High genetic similarity of *Salmonella* Enteritidis as a predominant serovar by an independent survey in 3 large-scale chicken farms in China

Jian Zhang,[*,†,1] Wei Luo,[*,†,1] Guifeng Liu,[*,†] Yang Wang,[*,†] Shizhong Geng,[*,†,2] Zhiming Pan,[*,†] and Xin'an Jiao[*,†]

*Key Laboratory of Prevention and Control of Biological Hazard Factors (Animal Origin) For Agrifood Safety and Quality, Ministry of Agriculture of China, and Jiangsu Key Laboratory of Zoonosis, Yangzhou University, Yangzhou, Jiangsu 225009, China; and †Jiangsu Co-Innovation Center For Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Joint International Research Laboratory of Agriculture and Agri-product Safety of the Ministry of Education, Yangzhou University, Yangzhou, Jiangsu 225009, China

ABSTRACT *Salmonella* Enteritidis (**SE**) are important zoonotic pathogens, and can be easily transferred to humans by contaminated animal products. Epidemic surveys of SE are necessary in current modern large-scale chicken farms. In this study, *Salmonella* strains were isolated from possibly infected samples collected at 3 independent farms, and their serotype, drug resistances, virulence genes, and genetic similarity were analyzed by molecular genetic analysis technologies including multilocus sequence typing (**MLST**), clustered regularly interspaced short palindromic repeats (**CRISPR**), pulsed-field gel electrophoresis (**PFGE**), and whole-genome sequencing (**WGS**). A total of 346 *Salmonella* strains were isolated from 3,598 samples (9.61%); 329 isolates were identified as SE (95.09%) and 308 isolates were multidrug resistant (93.62%). Virulotyping based on 6 virulence genes showed high similarity in SE isolates of each farm, with the exception of 2 isolates. All SE isolates were found to be the same ST11 type by MLST, and 22 strains of 150 SE isolates selected at random were found to belong to 1 cluster by PFGE and the same SET1 type by CRISPR. WGS results further revealed that these isolates belonged to the same clonal cluster, with high genetic similarity of 99.80 to 100.00%. All these results indicated that these SE isolates were overwhelmingly dominant and demonstrated high genetic similarity, which revealed that the same SE clone might be transmitted in these farms.

Key words: *Salmonella* Enteritidis, antimicrobial susceptibility, virulotype, genetic similarity, molecular genetic analysis technology

2021 Poultry Science 100:100941
https://doi.org/10.1016/j.psj.2020.12.038

INTRODUCTION

Food safety is of high concern worldwide, with a major focus on pathogenic microbes. *Salmonella* spp. are among the most frequently isolated foodborne pathogens (Bounar-Kechih et al., 2012), ranking second among the 31 major pathogens in the United States of America (Scallan et al., 2011). To date, over 2,600 *Salmonella* serotypes have been identified, more than half of which belong to *Salmonella enterica* subsp. *enterica* (Li et al., 2018), accounting for the majority of *Salmonella* infections in humans (Ren et al., 2016). Moreover, *Salmonella* spp. are important zoonotic pathogens worldwide and are responsible for 93.8 million foodborne illnesses and 155,000 deaths per year globally (Taylor et al., 2018). In 2013, 82,694 confirmed salmonellosis cases were reported by 27 European Union member states, resulting in a notification rate of 20.4 cases per 100,000 people (Kwambana-Adams et al., 2015). As in previous years, the 2 most commonly reported *Salmonella* serovars in 2013 were *Salmonella* Enteritidis (**SE**) and *Salmonella* Typhimurium, which represented 39.5 and 20.2%, respectively, of all reported serovars associated with confirmed human cases.

Salmonella spp. can be transmitted to humans through the food chain (Angulo et al., 2004; Kilonzo-Nthenge et al., 2008; Marshall and Levy, 2011), and

© 2021 Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Received October 7, 2020.
Accepted December 15, 2020.

[1]These authors contributed equally to this work.
[2]Corresponding author: gszhong@yzu.edu.cn

these species are predominantly found in poultry, eggs, and dairy products (Silva and Aguiar, 2011; Uche et al., 2017; Velasquez et al., 2018). Specifically, chicken and by-products are a major source of *Salmonella* infection in clinical and nonclinical settings. There are serious consequences when chickens on a farm are infected by *Salmonella*. In August 2010, 0.5 billion eggs from the California-based poultry producer Foster Farms, 0.17 billion eggs from the Iowa-based poultry producer Hillandale Farms, and 0.38 billion eggs from Wright County Egg Farms were recalled. These recalls were ordered because the eggs might have been contaminated with *Salmonella*, and 1,000 cases of human salmonellosis had been caused by eggs that had been sold. In 2014, the Centers for Disease Control and Prevention reported a multistate outbreak of human *Salmonella* infection linked to live poultry in the backyard flocks in Atlanta, GA. In 2012, 300 cases of human salmonellosis were reported, and 80% of the reported ill people had contact with live poultry 1 wk before the illness began (CDC, USA). These *Salmonella* incidents caused social panic in addition to severe economic losses and human deaths.

In China, an increasing number of chickens are raised on closed large-scale modern farms, as opposed to on a small scale in backyards. These chickens and their products are currently the main sources of food supplies and will continue to be in the future, as in the United States. The safety of chickens with regard to *Salmonella* is also of high concern to the Chinese government. Thus, it is necessary to examine *Salmonella* infections on modern large-scale chicken farms. Such investigations will offer information regarding control strategies and *Salmonella* clearance for different serotypes to reduce the incidence of salmonellosis in humans, which will meet government requirements for food safety.

In this study, we investigated *Salmonella* infections on 3 modern large-scale chicken farms in different provinces of China. Based on the drug resistance of SE isolates, certain virulence genes, and their molecular characteristics, including multilocus sequence typing (**MLST**), clustered regularly interspaced short palindromic repeats (**CRISPR**), pulsed-field gel electrophoresis (**PFGE**), and whole-genome sequencing (**WGS**) assessments, we found SE to be a predominant serovar, with a very close relationship with regard to genetic evolution of SE among the 3 farms.

