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Subtyping of *Salmonella* isolates on retail raw chicken in China by pulsed-field gel electrophoresis and plasmid analysis

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ABSTRACT

Salmonellosis is one of the most common bacterial foodborne diseases worldwide. Poultry and poultry products are considered significant vehicles for foodborne salmonellosis transmission. We subtyped 725 *Salmonella* isolates recovered from 1152 retail raw chickens using pulsed-field gel electrophoresis (PFGE), and 448 ampicillin-resistant ones among 725 isolates using plasmid analysis in this study. Based on 80% similarity, 126 PFGE clusters were identified among 725 *Salmonella* isolates. Sixty one (8.4%) of 725 isolates belonged to PFGE cluster C96, followed by cluster C5 (38/725, 5.2%), and cluster C18 (38/725, 5.2%). Fifty two distinct PFGE patterns were detected among the isolates in Shaanxi Province, and 67, 64, 82, 67, 55, 88, and 47 patterns were identified in isolates in Henan, Sichuan, Beijing, Guangxi, Shanghai, Guangdong, and Fujian, respectively. Plasmids were detected in 331 (73.9%) of the 448 ampicillin-resistant *Salmonella* isolates. One hundred and twenty three plasmid profiles were identified, and P3, P11, P30, P12, were defined as predominant ones. Combination of PFGE and plasmid analysis was an effective way to enhance the genotypic discrimination ability to *Salmonella*, especially for *S.* Typhimurium isolates. Our results indicated that multiple *Salmonella* subtypes were prevalent in retail raw chicken in China, and they were potential hazard for food safety.

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1. Introduction

Salmonellosis is one of the most common bacterial foodborne diseases worldwide (Rivoal et al., 2009; Tauxe, 2002; White et al., 2001; Yang et al., 2010). Food contaminated with *Salmonella* causes serious public-health and economic problems (Stevens et al., 2008). In the United States, the number of *Salmonella* infections in humans is over 1.4 million each year, and 95% of which were foodborne ones (http://wwwnc.cdc.gov/eid/article/17/1/p1-1101_article.htm). Previous surveillance data indicated that *Salmonella* infections have been the most frequently illness, and poultry and poultry products have been incriminated as primary foods in the outbreaks of human Salmonellosis (Stevens et al., 2008; Yang et al., 2010).

Microbial subtyping is an effective way used to attribute foodborne infections to their sources (Barco, Barrucci, Olsen, & Ricci, 2013). However, in most cases, pathogens that caused human illnesses are indistinguishable or have similar genetic profiles compared to those in original source and unrelated settings (Hyytiä-Trees, Cooper, Ribot, & Gerner-Smidt, 2007). To enhance the discrimination ability, a number of molecular genotyping methods including plasmid typing, Multi-Locus Sequence Type (MLST), random amplified polymorphic DNA (RAPD), single nucleotide polymorphism (SNP), and pulse field gel electrophoresis (PFGE) have been developed (Akatas et al., 2007; Boonmar et al., 1998; Castilla et al., 2012; Chiu, Pang, Chen, & Tsen, 2011; Laconcha et al., 1998). Among these methods, MLST is a molecular analysis tool based on the sequence of related house-keeping genes. Although the method is relatively straightforward, it is limited in the ability to discriminate the same serotype strains (Ryan, Julie, Shana, & Catherine, 2011). Based on PCR, RAPD is widely used for subtyping various foodborne pathogens, and this method can distinguish different strains with a few nucleotide differences, so it has been used for both gram-positive and gram-

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negative pathogen, especially closely related ones or epidemiologically related, however, it is hard to maintain its reproducibility (Chen, Brown, & Knabel, 2011). Similar to RAPD, SNP is more rapid and cost effective than MLST-based schemes, the high-throughput formats of SNP typing make it more discriminatory than PFGE and MLST. (Dearlove et al., 2002; Sobrino, Brion, & Carracedo, 2005). Furthermore, the high cost may compromise its application, and the huge genome information may confuse the targeting information, especially for epidemiologic typing purposes (Wassenaar, 2003). PFGE (http://www.cdc.gov/pulsenet/protocols/ecoli-salmonella-shigella protocols.pdf) has been recognized as golden standard method in epidemiological analysis of foodborne bacteria for its good repeatability, and sensitive discrimination power (Favier, Cecilia, & Estrada, 2013; Xia et al., 2009). After has been standardized by the Centers for Disease Control and Prevention (CDC) of the United States, this method was widely accepted for *Salmonella* subtyping and contamination tracing in food contamination (Laconcha et al., 1998; Pang et al., 2007; Rivoal et al., 2009). Since PFGE has its own limitation, plasmid analysis maybe a good compensation to enhance its discrimination ability. However, most of the previous similar studies mainly focused on comparing the advantages and disadvantages of these two methods or the combination of PFGE with other subtyping methods, such as phage typing (Aktas, Day, Kayacan, Diren, & Threlfall, 2007; Rychlik, Svestkova, & Karpiskova, 2000). Although many factors might influence the results of plasmid analysis, it is widely accepted as a cheaper and quicker method for pathogenic bacteria subtyping and used in many previous studies with high discrimination power (Chmielarczyk et al., 2013; Chu et al., 2009; Kariuki et al., 1996), and study on combination of these two methods used for pathogens subtyping was not well documented. The objective of this study is to reveal the advantages of the combination of the two methods for *Salmonella* subtyping.

