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Antimicrobial susceptibility, virulence gene and pulsed-field gel electrophoresis profiles of *Salmonella enterica* serovar Typhimurium recovered from retail raw chickens, China

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ABSTRACT

A total of 138 *Salmonella enterica* serovar Typhimurium isolates recovered from retail raw chickens in seven provinces of China from 2007 to 2012 were characterized for antimicrobial susceptibility, genotype and presence of virulence genes. Resistance was most frequently detected to sulfisoxazole (90.6%), tetracycline (73.2%), nalidixic acid (65.9%), ceftiofur (59.4%), ampicillin (54.3%) and kanamycin (34.8%). Resistance to cefoxitin (6.5%), amikacin (5.8%) and ceftriaxone (3.6%) was less frequently detected. One hundred and thirty seven (99.3%) isolates were resistant to at least one antibiotic, and 82 (59.4%) to more than five antibiotics. Pulsed-field gel electrophoresis (PFGE) subtyping generated 66 PFGE patterns and 12 clusters. Isolates with similar PFGE patterns in the same cluster were all resistant to similar categories and numbers of antibiotics and harbored similar virulence genes. Among 30 virulence genes we screened for, the top five genes were *pag*K (82.6%), *sod*C1 (66.7%), *sii*E (58.7%), *pef*A (58.7%) and *mar*T (50.7%). All isolates were identified harboring at least one virulence gene, 80 (58.0%) and 16 (11.6%) were simultaneously positive for at least nine and seventeen virulence genes, respectively. Twenty four (17.4%) isolates harbored at least ten virulence genes and exhibited resistance to at least ten antibiotics. Our results revealed that *S.* Typhimurium isolates in retail raw chickens were not only genotype multiform, but majority of them were multidrug resistant and co-carried multiple virulence genes, which may pose great potential hazard to public health.

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1. Introduction

Salmonellosis continues to be a major public health problem worldwide. It is estimated that 9,380,000 human infections and 155,000 deaths are caused by *Salmonella* worldwide annually (Majowicz et al., 2010). In the United States, more than 1.03 million illnesses, 19,500 hospitalizations and 378 deaths are caused by *Salmonella* annually (Scallan et al., 2011). In China, approximately 70%–80% of foodborne bacterial outbreaks were caused by *Salmonella* (Wang, Zheng, & Wang, 2007). Contaminated foods, especially retail chickens, are common vehicles for *Salmonella* transmission and infection.

Although more than 2610 *Salmonella* serovars have been identified, human infections have been caused by a limited number of serotypes with Typhimurium being one of the most common ones (Mezal, Stefanova, & Khan, 2013; Zhao et al., 2006). Moreover, the emergence of multidrug-resistant (MDR) *Salmonella*, particularly serovar Typhimurium, has imposed enormous challenges to the medical communities to treat infections (Carlet et al., 2012; Threlfall, Ward, Frost, & Willshaw, 2000). Additionally, the presence of virulence genes that commonly carried by plasmids, prophages and *Salmonella* pathogenicity islands (SPIs) that can transfer among these bacteria represents another potential human health concern. A number of severe *Salmonella* infections associated with these virulent elements were reported (Darwin & Miller, 1999; Litrup et al., 2010a; Tamang et al., 2014). Although many studies have revealed the presence of foodborne *S.* Typhimurium in chicken meats in China, the virulence genes carried by this pathogen were seldom investigated. The objective of this study was to

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determine the antimicrobial susceptibility, PFGE profiles and presence virulence genes in 138 *S.* Typhimurium isolates recovered from retail raw chickens in seven provinces in China.

2. Material and methods

2.1. Bacteria strains

A total of 138 *S.* Typhimurium isolates recovered from retail chicken samples were used in this study. Detailed information on sample collection, *Salmonella* isolation and identification was described elsewhere (Yang et al., 2010, 2011). The distribution of the 138 *S.* Typhimurium isolates by location (i.e., province), retail market type (i.e., wet market and supermarket), and retail market storage temperature at time of collection (frozen, chilled, and ambient) is shown in Table 1. *Salmonella* isolates were serotyped at Henan Center for Disease Control and Prevention (Zhengzhou, Henan, China). *Salmonella* O and H antigens were characterized using slide agglutination with hyperimmune sera (S&A Company, Bangkok, Thailand). The serotype was assigned following the Kauffmann-White scheme and manufacturer's instructions. Isolates were stored in Luria-Bertani/glycerol (V/V, 50%/50%) (LB; Difco, Cockeysville, MD) at −80 °C until use.