MATERIALS AND METHODS

Farms Selection and Samples Collection

This *Salmonella* survey was carried out on 3 modern large-scale chicken farms A, B, and C, in Shandong, Jiangsu, and Hebei provinces, respectively, with more than 10 million chickens. Suspected *Salmonella*-infected samples were continuously collected between September 2016 and July 2018 from 3 different breeder farms, once every 2 mo; samples included eggs, dead embryos, and sick and dead chickens, as well as drag samples from hatcheries according to the method described by Bailey et al. (2001). The chicken carcasses and samples were stored at 4°C and transported to the laboratory within 48 h in an insulated ice chest containing ice packs. Dead embryos were transferred to the laboratory within 24 h. Microbial analysis was performed immediately upon arrival of samples in the laboratory.

Salmonella Isolation and Serotype Identification

Liver and yolk sac samples were aseptically collected from the sick and dead chickens and dead embryos, respectively. Approximately 10 g liver tissue or yolk sac was suspended in 100 mL buffered peptone water and incubated at 37°C for 16 to 20 h; 1-mL aliquots of these pre-enriched cultures were inoculated into 10 mL Rappaport-Vassiliadis enrichment broth (BD Difco, Sparks, MD) and continuously cultured at 42°C for 24 h. The broth cultures were streaked onto xylose lysine Tergitol 4 (BD Difco) agar plates, which were incubated at 37°C for 24 h. Isolated strains exhibiting a typical *Salmonella* phenotype were selected, and their biochemical characteristics were confirmed using an API 20E test kit (bioMerieux, Marcy I'Etoile, France). *Salmonella* isolates were serotyped by slide agglutination for O and H antigens using commercially available antisera (Tianrun Bio-Pharmaceutical, Ningbo, China) according to the manufacturer's instructions.

Table 1. Primers for virulence gene.

Gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Amplicon (bp)	Reference
prgH	F: GCCCGAGCAGCCTGAGAAGTTAGAAA R: TGAAATGAGCGCCCCTTGAGCCAGTC	55	755	Manning et al., (2015)
sopB	F: GAAGACTACCAGGCGCACTT R: TTGTGGATGTCCACGGTGAG	55	804	Gole et al., (2017)
spiC	F: CCTGGATAATGACTATTGAT R: AGTTTATGGTGATTGCGTAT	56	300	Manning et al., (2015)
orfL	F: GGAGTATCGATAAAGATGTT R: GCGCGTAACGTCAGAATCAA	56	331	Manning et al., (2015)
pefA	F: GCGCCGCTCAGCCGAACCAG R: CAGCAGAAGCCCAGGAAACAGTG	58	154	Manning et al., (2015)
spvC	F: TCTCTGCATTTCGCCACCAT R: TGCACAACCAAATGCGGAAG	58	563	Manning et al., (2015)

Table 2. Amplification and sequencing primers of *Salmonella* for MLST.

Locus	Sequence of primers (5′–3′)		Size of products (bp)
	Amplification primers	Sequencing primers	
aroC	F: CCTGGCACCTCGCGCTATAC R: CCACACACGGATCGTGGCG	F: GGCACCAGTATTGGCCTGCT R: CATATGCGCCACAATGTGTTG	826
dnaN	F: ATGAAATTTACCGTTGAACGTGA R: AATTTCTCATTCGAGAGGATTGC	F: CCGATTCTCGGTAACCTGCT R: CCATCCACCAGCTTCGAGGT	833
hemD	F: ATGAGTATTCTGATCACCCG R: ATCAGCGACCTTAATATCTTGCCA	F: GTGGCCTGGAGTTTTCCACT R: GACCAATAGCCGACAGCGTAG	666
hisD	F: GAAACGTTCCATTCCGCGCAGAC R: CTGAACGGTCATCCGTTTCTG	F: GTCGGTCTGTATATTCCCGG R: GGTAATCGCATCCACCAAATC	894
purE	F: ATGTCTTCCCGCAATAATCC R: TCATAGCGTCCCCCGCGGATC	F: CGCATTATTCCGGCGCGTGT R: CGCGGATCGGGATTTTCCAG	510
sucA	F: AGCACCGAAGAGAAACGCTG R: GGTTGTTGATAACGATACGTAC	F: AGCACCGAAGAGAAACGCTG R: GGTTGTTGATAACGATACGTAC	643
thrA	F: GTCACGGTGATCGATCCGGT R: CACGATATTGATATTAGCCCG	F: ATCCCGGCCGATCACATGAT R: CTCCAGCAGCCCCTCTTTCAG	852

Abbreviation: MLST, multilocus sequence typing.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility test was performed according to the guidelines of the Clinical and Laboratory Standards Institute (Liljebjelke et al., 2017). Agar diffusion assays were performed using Mueller-Hinton agar with disks containing 7 classes of antimicrobial agents (Brain Heart Infusion, Oxoid, Basingstoke, UK): ampicillin, 10 μg; amoxicillin/clavulanic acid, 20/10 μg; cefazolin, 30 μg; meropenem, 10 μg; aztreonam, 30 μg; kanamycin, 30 μg; gentamicin, 10 μg; streptomycin, 10 μg; amikacin, 30 μg; ciprofloxacin, 5 μg; enrofloxacin, 5 μg; nalidixic acid, 30 μg; trimethoprim/sulfamethoxazole, 1.25/23 μg; chloramphenicol, 30 μg; and nitrofurantoin, 300 μg. All *Salmonella* isolates were tested for susceptibility to each of these antibiotics.

