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≪Research Note≫

Salmonella Contamination in Layer Farms in China: Detection and Genetic Analysis

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Salmonella is the most common cause of foodborne illnesses worldwide. Poultry eggs are a major contamination source of *Salmonella*. The prevalence of *Salmonella* has been effectively reduced since a series of measures were taken to reduce contamination in egg-laying houses. In the present study, 1,512 environmental samples obtained from layer farms of different production scales were screened in a voluntary *Salmonella* survey study. Contaminations were detected using a PCR method. Genetic relationships among *Salmonella* samples were specified using molecular typing by enterobacterial repetitive intergenic consensus (ERIC)-PCR. The survey results showed that two layer farms, located in the Shandong and Hebei provinces, were contaminated with *Salmonella*. Thirty-one samples from these two farms, including feed, drinking nipples, egg collection belt, air inlets and outlets, air, overshoes, and eggshells, were identified as *Salmonella*-positive. It was observed that certain samples within the henhouses as well as in the egg collecting areas showed relatively high genetic similarities. The survey conclusively revealed minor *Salmonella* contamination in northern China. Moreover, various areas within the layer farms were identified as part of the propagation chain of *Salmonella*. Furthermore, evidence of cross-contamination of *Salmonella* was found in the laying houses and egg collection areas, even between these two regions. Therefore, it is necessary to establish routine *Salmonella* detection and subsequent environmental control measures in order to decrease the prevalence of *Salmonella*.

Key words: environment, ERIC-PCR, layer farm, PCR, *Salmonella*

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Introduction

With the fast growth of the Chinese economy, the chicken industry in China has been on the rise over the last 30 years, and has achieved great production values. According to the Food and Agriculture Organization of the United Nations (FAO), the total number of chickens was 4,539 billion in 2014, and total egg production reached 24,446 million metric tons in 2013 (FAO, 2016a, b). According to the data published in the annual report of the National Bureau of Statistics of the People's Republic of China, in 2014, the provinces of Shandong, Liaoning, Henan, and Hebei accounted for nearly half of the countrywide egg production capacity (National Bureau of Statistics of China, 2015). There have only been limited reported investigations of *Salmonella* contamination in poultry flocks and the environment (Li *et al*., 2013; Gong *et al*., 2014). However, the worldwide epidemic of infectious diseases known to be caused by various serotypes of *Salmonella* has brought the public health hazard to the forefront (Morpeth *et al*., 2009; Arnold *et al*., 2010; Raufu *et al*., 2013). Although the egg industry has taken measures, such as cleaning and disinfection, to decrease the prevalence of *Salmonella* and a large reduction in infections in poultry and humans was subsequently reported, residual contaminations still exist in laying houses (Carrique-Mas *et al*., 2009; O'Brien, 2013; Gosling *et al*., 2014).

Salmonella is primarily spread through the food production chain, particularly through contaminated eggs. *Salmonella* on the surface of eggshells poses a potential threat to the public (De Buck *et al*., 2004; Schulz *et al*., 2011). Previous studies have indicated that most *Salmonella* infections are a consequence of re-invasion, rather than introduction, of pathogens from the farm environment (Van Hoorebeke *et al*., 2011). Asymptomatic carrier birds may also facilitate the spread of the bacteria (Gast and Holt, 1998; Guard-Petter, 2001). Therefore, it is of utmost importance to detect and monitor *Salmonella* contaminations in laying house environments. In this study, we conducted a voluntary survey to gain insight into the general *Salmonella* contamination status

