2 – Sensitivity of PCR identification of *Salmonella* Enteritidis. The detection limit was determined using 10-fold dilutions of the reference strains. CFU level was measured using the plate count method. Lane N: negative control, lane M: Marker 1. (A). Detection limit with of extracted DNA templates after enrichment procedures. Lanes 1-9: 10^2 CFU/L to 10^{10} CFU/L. (B). Detection limit with bacterial liquid templates. Lanes 1-4: 8×10^5 CFU/L to 8×10^8 CFU/L. (C). Processed band of lane 1 in Figure 2B with ImageJ software.

Environmental contamination of SE on layer farms

According to the *Prot6e* 5 (F) & 6 (R) results, non-contaminated samples were confirmed as SE-negative by *Sdf I* primer pairs in seven of the eight farms evaluated. Only samples from farm H were contaminated with *Salmonella* Enteritidis (Table 4). The contamination rates of the different sample types varied between 3.7% and 16.7%, with a total contamination prevalence of SE-positive samples of 4.8%. In the layer house, contamination was detected in three water samples collected from the drinking nipples, which were located on different pipelines and distant from each other. An egg collection belt sample was positive for SE at the bottom tier, near the air inlet. One sample spot on the top of the air inlet and one overshoe swab sample of the left foot were also contaminated. In the egg collecting area, only two eggshell samples were detected as positive for SE.



Table 4 – *Salmonella* Enteritidis contamination of samples collected in layer farms in northern China

Location	Contaminated samples/ Examined samples	
	Farm A - Farm G	Farm H
Henhouse	Feed	0/27
	Drinking nipple	3/27
	Egg collection belt	1/27
	Air inlet	1/9
	Air outlet	0/9
	Air	1/9
	Overshoe	1/6
	Eggshell	0/30
	Air	0/9
Egg collecting area	Overshoe	0/6
	Eggshell	2/30
Total percentage		4.8%

¹⁾ Contamination of *Salmonella* Enteritidis was not detected in tested samples.

DISCUSSION

PCR is an efficient method for screening SE contamination in poultry environmental samples, considering factors such as cost, time, and accuracy. It is well known that primer-targeted sequences can influence PCR specificity (Seo *et al.*, 2004). *Salmonella* serovars are extremely similar within conserved regions even with other bacterial species like *Escherichia coli* (*E. coli*), and therefore, it is essential to select the appropriate oligonucleotide primers for subsequent identification process (Boyd *et al.*, 1996; McClelland *et al.*, 2001; Edwards *et al.*, 2002). Unlike other primer design software programs, Primer-BLAST is an online tool that allows checking the specificity checking of pre-existing primers. It combines the BLAST program with the Needleman-Wunsch (NW) global alignment algorithm to ensure full alignments, and this strategy is sufficiently sensitive to identify targets that differ from the original primers (Needleman & Wunsch, 1970; Ye *et al.*, 2012). Using the giant and burgeoning database owned by NCBI, primer specificity tests are faster and more accurate. In the present study, three candidate primer pairs were targeted after sequence alignment via Primer-BLAST. After further testing of farm samples with PCR, the *Prot6e*-5 (F) & *Prot6e*-6 (R) primers located on *Prot6e* gene were eventually adopted. This gene may play a role in biosynthesis of the surface fimbriae and alter their interaction with egg albumen components (Clavijo *et al.*, 2006). Malorny *et al.* (2007) tested a panel of 119 non-Enteritidis SE

strains comprising 54 serovars, as well as 79 SE strains comprising 19 different phage types, and verified that none of these non-Enteritidis strains were positive for the *Prot6e* gene. Moreover, those researchers found that nearly all SE strains analyzed presented a specific 60-kb virulence plasmid, and inferred that plasmid-free strains were less virulent. Although chromosomal-located fragment gene *SdfI* also showed high exclusivity, which was employed to confirm the detection results in the present study, phage types 6A, 9A, 11, 16, 20, and 27 did not present such gene (Agron *et al.*, 2001).