Virulotyping Based on Selected Virulence Genes

Salmonella genomic DNA was extracted according to the QIAamp DNA Mini kit protocol (Qiagen GmbH, Hilden, Germany). The extracted genomic DNA was stored at 70°C for later use.

PCR was conducted in individual reactions using primers targeting the following genes: *prgH*, *sopB*, *spiC*, *orfL*, *pefA*, and *spvC* (Manning et al., 2015; Gole et al., 2017). The specific primer sequences are shown in Table 1. Fifty SE isolates randomly selected from each farm were tested.

Molecular Genetic Analysis by Multi-Technologies

MLST Seven housekeeping genes, *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA* (primers are shown in Table 2), recommended by the University College Cork (http://mst.ucc.ie) were assessed in the MLST assay (Sukhnanand et al., 2005). The amplified PCR products were sent to GenScript (Nanjing) Co., Ltd. for sequencing. The sequencing results were evaluated using MEGA v7.0 (Kumar et al., 2016), and the corresponding minimum spanning tree diagram was generated using BioNumerics v7.5 (Applied Maths NV, Sint-Martens-Latem, Belgium).
CRISPR Primers for CRISPR are listed in Table 3 (Fei et al., 2017). The PCR products were sent to GenScript (Nanjing) Co., Ltd. for sequencing. The sequencing results were uploaded to a website (http://crispr.i2bc.paris-saclay.fr/Server/) for processing to determine the spacer arrangement of SE and to export the files in binary form. A corresponding color map was completed.
PFGE PFGE was conducted as a part of routine surveillance. PFGE for *Salmonella* strains was performed according to the PulseNet protocol using *Xba* I as the restriction enzyme (New England Biolabs, Leusden, The Netherlands). Cluster analysis was performed with BioNumerics 5.1 using the Dice similarity coefficient and the unweighted pair group method using average linkages dendrogram type (optimization 0.5%, position tolerance 1.5%).
WGS Genomic DNA of the selected *Salmonella* isolates was extracted using the QIAamp DNA Mini kit (Qiagen

Table 3. Amplification and sequencing primers of *Salmonella* for CRISPR.

Locus	Primer sequence (5′–3′)	Size of products (bp)
CRISPR1	F: GCTGGTGAAACGTGTTTATCC R: ATTCCGGTAGATYTKGATGGAC	1,000–2,000
CRISPR2	F: AACGCCATGGCCTCCTCCTG R: CAAAATCAGYAAATTAGCTGCTGTTC	1,000–2,000

Abbreviation: CRISPR, clustered regularly interspaced short palindromic repeats.

Table 4. *Salmonella* isolation from different samples in 3 farms.

Samples	*Salmonella* positive/samples (% ratio)												Total
	A (Shandong)												
Eggs	0/17	2/26 (7.7)	1/23 (4.3)	3/32 (9.4)	1/25 (4)	1/29 (3.4)	2/24 (8.3)	0/19	2/21 (9.5)	0/25	4/25 (16)	0/21	16/287 (5.6)
Dead embryos	11/34 (32.4)	9/30 (30)	11/37 (29.7)	7/27 (25.9)	7/29 (24.1)	7/25 (28)	10/33 (30.3)	11/35 (31.4)	9/32 (28.1)	8/29 (27.6)	7/23 (30.4)	11/36 (30.6)	108/370 (29.2)
Sick/dead chickens	-	0/1	1/1 (100)	-	1/3 (33.3)	0/2	-	1/3 (33.3)	1/2 (50)	-	1/2 (50)	0/1	5/15 (33.3)
Drag samples	2/28 (7.1)	2/37 (5.4)	1/35 (2.9)	0/35	5/39 (12.8)	2/36 (5.6)	2/33 (6.1)	0/29	3/35 (8.6)	2/32 (6.3)	2/34 (5.9)	1/32 (3.1)	22/405 (5.4)
Total	151/1,077 (14.02)												
	B (Jiangsu)												
Eggs	2/40 (5)	0/43	3/52 (5.8)	0/36	1/39 (2.6)	2/41 (4.9)	1/43 (2.3)	0/33	2/40 (5)	0/39	2/42 (4.8)	1/36 (2.8)	14/484 (2.9)
Dead embryos	5/28 (17.9)	6/31 (19.4)	4/23 (17.4)	4/25 (16)	3/22 (13.6)	4/27 (14.8)	6/28 (21.4)	4/25 (16)	3/23 (13.0)	3/25 (12)	4/22 (18.2)	3/23 (13.0)	49/302 (16.2)
Sick/dead chickens	0/2	-	-	0/2	1/1 (100)	0/1	-	0/1	-	1/2 (50)	0/2	0/1	2/12 (16.7)
Drag samples	0/37	0/41	0/40	1/35 (2.9)	1/42 (2.4)	0/41	2/43 (4.7)	1/36 (2.8)	0/42	1/40 (2.5)	1/37 (2.7)	0/39	7/473 (1.5)
Total							72/1,271 (5.66)						
	C (Hebei)												
Eggs	2/41 (4.9)	0/37	3/30 (10)	3/43 (7.0)	4/40 (10)	2/38 (5.3)	3/42 (7.1)	1/40 (2.5)	4/39 (10.3)	2/43 (4.7)	1/36 (2.8)	3/39 (7.7)	28/468 (6.0)
Dead embryos	6/21 (28.6)	8/24 (33.3)	5/22 (22.7)	8/26 (30.8)	6/26 (23.1)	6/20 (30)	6/23 (26.1)	5/21 (23.8)	7/25 (28)	6/23 (26.1)	5/22 (22.7)	5/24 (20.1)	73/277 (26.4)
Sick/dead chickens	-	-	0/1	-	0/2	-	1/2 (50)	1/1 (100)	0/3	2/2 (100)	0/2	0/3	4/16 (25)
Drag samples	2/42 (4.8)	0/43	0/39	4/36 (11.1)	2/42 (4.8)	3/46 (6.5)	1/41 (2.4)	0/35	0/45	4/40 (10)	0/41	2/39 (5.1)	18/489 (3.7)
Total							123/1,250 (9.84)						

Table 5. Classification of *Salmonella* serovars in 3 farms.