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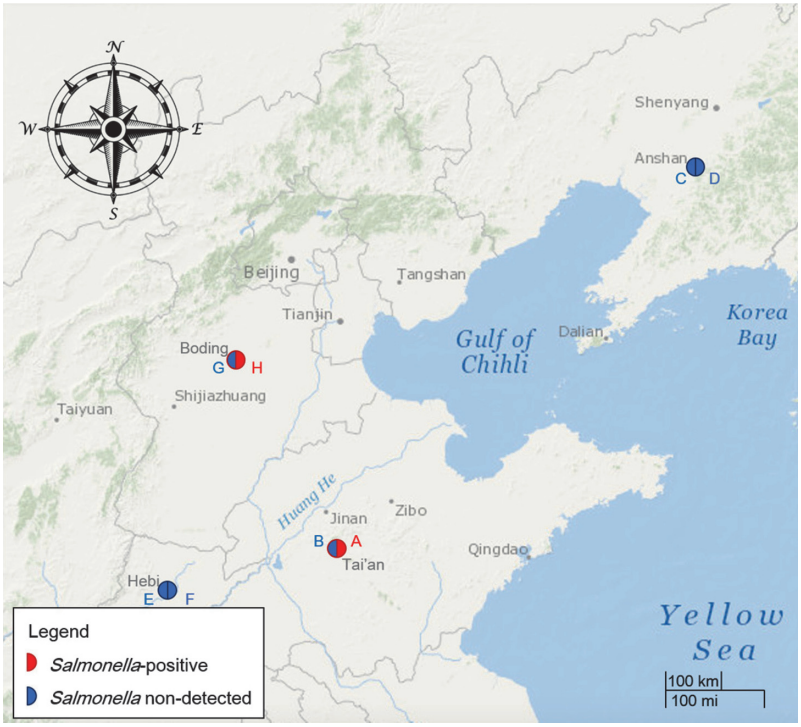

Fig. 1. **Geographical map showing the locations of the farms in four provinces in northern China where environmental samples were collected.** The eight farm locations are marked with red or blue semicircles. Red semicircles represent farms that were contaminated with *Salmonella*, blue semicircles represent contamination-free farms. Farms are indicated with capital letters, which correspond to those in Table 2.

of small- and large-scale layer farms in the main egg production areas of China, and potential cross contamination among the confirmed positive eggs and poultry environmental sites was analyzed.

Materials and Methods

Environmental Samples

In this study, environmental samples were collected from eight layer farms located in four provinces (Shandong, Liaoning, Henan, and Hebei) of northern China (Fig. 1). In each province, one large-scale (>50,000 cages) and one small-scale (<50,000 cages) farm were selected. Sampling was done between August and September 2014. More detailed information on each of the farms is shown in Table 1.

Cotton swabs used for sampling were sterilized by autoclaving prior to use and were then kept in sealed tubes. The sterile cotton swabs were moistened with buffered peptone water (BPW) before sampling. The person collecting the samples wore sterile gloves when handling and moistening the swabs. A 70% ethanol solution was used to decontaminate the top of the BPW-containing tube before opening. Following sampling, each swab was placed in an individual sample tube with 10 mL of BPW. Samples were not pooled. Sampling was carried out under the guidelines of the US Food and Drug Administration (FDA) and the National Institute of Agricultural Research (FDA, 2009; Nys *et al*., 2011). All samples were collected using sterile cotton swabs, and the same standards were maintained. The sampling process was deemed completed when the surfaces of swabs were fully covered with the targeted samples. The tubes were stored in a cooler box at approximately 4℃ and transported back to the laboratory when the sampling process was completed.

Samples obtained from egg collection belts and overshoes were regarded representative environmental samples. The egg collection belts in each henhouse were hand-swabbed at both ends and the central portion of the tire. Three swabs were taken from three adjacent sampling cages, without overlap. This process was continued until all three tires on one side of the bank in the middle of the henhouse had been sampled. Wearing overshoes, a walk was taken along the entire length of each walkway and back. Three random spots were sampled for each overshoe by using sterile cotton swabs. Feed and drinking nipples were sampled as described for the egg collection belt. Samples from the air inlets and outlets were separately taken at nine random locations. Nine air samples were randomly collected by placing moistened swabs on sterile petri dishes close to the ground for 1 h.

Table 1. **Information on the layer farms of different scale in the main egg production area in northern China evaluated in this study**

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	CAU-3		Semi-open
	F	250,000	CAU-3		Closed
Hebei	G	4,000	Hy-Line Sonia		Open
	H	100,000	Hy-Line Brown		Closed

[1] Farms were named alphabetically;
[2] Same chicken breed was kept in each laying house

Thirty eggs were randomly sampled at different positions in the henhouse. Finally, in the egg collection areas, six overshoe, nine air, and 30 egg samples were collected, using the same protocol as that described for henhouse sampling.