The pre-enrichment and selective enrichment steps before the PCR assay were performed to dilute PCR-inhibitory substances as well as to improve detection sensitivity, and proved to be more sensitive than the traditional culture method and direct amplification (Hsu *et al.*, 2011). Both enrichment steps may effectively dilute PCR inhibitors present in environmental matrix. The overnight pre-enrichment step increases the number of intact bacterial cells, and the selective enrichment step suppresses the growth of background flora (O'Regan *et al.*, 2008). The testing procedure was based on thermal-lysed cell DNA extraction method (Wang *et al.*, 1996; Theron *et al.*, 2001; Fan *et al.*, 2014). Double washing steps with ddH₂O prior to DNA extraction and the subsequent washing step with anhydrous ethanol were adopted as part of the process. Hyeon *et al.* (2010) reported that enrichment media residue still exerted inhibitory effects after a single washing step, while significant improvement in SE detection was observed after an additional washing step. Ethyl alcohol removes water-insoluble inhibitory substances remaining in the matrix. The sensitivity test in this study was conducted using cell lysates from pure cultures, and therefore, the actual detection sensitivity may be a little higher when screening samples from various sorts of environments.

In the conventional PCR assay, high annealing temperature guarantees detection specificity. However, amplification efficiency simultaneously decreases (Rychlik *et al.*, 1990; Shen *et al.*, 2007). The adopted annealing temperature of 58°C determined in the preliminary experiment prevented nonspecific reactions, and maintained detection sensitivity. An alternative to increase PCR efficiency without losing its specificity requires using amplification facilitators (Szmolka *et al.*, 2006). The use of bovine thrombin (BT) as an additive in PCR enhances its efficiency and specificity (Zhang *et al.*, 2014), as it has the remarkable ability of effectively reducing PCR inhibition even at nanoscale sizes. Another approach involves the replacement of conventional *Taq* DNA polymerase.



A novel *Taq* DNA polymerase mutant (SD DNA polymerase) produces better PCR amplification results with a DNA template of complex secondary structure or a GC-rich template (Ignatov *et al.*, 2014). As for the electrophoretic-gel processing step shown in Figure 2C, a Java-based software of ImageJ allowed adjusting gel images to a higher discrimination capacity by human eyes (Schneider *et al.*, 2012).

Northern China is the main egg production region of that country. The annual report of the National Bureau of Statistics of the People's Republic of China (NBS) showed that the egg production of Henan, Shandong, Hebei, and Liaoning provinces accounted for half of the countrywide capacity in 2014 (National Bureau of Statistics of China, 2015). Another survey conducted by the National Laying Hen Industrial Technology System showed that most layer farms, up to 88.28%, housed 2,000 to 50,000 laying hens, and small-household farming systems produce most of the eggs. In the present study, on-farm surveillance was conducted to evaluate the SE-contamination status of layer farms of two different scales. No SE contamination was found in small household farms, and the only contaminated samples were collected on a single large-scale layer farm. SE outbreaks are rarely reported in China, which maybe partly due to Chinese dietary habit of eating only cooked eggs. Contamination of SE is ubiquitous in different housing systems (Holt *et al.*, 2011), and we focused the study on the battery-cage systems, which are predominant in many regions of China. Cloacal and fecal samples were not collected due to intermittent SE excretion by infected hens (Gast *et al.*, 2015). On the SE-positive farm, contaminated water samples were collected from drinking nipples scattered on different pipelines, which indicates that SE may not originate from the water reservoir, whereas the physical structure of the drinking nipple may effectively prevent the ascending contamination of pathogenic bacteria. Airborne transmission route is one of the SE propagation paths, and accordingly, contamination was also found in an air sample. Se-positive samples collected from eggshells in the egg collecting area highlight the necessity of disinfection and sterilization during the transport of poultry eggs. In a previous study, Dewaele *et al.* (2012) observed persistent SE contamination of the environment during the entire laying period. Interestingly, on farm H, all *Salmonella*-positive samples were contaminated with SE (Li X. Z., 2016, unpublished data), demonstrating the competitive exclusion between SE and other *Salmonella* serotypes to some extent (Rabsch *et al.*, 2000). Due to the governmental enforcement and to the objectives of "establishing

standardized poultry farms, disease decontamination, and biosecurity measures" by the egg industry, strict self-discipline is exercised in layer farms. The results of the present study indicate that the established normative control measures and disease control and prevention systems have effectively inhibited the dissemination of SE in the farm environment in China, independently of farm scale.

CONCLUSION

The PCR method applied for screening SE in farm samples is simple, inexpensive, as well as effective. Using the specific single-primer PCR method, minor environmental contamination with *Salmonella* Enteritidis was detected on layer farms in northern China. Several areas within layer farms may contribute for SE dissemination.

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