Farm	No. of samples	No. of *Salmonella* isolates (%)	No. of *Salmonella* isolates (%)				
			SE	ST	SIND	SINF	SD
A (Shandong)	1,077	151 (14.02)	144 (95.36)	6 (3.97)	1 (0.67)		
B (Jiangsu)	1,271	72 (5.66)	70 (97.22)	2 (2.78)			
C (Hebei)	1,250	123 (9.84)	115 (93.50)	4 (3.25)		2 (1.62)	2 (1.62)
Total	3,598	346 (9.61)	329 (95.09)	12 (3.46)	1 (0.29)	2 (0.58)	2 (0.58)

Abbreviations: SD, *Salmonella* Derby; SE, *Salmonella* Enteritidis; SIND, *Salmonella* Indiana; SINF, *Salmonella* Infantis; ST, *Salmonella* Typhimurium.

GmbH), and WGS was performed by Novogene Co. Ltd., Beijing, China. The genomic DNA sequencing results were analyzed using the Illumina HiSeq platform to generate 150-bp parallel (Almeida et al., 2018) reads with a data volume of approximately 150 Gb/strain clean reads (Thermo Fisher Scientific, Waltham, MA). Gene fragments were assembled using SPAdes v3.11 (Illumina, Inc., San Diego, CA) with the parameter para: –careful–cov cutoff auto. Genomic similarity analysis was performed on the assembly results using the Mash assay (Lindsay et al., 2018).

RESULTS

High Prevalence of SE as a Predominant Serovar

The prevalence of *Salmonella* on the breeder farms is summarized in Table 4. Overall, the *Salmonella* isolation rate of farm A (Shandong) was the highest (14.02%), followed by farm C (Hebei) (9.84%), and farm B (Jiangsu) scored the lowest (5.66%). A total of 346 *Salmonella* isolates were recovered from 3,598 samples (9.61%). These *Salmonella* isolates showed low diversity, with 95.09% being identified as SE (Table 5).

Little Difference in Antimicrobial Susceptibility

The antimicrobial susceptibilities or drug resistances observed are summarized in Table 6. A total of 329 SE isolates were resistant to at least 1 antimicrobial agent, and 308 SE isolates exhibited multidrug resistance (93.62%). The highest resistance rates recorded corresponded to nalidixic acid (96.35%), ampicillin (57.75%), and streptomycin (53.19%). There was little difference in the drug resistance of SE isolates among the 3 breeder farms.

High Similarity in Virulotype

Salmonella virulence was assessed by PCR using 50 SE isolates randomly selected from each farm. All SE isolates tested positive for the *pefA* and *spvC* genes (Table 7). Only 2 isolates differed: 1 SE isolate from the Shandong A farm carried only *orfL*, *spiC*, *pefA*, and *spvC* genes without *prgH* and *sopB* genes; and 1 SE isolate from the Jiangsu B farm harbored only *pefA* and *spvC* genes without *prgH*, *sopB*, *orfL*, and *spiC* genes. The rate of virulence gene positivity among the remaining SE isolates tested was 100%.

Table 6. Antibiotic susceptibility among 329 SE isolates in 3 breeder farms.

Drugs		No. of drug-resistant strains (%)			
		A $_{n=144}$	B $_{n=70}$	C $_{n=115}$	Total $_{n=329}$
β-Lactams	AMP	93 (64.58)	52 (74.29)	45 (39.13)	190 (57.75)
	AMC	68 (47.22)	14 (20.00)	1 (0.87)	83 (25.23)
	CZO	15 (10.42)	4 (5.71)	8 (6.96)	27 (8.21)
	MEM	3 (2.08)	3 (4.29)	0 (0)	6 (1.82)
	ATM	14 (9.72)	3 (4.29)	7 (6.09)	24 (7.29)
Aminoglycosides	KAN	0 (0)	4 (5.71)	6 (5.22)	10 (3.04)
	GEN	0 (0)	4 (5.71)	1 (0.87)	5 (1.52)
	STR	92 (63.89)	50 (71.43)	33 (28.70)	175 (53.19)
	AMK	0 (0)	4 (5.71)	0 (0)	4 (1.22)
Quinolones	CIP	15 (10.42)	57 (81.43)	3 (2.61)	75 (22.80)
	ENR	66 (45.83)	33 (47.14)	3 (2.61)	102 (31.00)
	NAL	143 (99.31)	69 (98.57)	105 (91.30)	317 (96.35)
Sulfonamides	SXT	3 (2.08)	4 (5.71)	3 (2.61)	10 (3.04)
Chloramphenicols	CHL	1 (0.69)	5 (7.14)	13 (11.30)	19 (5.78)
Nitrofurantoin	NIT	134 (93.06)	12 (17.14)	2 (1.74)	148 (44.98)

Abbreviations: AMC, amoxicillin/clavulanic acid; AMK, amikacin; AMP, ampicillin; ATM, aztreonam; CHL, chloramphenicol; CIP, ciprofloxacin; CZO, cefazolin; ENR, enrofloxacin; GEN, gentamicin; KAN, kanamycin; MEM, meropenem; NAL, nalidixic acid; NIT, nitrofurantoin; SE, *Salmonella* Enteritidis; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole.

Table 7. PCR results for the presence of virulence genes in SE isolates in 3 breeder farms.

Farm	PCR results	No. of resistant strains
A (Shandong)	*prgH-sopB-orfL-spiC-pefA-spvC*	49
	orfL-spiC-pefA-spvC	1
B (Jiangsu)	*prgH-sopB-orfL-spiC-pefA-spvC*	49
	pefA-spvC	1
C (Hebei)	*prgH-sopB-orfL-spiC-pefA-spvC*	50

Abbreviation: SE, *Salmonella* Enteritidis.