Culture Condition and Genomic DNA Extraction

In the laboratory, swab samples in BPW were incubated at 37℃ for 18 h with shaking at 170 rpm. Then, 1 mL of bacterial suspension was transferred to 9 mL of selenite cystine broth *Salmonella* enrichment medium and incubated at 37℃ for 18 h with shaking at 170 rpm. Then, the bacterial suspension was stored at 4℃ for further analysis.

For genomic DNA extraction, 1 mL of selectively enriched sample was transferred to a microcentrifuge tube and centrifuged at 4℃ for 5 min at 12,000 rpm using a Centrifuge 5424R (Eppendorf, Hamburg, Germany). The pellet was washed twice with ddH_2O taken from a Millipore Milli-Q® Academic Water Purification System (Merck KGaA, Darmstadt, Germany) and then by suspending it in 1 mL of anhydrous ethanol. The cells were air-dried and then resuspended in 200 μL of ddH_2O. Then, the micro-centrifuge tube was boiled for 15 min and immediately chilled on ice for 2 min. The tube was centrifuged at 12,000 rpm at 4℃ for 15 min. The supernatant was transferred to a new microcentrifuge tube and used as a DNA template for PCR.

Salmonella Detection Via PCR

The sequences of the primer pair targeting the *invA* gene used for the identification of *Salmonella* contamination were as follows: 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and 5′-TCATCGCACCGTCAAAGGAACC-3′ (Rahn *et al*., 1992). For confirmation, all environmental samples were screened using primers targeting *hilA*: 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and 5′-TCATCGCACCGTCAAAGGAACC-3′ (Craciunas *et al*., 2012). Amplification was carried out in a 20 μL reaction mixture (10 μL 2× Power Taq PCR MasterMix [BioTeke Corporation, Beijing, China], 1 μL of 10 μM of each of the primers, 2 μL of DNA template, and 6 μL of ddH_2O) in an Applied Biosystems Veriti 96-well thermal cycler (Life Technologies, CA, USA). The thermal cycles were: initial denaturation at 95℃ for 5 min, 35 cycles of 95℃ for 30 s, 58℃ for 30 s, 72℃ for 30 s, and final extension at 72℃ for 10 min.

Genomic DNA of *Salmonella* strains and ddH_2O were systematically included as positive and negative controls in each assay, respectively. Five-microliter aliquots of PCR product were analyzed by electrophoresis on 2% (w/v) agarose gel (BIOWEST, Nuaillé, France) using a PowerPac Basic Power Supply (Bio-Rad Laboratories, CA, USA). Gels were stained with GelStain (TransGen Biotech, Beijing, China) and visualized under UV light using an ImageQuant 300 Imager (GE Healthcare, Buckinghamshire, UK).

Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR Typing

One milliliter of *Salmonella*-positive bacterial solution in selenite cystine broth was transferred to 9 mL of BPW and incubated overnight for recovery and multiplication. Then, 1 mL of cell solution was pelleted by centrifugation. After the supernatant was discarded, 700 μL of cell lysis buffer containing 20 mM of Tris, 5 mM of EDTA, 400 mM of NaCl and 1% (w/v) of SDS (Sigma-Aldrich, Darmstadt, Germany) was added with 30 μL 2% (w/v) of proteinase K, and 20 μL 1% (w/v) RNase (TransGen Biotech). The mixture was placed in a 55℃ water bath and shaken at 220 rpm overnight. Genomic DNA was extracted using a standard phenol-chloroform method, air-dried, and dissolved in 200 μL of ddH_2O for further utilization. DNA quality was checked by electrophoresis and spectrophotometrically.

The primers used for ERIC-PCR were: ERIC-1R (5′-ATGTAAGCTCCTGG-GGATTCAC-3′) and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′), as previously described (Hulton *et al*., 1991; Campioni *et al*., 2014). The PCR mixture (20 μL) consisted of the following: 10 μL of One *Taq* 2× Master with Standard Buffer (New England Biolabs, MA, USA), 0.4 μL of 10 μM of each primer, 2 μL of DNA template, and 7.2 μL of ddH_2O. The PCR amplification program was as follows: initial denaturation at 94℃ for 30 s, 30 cycles of 94℃ for 30 s, 52℃ for 1 min, 68℃ for 2 min, and final extension at 68℃ for 5 min. PCR products were electrophoresed on 1% (w/v) agarose gel at 30 V for 3 h using the equipment mentioned above. To avoid any misinterpretations due to interassay variation, a standard 1-kb ladder (Dongsheng Biotech, Guangdong, China) was added in three lanes on each gel for normalization as well as to