Recently, we acquired 725 *Salmonella* isolates after completing a study to determine the baseline prevalence of *Salmonella* on retail raw chicken in six provinces and two National cities in China (Yang et al., 2011). In this study, these isolates were subtyped using PFGE and plasmid analysis to better understand their genetic and plasmid subtypes for food safety prevention.

2. Materials and methods

2.1. Bacterial strains

Seven hundred and twenty five *Salmonella* isolates were used in this study. The isolates were recovered from 1152 retail raw whole chickens collected from 192 supermarkets and 96 wet markets in Henan, Shaanxi, Sichuan, Guangdong, Guangxi, Fujian provinces, and Beijing and Shanghai cities in China, from March to December 2010. Detailed information on sample collection, *Salmonella* isolation and identification were as previous described (Yang et al., 2011). Seven hundred and twenty five *Salmonella* isolates (Henan, $n = 89$; Shaanxi, $n = 70$; Sichuan, $n = 71$; Beijing, $n = 133$; Guangdong, $n = 117$; Guangxi, $n = 89$; Fujian, $n = 80$; Shanghai, $n = 76$) were recovered in total. O and H hyperimmune seras (Statens Serum Institut, Artilerivej, Denmark) and slide agglutination method were employed to determine the serotypes of the isolates according to the manufacturer's instructions in Henan Centers for Disease Control and Prevention, China. The isolates were stored in Luria–Bertani/glycerol (V/V, 50%/50%) (Difco, Cockeysville, MD) culture at −80 °C for future transfer and growth on Tryptic Soy Agar (TSA; Difco).

2.2. Pulse-field gel electrophoresis (PFGE)

PFGE was performed to determine the genotypic relatedness of the isolates as previously reported (Ribot et al., 2006). Briefly, agarose-embedded DNA was digested with 50 U of *Xba*I (TaKaRa, Dalian, China) for 1.5–2 h in a water bath at 37 °C. The restricted fragments were separated by electrophoresis in 0.5×Tris-borate-EDTA (TBE) buffer at 14 °C for 19 h using a Chef-Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16–63.8 s. *Salmonella* Braenderup H9812 was used as the standard control strain. The gels were stained with ethidium bromide, and DNA bands were visualized with UV transillumination (Bio-Rad). PFGE results were analyzed using the BioNumerics Software (Applied-Maths, Kortrijk, Belgium) manually, the genotypic relatedness was determinated by use of the Jeffrey's coefficient and clustering was based on the Complete linkage method with 80% similarity when PFGE patterns and clusters were assigned in the study. Profiles were considered to be different if they differed by one band.

2.3. Plasmid analysis

Plasmid DNA was extracted from 448 (61.8%) ampicillin-resistant *Salmonella* isolates of the 725 isolates using a Bio-tech MiniBEST plasmid purification kit (Bio-tech, Beijing, China) according to the manufacture's instruction. The profiles of plasmid DNA were determined based on its number and size as previously documented (Aktas et al., 2007; Mohan, Sharma, Agarwal, Purnima, & Pillai, 1995). Extracted plasmid DNA was electrophoresed for 60 min at 80 V in 0.8% agarose gel in 0.5×TBE buffer, the number and size of the DNA were visualized and recorded after staining with ethidium bromide and UV irradiation (Bio-Rad).λ-phage HindIII digestions and Supercoiled DNA Ladders (TaKaRa) were used as molecular weight markers.

2.4. Statistic analysis

The Simpson index (D) was calculated to assess the genetic diversity of the *Salmonella* isolates as following (Denis et al., 2009; Hunter, 1990; Rivoal et al., 2010).

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

N: number of isolates tested
S: number of different genotypes
Nj: number of isolates belonging to type j

The Simpson index (D) of PFGE and plasmid analysis results was calculated to assess the genetic diversity, respectively. The higher D value, the higher resolution was. For combination analysis, the overall D value was calculated and compared with the D value of PFGE and plasmid analysis to assess the increase of discrimination ability.

The significant difference between different subtyping methods (Table 2) was determined by Duncan's multiple range tests. Threshold for significant difference was $P < 0.05$ and extreme significant difference was $P < 0.01$. All statistical analysis was performed by Data Processing System software (Data Processing System version 6.5, Zhejiang University, CHN).

3. Results

Eleven to 15 DNA bands could be found in each lane after PFGE. Digested fragments of genomic DNA of the 725 *Salmonella* isolates

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