High Genetic Similarity by Multi-Technologies

MLST MLST was performed on the *Salmonella* isolates, and the sequencing results showed 95.09% (329 out of 346) of these *Salmonella* isolates to be ST11, indicating that they are SE. MLST results for the SE isolates showed that the nucleotide sequence ranges of the *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA* sites were completely identical: 5, 2, 3, 7, 6, 6, and 11, respectively (Figure 1).

CRISPR All SE isolates from the 3 breeder farms were subjected to CRISPR typing (Fei et al., 2017). Specific results with the same SET1 CRISPR type are presented in Figure 2. CRISPR1-Spacer had 8 spacers, including Ent1, Ent2, Ent3, Ent4, Ent5, Ent6, Ent7, and Ent8. The CRISPR2 segments had 10 spacers, including EntB0 and EntB1, EntB2, EntB3, EntB4, EntB5, EntB6, EntB7, EntB8, and EntB9. The spacer composition and arrangement of CRISPR types revealed that all the tested SE isolates from the 3 breeder farms were of the SET1 type (Figure 3), which was the same as the CRISPR type of SE P125109 (submission number: AM933172) in the National Center for Biotechnology Information library.

PFGE The same 22 SE isolates from the 3 breeder farms were subjected to PFGE subtyping. The Dice coefficient was greater than 0.85 for the same cluster, and *Salmonella* in the same cluster was considered homologous. A homology of 100% was considered to represent the same PFGE subtype, and the 22 SE strains were divided into 1 cluster and 8 PFGE subtypes (Figure 4). The similarity of the bands was high, indicating that these SE isolates on the 3 chicken farms had very similar genetic relationships, though a few bands differed significantly.

WGS Genomic DNA sequencing results showed that the similarity among the 22 representative SE isolates from the 3 breeder farms was 99.80 to 100.00% (Figure 5); these isolates also belonged to the same

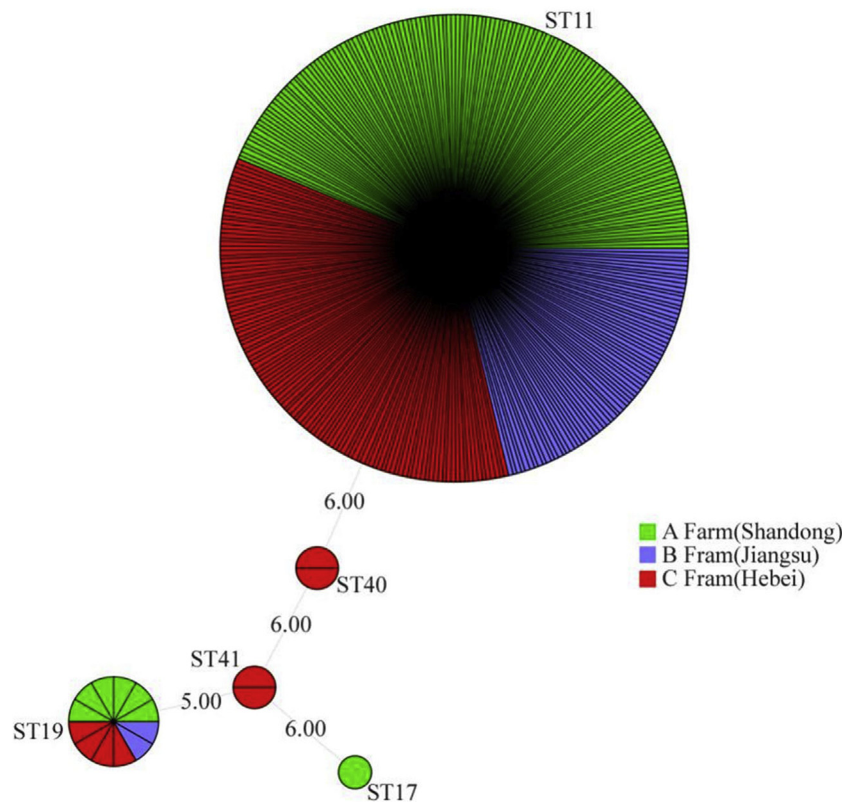

Figure 1. Three hundred and twenty nine of 346 isolates from 3 breeder farms were ST11-type (SE) based on MLST. Abbreviations: MLST, multilocus sequence typing; SE, *Salmonella* Enteritidis.

SE	CRISPR cluster																	
	CRISPR1-Spacer								CRISPR2-Spacer									
	Ent1	Ent2	Ent3	Ent4	Ent5	Ent6	Ent7	Ent8	EntB0	EntB1	EntB2	EntB3	EntB4	EntB5	EntB6	EntB7	EntB8	EntB9
P125109	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
SET1(n=22)	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

Note: "o" is positive

Figure 2. CRISPR typing dendrogram of SE isolates in 3 breeder farms.

clonal cluster. WGS analysis further indicated the possibility that the same SE strain was pandemic on the 3 breeder farms.

DISCUSSION

Salmonella infection remains a major public health concern worldwide (Flockhart et al., 2017), contributing to the economic burden of both industrialized and underdeveloped countries due to the costs associated with surveillance, prevention, and treatment of salmonellosis (Uche et al., 2017). In China, an increasing number of chickens are raised on closed large-scale modern farms and not on a small scale in backyards. These chickens and their by-products are major sources of food and will continue to be a major source in the future, as in the western developed countries (Besser, 2018).