Table 2. **Origins of *Salmonella*-positive samples in contaminated layer farms**

Locations		Farm A		Farm H	
		Contamination rate[1]	Key[2]	Contamination rate	Key
Henhouses	Feed	1/27 (3.7%)	1	0/27	—
	Drinking nipples	1/27 (3.7%)	2	3/27 (11.1%)	23–25
	Egg belt	0/27	—	1/27 (3.7%)	26
	Air inlets	2/9 (22.2%)	3, 4	1/9 (11.1%)	27
	Air outlets	3/9 (33.3%)	5–7	0/9	—
	Air	2/9 (22.2%)	8, 9	1/9 (11.1%)	28
	Overshoes	2/6 (33.3%)	10, 11	1/6 (16.7%)	29
	Eggshells	5/30 (16.7%)	12–16	0/30	—
Egg collecting areas	Air	2/9 (22.2%)	17, 18	0/9	—
	Overshoe	0/6	—	0/6	—
	Eggshell	4/30 (13.3%)	19–22	2/30 (6.7%)	30, 31
Total percentage		11.6%		4.8%	

[1] Number of contaminated samples/number of detected samples for each sample type are shown, with the percentage in parentheses;
[2] Contaminated samples were assigned unique key values.

facilitate comparison of fingerprints between different gels. All samples were assessed simultaneously and under similar conditions.

Data Analysis

The ERIC-PCR fingerprint patterns, in the form of TIFF images, were analyzed using GelCompar II software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). Dice similarity coefficients between samples were calculated with optimization, and the "band matching tolerance" was set as 0.5. A dendrogram was obtained from the resulting similarity matrix by means of an Unweighted Pair Group Method with Arithmetic Averages (UPGMA) clustering algorithm. Default parameters were adopted in accordance with the manufacturer's instruction, except for curve extraction and band definition, where "Averaging thickness" was set as 20 pts, "Min. profiling" was set as 4.00%, and "Shoulder sens." was set as 3. The discriminatory power of the ERIC-PCR was calculated using Simpson's Index of Diversity by the Hunter-Gaston Diversity Index (Hunter and Gaston, 1988).

Results

***Salmonella* Environmental Contamination in Layer Farms**

In total, 1,512 environmental samples were collected from eight layer farms. *Salmonella* was detected by PCR using primers targeting *invA* and primers targeting *hilA* for confirmation. Samples from six farms were negative for *Salmonella*. On farms A and H, however, *Salmonella* was detected. Details on the contamination sources in these two farms are summarized in Table 2.

Farm A, located in Shandong Province, was a small-scale farm with 30,000 laying hens. Nearly all types of environmental samples from this farm were *Salmonella*-positive, at levels ranging from 3.7% to 33.3%, with an average of 11.6 %. In the henhouse area, positive samples included: one feed sample taken at the top of the tier close to the air inlet; one water sample from a drinking nipple at the middle of the tier near the air outlet; two samples taken at the bottom of the air inlet; two samples from the middle and one from the bottom of the air outlet; two air samples from locations close to the air inlet and in the middle of the house; two overshoe samples (from both feet); and five eggshells from the top, middle, and bottom of the tier. In the egg collection area, two air samples and four eggshell samples were found to be *Salmonella*-positive.

Farm H, located in Hebei Province, was a large-scale farm with 100,000 laying hens. Six types of environmental samples were contaminated with *Salmonella* at levels ranging between 3.7% and 16.7%, with an average of 4.8%. In the henhouse area, positive samples included: three water samples from drinking nipples scattered over three pipelines located at different tiers, away from each other; one sample from the egg collection belt at the bottom of the tier near the air inlet; one sample taken at the top of the air inlet; and one overshoe sample. In the egg collecting area, two eggshells were contaminated with *Salmonella*.