2.2. Antimicrobial susceptibility test

All isolates were tested for susceptibility to 15 antibiotics via agar dilution method using Mueller-Hinton agar (Beijing Land Bridge Technology C., Ltd, Beijing, China) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2013). The antibiotics were selected according to those used for susceptibility testing of *Salmonella* and *Escherichia coli* in the National Antimicrobial Resistance Monitoring System (NARMS) managed by the U.S. Food and Drug Administration (FDA), the Centers for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA). *Escherichia coli* ATCC 25922 and ATCC 35218, *Enterococcus faecalis* ATCC 29212 were used as quality control strains during minimal inhibitory concentrations (MIC) determination. The breakpoints used for the interpretation of susceptibility and resistance were determined according to the CLSI guidelines except streptomycin, for which provisional breakpoints by NARMS were used. Isolates with intermediate MICs were classified as susceptible.

Table 1
The distribution of the 138 *S.* Typhimurium isolates by location, retail market type and retail market storage condition at time of collection.

Province	Year of isolation	Market type[a]	Storage condition[b]	Total
Shaanxi	2007	Wet market	Ambient	17
		Supermarket	Ambient	9
	2008	Supermarket	Ambient	4
	2012	Wet market	Ambient	1
		Supermarket	Chilled	11
		Supermarket	Frozen	5
Sichuan	2010	Wet market	Ambient	1
	2012	Wet market	Ambient	6
Guangdong	2010	Wet market	Ambient	9
		Wet market	Frozen	4
		Supermarket	Chilled	11
	2011	Wet market	Ambient	11
		Supermarket	Chilled	19
Fujian	2010	Wet market	Ambient	7
		Supermarket	Chilled	3
Guangxi	2010	Wet market	Ambient	1
		Supermarket	Chilled	7
Beijing	2010	Supermarket	Ambient	2
Shanghai	2010	Wet market	Ambient	8
		Supermarket	Chilled	1
		Supermarket	Frozen	1

Denote.
[a] Supermarkets were self-service shops offering a wide range of food and chicken meat was sold either refrigerated or frozen in the meat department. Wet markets were open food (mostly meat) markets and these markets included butcher shops for live poultry and fish stalls, as well as other stands for fruit and vegetables.
[b] Ambient chickens were stored at 20–30 °C, chilled chickens were stored at 4–10 °C, and frozen chickens were stored at −10 to −20 °C.

2.3. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed to analyze genetic relatedness of *S.* Typhimurium isolates with *Xba*I according to the protocol described previously (Ribot et al., 2006). Briefly, after the SeaKem Gold Agarose embedded DNA was digested with 50 U of restriction endonuclease *Xba*I (TaKaRa, Dalian, China) for 1.5–2 h in water bath at 37 °C, DNA fragments were electrophoresed in 0.5 × TBE buffer at 14 °C for 18 h in Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16 s–63.8 s. *Salmonella* Braenderup H9812 was used as DNA ladder control strain. After DNAs were separated, the gel was stained with ethidium bromide, the DNA patterns were visualized with UV *trans*-illumination (Bio-Rad). PFGE results were analyzed using the BioNumerics Software (Applied-Maths, Kortrijk, Belgium). The genotypic relatedness was determined by the Jeffrey's coefficient and clustering based on the Complete linkage method with 90% similarity when PFGE patterns and clusters were assigned. Profiles were considered to be different if they differed by one band.

2.4. PCR amplification of virulence gene

All *S.* Typhimurium isolates were screened for 30 virulence genes by a simplex PCR. The location of the virulence genes was determined by checking the known genes' information in National Center for Biotechnology Information (NCBI http://www.ncbi.nlm.nih.gov/). Of the 30 virulence genes, eight (*avr*A, *sip*A, *sse*C, *mar*T, *rhu*M, *sii*E, *pip*A and *pip*D) are carried by *Salmonella* pathogenicity islands (SPIs) 1–5, five (*gog*B, *gtg*A, *sod*C1, *sse*I and *irs*A) are in prophages, three (*rck*, *spv*C and *spv*R) are in virulence plasmids, seven (*envR*, *sopE2*, *fhu*A, *msg*A, *pag*K, *srfj* and *stkc*) are in the islets, and seven (*fim*A, *lpf*D, *pef*A, *stc*C, *ste*B, *stj*B and *tcf*A) are in the fimbrial cluster. Primers used in the present study were designed using Primer Premier Software (Version 5.0, PREMIER Biosoft International, Canada) and synthesized by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China) (Table 2). A final 25 μl PCR mixture consisted of 5 μl of sample DNA template, 1 × PCR buffer, 250 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (TaKaRa) and 0.5 μM of forward and reverse primers. The amplification was carried out at 94 °C for 10 min for predenaturation, followed by 35 cycles of 94 °C for 30 s, at annealing temperatures for 30 s and 72 °C for 30 s, and an additional cycle of 72 °C for 7 min. PCR products were stained with ethidium bromide and visualized under UV light after gel electrophoresis in 1% agarose.

2.5. Statistical analysis

The relationships among the proportion of isolates resistant to each of the antibiotics was compared by market type (supermarket and wet market) and storage temperatures (frozen, chilled and ambient) using two-sided Fisher's exact test with $P < 0.05$ considered as statistically significant in SPSS software (Version 12.0; SPSS Inc., Chicago, IL).

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