In the past, chickens were raised on a small scale in the backyard, and many different serotypes of *Salmonella* were isolated, with rich diversity, including *Salmonella* Pullorum, *Salmonella* Gallinarum, SE, and *Salmonella* Typhimurium (Ford et al., 2018). Although SE can also infect chickens, the birds show no clinical symptoms, and few die under normal feeding conditions. Thus, SE has been easily neglected. In addition, *Salmonella* has a placeholder effect: once SE becomes established on a farm, it diffuses via horizontal and vertical transmission. Vertical transmission of SE can lead to infection of eggs and offspring to cause generational magnification, persisting on a farm for long time (Taylor et al., 2018). In this survey, *Salmonella* strains were isolated from many eggs, dead embryos, and sick and dead chickens (Bailey et al., 2001; Fei et al., 2017), though we cannot conclude which *Salmonella* strain was the main factor causing death among embryos and

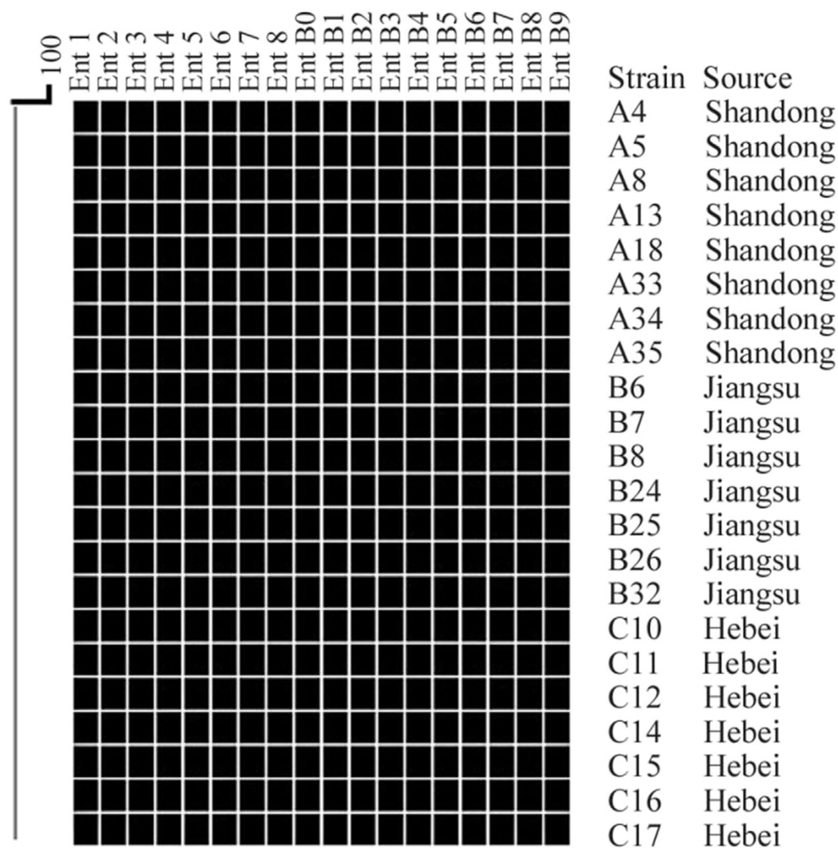

Figure 3. Twenty two representative SE isolates were SET1-type based on CRISPR typing. Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; SE, *Salmonella* Enteritidis.

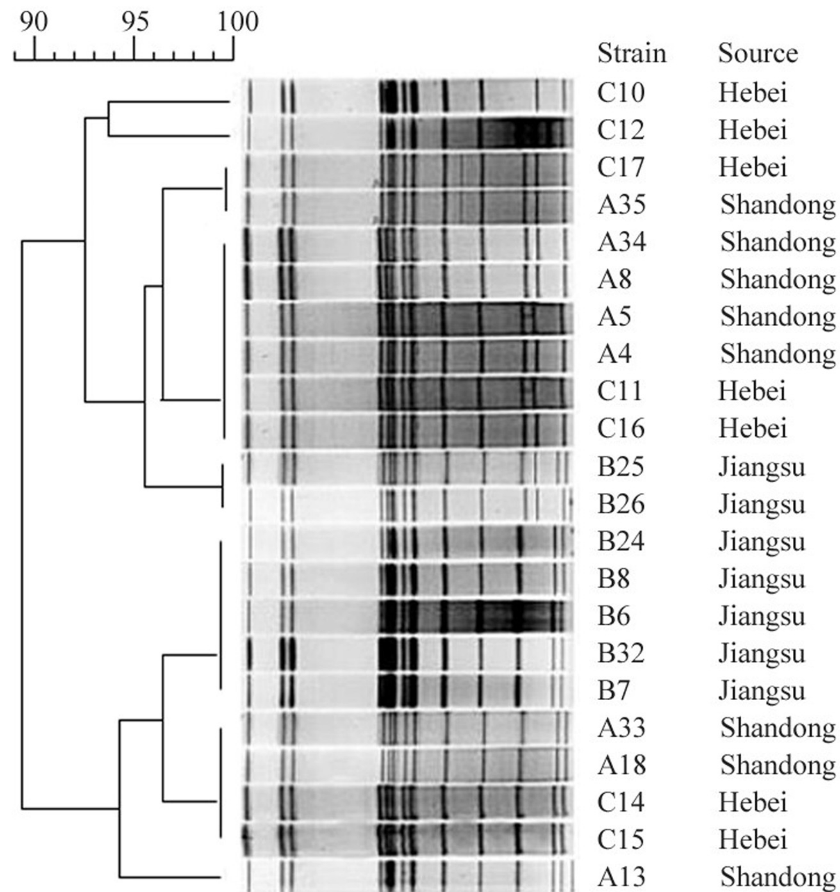

Figure 4. Twenty two representative SE isolates belonged to 1 same cluster based on PFGE. Abbreviations: PFGE, pulsed-field gel electrophoresis; SE, *Salmonella* Enteritidis.

chickens. As SE in chickens can exclude other *Salmonella* serotypes, SE can readily become the predominant serotype.