ERIC-PCR Fingerprinting and Clustering Analysis

Based on a similarity matrix calculated on the basis of ERIC-PCR patterns, a dendrogram was constructed that shows the genetic relationships between *Salmonella* isolates in positive samples (Fig. 2). The discrimination index of ERIC-PCR typing was calculated to be 99.60% in the present analysis. In each gel lane, 1 to 15 major bands were identified, and amplicons varied in size from 100 bp to more than 10 kb. Based on the ERIC-PCR profiles, three major clusters were distinguished, designated as C1 (keys 02 to 07), C2 (keys 28 to 24), and C3 (keys 13 to 06). Samples from farm A (9/22), which were clustered into C1, showed 25–75% similarity. C2, which covered almost all of the *Salmonella*-positive samples from farm H (7/9) and six out of 22 samples from farm A, exhibited 30–100% similarity. The

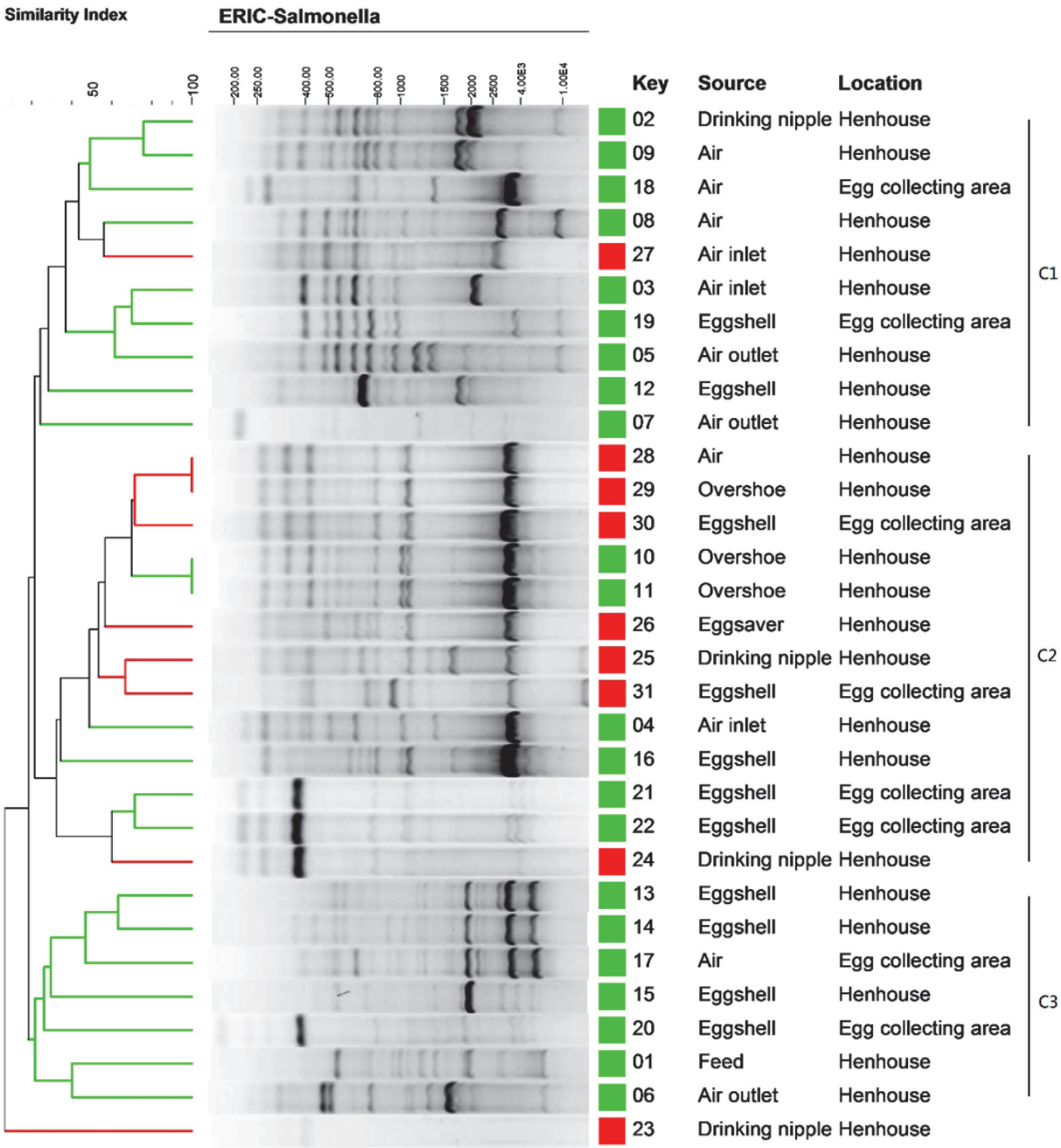

Fig. 2. **Dendrogram produced by cluster analysis of the ERIC-PCR fingerprinting data (UPGMA) of the *Salmonella*-positive samples, based on a Dice coefficient.** Three major clusters (C1 to C3) of related samples were defined. Samples from farm A and farm H are marked with green and red squares, respectively. Each key refers to a unique sample and corresponds to the keys in Table 2. For each ERIC-PCR fingerprint, band sizes, sources, and locations of the samples are shown.

samples that were grouped in C3 all originated from farm A (7/22), and displayed 20−60% similarity.

Discussion

Since the late 1990s, the improved biosecurity and hygiene

in chicken farms, along with the vaccination of laying hens, have significantly reduced the incidents of *Salmonella* in poultry and humans (Marcus *et al*., 2004; Barrow *et al*., 2012). However, *Salmonella* infections in laying flocks are still occasionally reported (Gast *et al*., 2015; Jones *et al*., 2016; Pande *et al*., 2016). Environmental contamination is one of the primary problems on layer farms (Wales *et al*., 2007). Infections of laying hens are difficult to efficiently eliminate by vaccination and other interventions, and are especially challenged by heavy environmental burdens (Barrow, 2007). *Salmonella* contaminations in laying hens differ depending on the housing systems (Van Hoorebeke *et al*., 2011). For example, in the United States, with the public awareness of farm animal welfare issues, layers are raised on large ranches where environmental parameters are controlled (Mench, 2008). USDA-graded eggs have been washed and sanitized at processing plants. Eggs packaged for consumption are to be stored and transported under refrigeration, at an ambient air temperature not exceeding 7℃, as per the FDA Egg Safety Rule. In the European Union, the housing of laying hens in conventional battery cages was forbidden in 2012, after which only enriched cages and non-cage housing systems are allowed (Appleby, 2003). The European Union legislation prohibits the washing of class A eggs, partly because chilled, washed shell eggs tend to rot when transported over long distances (Hutchison *et al*., 2003). In China, egg production has long been led by small-to-medium-scale housing systems with battery cages. A surveillance conducted in 2013 by the National Layer Production Technology System showed that the majority of layer farms (up to 88.28%) penned 2,000 to 50,000 laying hens. Compared with the sporadic reports of poultry *Salmonella* serovars from South Africa, Egypt, and other developing countries (Barbour *et al*., 2015), *Salmonella* infections in China have been hardly investigated. In the present study, 1,512 samples taken from eight layer farms in northern China during the late summer of 2014 were tested for *Salmonella* using a PCR method. Two farms in different provinces were found to be contaminated with *Salmonella*. However, the survey revealed the contaminations to be minor, although the samples were collected during an outbreak-prone season. Previous studies have revealed that *Salmonella* contaminations may fluctuate throughout the year because of seasonal effects, and that contaminations are generally relatively higher during summer (Zdragas *et al*., 2012; Sivaramalingam *et al*., 2013).

Environmental samples reportedly provide an accurate and representative risk indicator for *Salmonella* contamination in layer flocks and eggs (Namata *et al*., 2008; Denagamage *et al*., 2015). Certain serotypes of *Salmonella* spp., in particular for *S. enteritidis*, are commonly associated with the production environment of laying hens (Murakami *et al*., 2001). In the present study, *Salmonella*-positive samples were obtained from both the henhouse and egg collection area on both contaminated farms. These contaminations may not have been newly introduced, but the effects of re-invasions of the pathogen that might have been present in the farm environment long before the investigation (Van Hoorebeke *et al*., 2010). The contaminated status may persist over successive laying periods (Dewaele *et al*., 2012b), which poses a potential threat to the farm's entire flocks and eggs, especially during the molting period when the layers' resistance against disease tends to be lower, as contaminated hens are more likely to disseminate pathogens into the environment (Golden *et al*., 2008). This study revealed that almost all environmental sample types were positive for *Salmonella*, to varying degrees. These findings may have been strongly associated with the contaminations in layer flocks. The overshoe samples in the present study were positive for *Salmonella* on both of the contaminated farms, which was consistent with the results reported by Carrique-Mas *et al*. (2009) and Dewaele *et al*. (2012a, b), suggesting that samples of overshoes and floors are promising indicators for the presence or absence of *Salmonella* in layer flocks (O'Brien, 2013; Gosling *et al*., 2014). In the present study, the *Salmonella* contamination rate of eggshells was 2.29% (11/480). Other types of pathogens may be present on the surfaces of eggshells that may threaten public health (De Buck *et al*., 2004). Undercooked or raw eggs are known to be strongly associated with *Salmonella* infections in humans (Gillespie *et al*., 2005).