In this survey, *Salmonella* isolates exhibited low diversity, and SE was the predominant serotype on modern large-scale chicken farms in China, consistent with reports

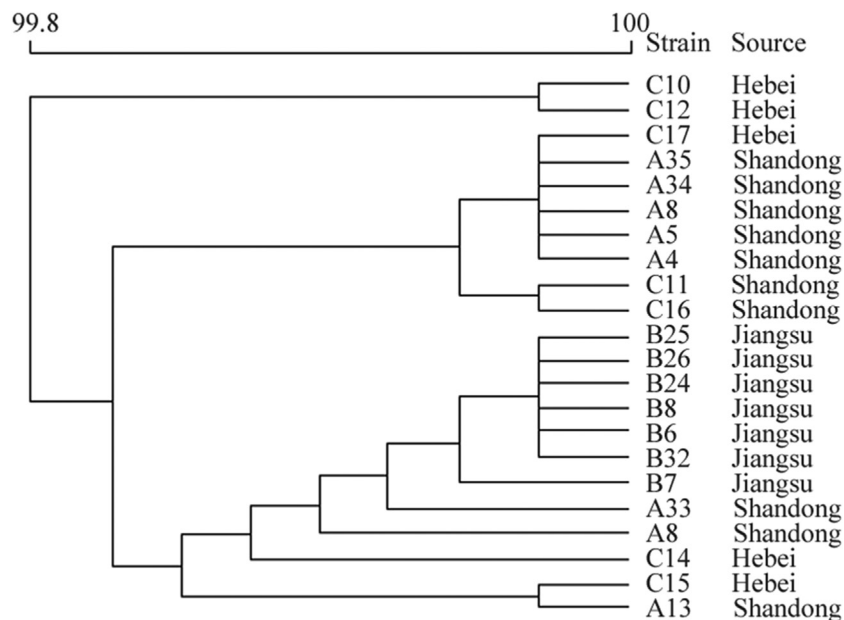

Figure 5. Twenty two representative SE isolates belonged to 1 same clonal cluster based on WGS. Abbreviations: SE, *Salmonella* Enteritidis; WGS, whole-genome sequencing.

from other countries (Silva et al., 2011; Uche et al., 2017). Additionally, a very close genetic relationship among the SE isolates from the 3 breeder farms in China was observed, and the same pandemic SE strain might be present on these farms. We sought to address why SE is prevalent on different remote poultry farms and has a very close genetic relationship, though it might be the same pandemic strain. This transition may be due to the introduction by breeders, which had been confirmed that SE was able to be transmitted following the chick supply chain (Fei et al., 2017). Some modern large-scale chicken farms have no self-cultivated ancestral chickens, with their chickens obtained from different domestic ancestral chicken farms. Conversely, some modern large-scale chicken farms have their own ancestral chickens, which were imported directly from farms abroad; nonetheless, it cannot be guaranteed in a commercial contract that these ancestral chickens were not infected with SE. According to many reports (Eriksson et al., 2018), *Salmonella* Pullorum has been purged from chicken farms in developed western countries, whereas SE has not been completely purged. Many of the breeder farms involved in this experiment were recorded as importing chickens from abroad. It is possible that *Salmonella* was introduced when chickens were imported, which may account for this type of SE. Regardless, there is a lack of sufficient evidence for this hypothesis that this large cluster of SE derives from the same source as the common SE in exporting countries, and further investigation is needed.

Four technologies, including MLST, CRISPR, PFGE, and WGS, were employed for genetic analysis, with the results of MLST and CRISPR being similar and those of PFGE and WGS showing high similarity. PFGE indicated that the representative SE isolates assessed belonged to 1 cluster, with a Dice coefficient greater than 0.85. WGS also showed that the isolates belonged to the same clonal cluster, with similarity of 99.80 to 100.00%. These 4 methods indicated that genetic differences among these SE isolates were very small, and the fact that the isolates have a close relationship suggests the same pandemic strain in these farms. If this is true, SE will become a main target during *Salmonella* clearance. Many targeted measures, such as a novel vaccine, can be used to control this type of SE, which is the main serotype in these farms.

However, antimicrobial susceptibility testing revealed little differences in drug resistance among these *Salmonella* isolates in different breeder farms. It is possible that the same bacterium can undergo changes in drug resistance to adapt to different environments and antibiotic pressures.

The severe problem of SE in modern large-scale chicken farms is not restricted to China; it is in fact a worldwide issue, and it is currently difficult to control this type of SE in these farms. However, researchers in China and other countries continue to study novel methods to protect poultry and livestock against SE. The high genetic similarity of SE as a predominant serotype in large-scale chicken farms in China will offer a new targeted strain to design vaccines.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program Special Project (2016YFD0501607), the Special Fund for Agro-scientific Research in the Public Interest (201403054), the National Natural Science Foundation of China (31320103907), and Open Project of State Key Laboratory of Genetically Engineered Veterinary Vaccines (AGVSKL-ZY-201803).

DISCLOSURES

No conflict of interest exists among authors during manuscript submission and publishing.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.12.038.

REFERENCES

Angulo, J. A., N. Angulo, and J. Yu. 2004. Antagonists of the neurokinin-1 or dopamine D1 receptors confer protection from methamphetamine on dopamine terminals of the mouse striatum. Ann. Ny. Acad. Sci. 1025:171–180.

Almeida, F., A. A. Seribelli, M. I. C. Medeiros, D. D. P. Rodrigues, A. D. MelloVarani, Y. Luo, M. W. Allard, and J. P. Falcão. 2018. Phylogenetic and antimicrobial resistance gene analysis of *Salmonella* Typhimurium strains isolated in Brazil by whole genome sequencing. PLoS One 13:e0201882.

Bounar-Kechih, S., T. M. Hamdi, L. Mezali, F. Assaous, and K. Rahal. 2012. Antimicrobial resistance of 100 Salmonella strains isolated from Gallus gallus in 4 wilayas of Algeria. Poult. Sci. 91:1179–1185.