Fewer contaminated spots were detected on farm H (which housed more than 50,000 laying hens) than on farm A. In general, intensive housing systems with effective environmental management can effectively control the prevalence of *Salmonella*. Meanwhile, environmental cleaning and control processes should cover the entire henhouses and egg collection areas, especially certain blind sites, in order to prevent the spreading of pathogenic bacteria. In farms A and H, some drinking nipples were contaminated with *Salmonella*. Previous studies have indicated that moist environments promote the multiplication of microflora (Rusin *et al*., 1998), and bacterial abundance on wet litter was found to more diverse than that in dry conditions (Dumas *et al*., 2011). Furthermore, the absence of dry cleaning between laying rounds reportedly worsens the *Salmonella* contamination status in conventional battery cages (Van Hoorebeke *et al*., 2010). We also detected contamination in air samples. Holt *et al*. (1998) suggested that *Salmonella enteritidis* can spread via airborne transmission when the pathogen is contained in aerosols. In addition, eggshells in the egg collection areas as well as in the henhouses were found to be contaminated with *Salmonella*. *Salmonella* is capable of growing rapidly in naturally contaminated egg content at room temperature (Humphrey and Whitehead, 1993). A similar proliferation process may occur on the surfaces of the eggshells.

The ERIC-PCR method used in this study has a higher discriminatory power than repetitive sequence-based PCR and classical phenotypic methods, such as serotyping, phage typing, pyocin typing, and biotyping (Wolska and Szweda, 2008; Waturangi *et al*., 2012). ERIC-PCR-based genotyping method is reproducible and as discriminative as pulsed field gel electrophoresis. It is fast, cheap, and requires no spe-

cialized equipment or reagents (Bishi *et al*., 2008; Kosek *et al*., 2012). In this study, two *Salmonella*-positive overshoe samples collected from one person on farm A yielded an identical gel pattern, further demonstrating the reliability and accuracy of the ERIC-PCR. The technique has been successfully applied to the typing of numerous bacterial strains (Dorneles *et al*., 2014; Silva *et al*., 2014) and to diversity analyses of bacterial communities, including *Salmonella* (Pang *et al*., 2007; Cao *et al*., 2008). In the present study, ERIC-PCR was used for tracing down the source of the contaminations. As shown in the dendrogram in Fig. 2, the three water samples collected from drinking nipples on farm H dispersed into separate branches. This suggested that the *Salmonella* contamination at these sites may not have stemmed from the water, but from other sources. Subsequent on-site investigation revealed that the waterlines had been misassembled. Thus, the ERIC-PCR results for the drinking nipples assisted the manager of farm H in adjusting the height of the waterline to solve the problem. However, different samples collected within the henhouse as well as in the egg collecting area held relatively high similarities within the strictly controlled parameter settings, as described in the Methods section, e.g., keys 28, 29, and 30 in Fig. 2. Previous studies have shown that the transfer process of eggs presents a potential risk for cross-contamination (Davies and Wray, 1994). Based on multilocus variable number of tandem repeat analysis-phage typing, Dewaele *et al*. (2012a) also suggested that cross-contamination may occur between the henhouse and egg collection areas.

In conclusion, based on *Salmonella* survey results, this study suggested that the *Salmonella* prevalence in the main egg production areas of China in 2014 was low. Some progress has been achieved in *Salmonella* control through a public-private partnership. Routine detection and subsequent adoption of effective environmental *Salmonella* control measures will contribute to the realization of the objective of preventing *Salmonella* contamination of eggs during production, storage, and transportation.

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