Bailey, J. S., N. J. Stern, P. Fedorka-Cray, S. E. Craven, N. A. Cox, D. E. Cosby, S. Ladely, and M. T. Musgrove. 2001. Sources and movement of Salmonella through integrated poultry operations: a multistate epidemiological investigation. J. Food Prot. 64:1690–1697.

Besser, J. M. 2018. *Salmonella* epidemiology: a whirlwind of change. Food Microbiol. 71:55–59.

Eriksson, H., R. Soderlund, L. Ernholm, L. Melin, and D. S. Jansson. 2018. Diagnostics, epidemiological observations and genomic subtyping in an outbreak of pullorum disease in non-commercial chickens. Vet. Microbiol. 217:47–52.

Fei, X., X. He, R. Guo, C. Yin, H. Geng, K. Wu, K. Yin, S. Geng, Z. Pan, Q. Li, and X. Jiao. 2017. Analysis of prevalence and CRISPR typing reveals persistent antimicrobial-resistant *Salmonella* infection across chicken breeder farm production stages. Food Control 77:102–109.

Flockhart, L., K. Pintar, A. Cook, S. McEwen, R. Friendship, D. Kelton, and F. Pollari. 2017. Distribution of *Salmonella* in humans, production animal operations and a watershed in a foodnet Canada sentinel site. Zoonoses. Public Hlth. 64:41–52.

Ford, L., C. R. M. Moffatt, E. Fearnley, M. Miller, J. Gregory, T. S. Sloan-Gardner, B. G. Polkinghorne, R. Bell, N. Franklin, D. A. Williamson, K. Glass, and M. D. Kirk. 2018. The epidemiology of *Salmonella enterica* outbreaks in Australia, 2001–2016. Front. Sustain. Food Syst. 2:86.

Gole, V. C., R. Woodhouse, C. Caraguel, T. Moyle, J. L. Rault, M. Sexton, and K. Chousalkar. 2017. Dynamics of *Salmonella* shedding and welfare of hens in free-range egg production systems. Appl. Environ. Microbiol. 83:3313–3316.

Kwambana-Adams, B., S. Darboe, H. Nabwera, E. Foster-Nyarko, U. N. Ikumapayi, O. Secka, M. Betts, R. Bradbury, R. Wegmüller,

and B. Lawal. 2015. *Salmonella* infections in the Gambia, 2005-2015. Clin. Infect. Dis. 61:S354–S362.

Kilonzo-Nthenge, A., S. N. Nahashon, F. Chen, and N. Adefope. 2008. Prevalence and antimicrobial resistance of pathogenic bacteria in chicken and Guinea fowl. Poult. Sci. 87:1841–1848.

Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 33:1870–1874.

Li, Q., X. Wang, K. Yin, Y. Hu, H. Xu, X. Xie, L. Xu, X. Fei, X. Chen, and X. Jiao. 2018. Genetic analysis and CRISPR typing of *Salmonella enterica* serovar Enteritidis from different sources revealed potential transmission from poultry and pig to human. Int. J. Food Microbiol. 266:119–125.

Liljebjelke, K. A., C. L. Hofacre, D. G. White, S. Ayers, M. D. Lee, and J. J. Maurer. 2017. Diversity of antimicrobial resistance phenotypes in *Salmonella* isolated from commercial poultry farms. Front. Vet. Sci. 4:96.

Lindsay, C., J. Flint, K. Lilly, K. Hope, and D. N. Durrheim. 2018. Retrospective use of whole genome sequencing to better understand an outbreak of *Salmonella enterica* serovar Mbandaka in New South Wales, Australia. Western. Pac. Surveill. Response J. 9:20–25.

Marshall, B. M., and S. B. Levy. 2011. Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24:718–733.

Manning, J., V. Gole, and K. Chousalkar. 2015. Screening for *Salmonella* in backyard chickens. Prev. Vet. Med. 120:241–245.

Ren, X., M. Li, C. Xu, K. Cui, Z. Feng, Y. Fu, J. Zhang, and M. Liao. 2016. Prevalence and molecular characterization of *Salmonella enterica* isolates throughout an integrated broiler supply chain in China. Epidemiol. Infect. 144:2989–2999.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. J. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States–major pathogens. Emerg. Infect. Dis. 17:7–15.

Silva, A. I., and H. G. Aguiar. 2011. Diversification in the first year of food life. Acta Med. Port. 24:1035–1040.

Sukhnanand, S., S. Alcaine, L. D. Warnick, W. Su, J. Hof, M. P. J. Craver, P. McDonough, K. J. Boor, and M. Wiedmann. 2005. DNA sequence-based subtyping and evolutionary analysis of selected *Salmonella enterica* serotypes. J. Clin. Microbiol. 43:3688–3698.

Silva, J., D. Leite, M. Fernandes, C. Mena, P. A. Gibbs, and P. Teixeira. 2011. *Campylobacter spp.* as a foodborne pathogen: a review. Front. Microbiol. 2:200.

Taylor, M., W. Cox, M. Otterstatter, N. de. With, and E. Galanis. 2018. Evaluation of agricultural interventions on human and poultry-related *Salmonella* Enteritidis in British columbia. Foodborne. Pathog. Dis. 15:39–43.

Uche, I. V., C. A. MacLennan, and A. Saul. 2017. A systematic review of the incidence, risk factors and case fatality rates of invasive nontyphoidal *Salmonella* (iNTS) disease in Africa (1966 to 2014). PLoS Neglect. Trop. D. 11:e0005118.

Velasquez, C. G., K. S. Macklin, S. Kumar, M. Bailey, P. E. Ebner, H. F. Oliver, F. S. Martin-Gonzalez, and M. Singh. 2018. Prevalence and antimicrobial resistance patterns of *Salmonella* isolated from poultry farms in southeastern United States. Poult. Sci. 97:2144–2152.