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Antimicrobial resistance and resistance genes in *Salmonella* strains isolated from broiler chickens along the slaughtering process in China

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Abstract: A total of 189 *Salmonella* isolates were recovered from 627 samples which were collected from cecal contents of broilers, chicken carcasses, chicken meat after cutting step and frozen broiler chicken products along the slaughtering process at a slaughterhouse in Sichuan province of China. The *Salmonella* isolates were subjected to antimicrobial susceptibility testing to 10 categories of antimicrobial agents using the Kirby–Bauer disk diffusion method. Those antibiotics-resistant isolates were further investigated for the occurrence of resistance genes, the presence of class 1 integron as well as the associated gene cassettes, and the mutations within the *gyrA* and *parC* genes. Consequently, the prevalence of *Salmonella* was 30.14% (47.96% for cecal content, 18.78% for chicken carcasses, 31.33% for cutting meat and 14.00% for frozen meat, respectively). The predominant serotypes were *S.* Typhimurium (15.34%) and *S.* Enteritidis (69.84%). High resistance rates to the following drugs were observed: nalidixic acid (99.5%), ampicillin (87.8%), tetracycline (51.9%), ciprofloxacin (48.7%), trimethoprim/sulfamethoxazole (48.1%), and spectinomycin (34.4%). Antimicrobial resistance profiling showed that 60.8% of isolates were multidrug resistant (MDR), and MDR strains increased from 44.7% to 78.6% along the slaughtering line. 94.6% (n=157) of beta-lactam-resistant isolates harbored at least one resistance gene of bla_{TEM} or $bla_{\text{CTX-M}}$. The relatively low prevalence of aminoglycoside resistance genes (*aac(3)-II*, *aac(3)-IV*, and *ant(2'')-I*) was found in 49 (66.2%) of antibiotic-resistant isolates. The tetracycline resistance genes (*tet*(A), *tet*(B), *tet*(C), and *tet*(G) and sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) were identified in 84 (85.7%) and 89 (97.8%) antibiotic-resistant isolates

respectively. *floR* was identified in 44 (97.8%) florfenicol-resistant isolates. Class 1 integron was detected in 37.4% (n=43) of the MDR isolates. Two different gene cassettes, *bla*$_{\text{OXA-30}}$-*aadA1* (19 isolates) and *bla*$_{\text{OXA-30}}$-*aadA1*/*drfA1-orfC* (2 isolates), were identified in class 1 integron-positive isolates. 97.9% (184/188) of quinolone-resistant isolates had at least one mutation within *gyrA* or *parC*. Overall, antimicrobial resistance showed an increasing trend along the slaughtering process. The results showed that broiler chicken products in the slaughterhouse were contaminated with MDR *Salmonella*, which might originate from food producing animals to some extent, and cross-contamination during slaughter, and facilitate the dissemination of the resistance genes to consumers along the production chain, which suggests importance of controlling *Salmonella* during slaughter for public health, underlying strict hygiene method and HACCP management to reduce cross-contamination.

Keywords: *Salmonella;* antimicrobial resistance; resistance gene; broiler chicken; slaughter; multidrug resistant (MDR)

1. Introduction

Salmonella is one of the most common foodborne pathogens, causing outbreaks of foodborne disease worldwide (Newell et al., 2010). Foodborne *Salmonella* infection typically causes a range of clinical syndromes in humans including typhoid fever, diarrhoeal disease, and may have a dramatically more severe systemic disease in the immunocompromised people (Gordon, 2008). Animal food products especially eggs and poultry meats, have been the most common vehicles of the *Salmonella* infections (Greig and Ravel, 2009). With the increasing consumption of poultry meat globally, bacterial pathogen *Salmonella*, as an important factor for affecting the safety of poultry and raw meat, will continue to receive growing attention (Henchion et al., 2014; Sofos, 2008).

Recently, there has been an increasing trend of antimicrobial resistance on a worldwide scale, especially for multidrug-resistant (MDR) *Salmonella* strains from food animals (Hur et al., 2012). In China, the usage of antimicrobial agents is greater than in most other countries; in a 2007 survey, nearly half of the 210, 000 tons of antibiotics produced in China, were used in livestock as therapeutic drugs and feed additives (Hvistendahl, 2012). Abuse of antimicrobial agents in food animal production is regarded as one of the important reasons for emergence of antimicrobial resistance in *Salmonella*; this resistance can be transmitted to the human population through the animal foodstuffs, which poses a serious threat to public health. Multidrug-resistant phenotypes in *Salmonella* of animal origin have been increasingly observed in China (Lai et al., 2014; Yang et al., 2013).

Various factors from farm to fork along the food chain heavily influence the microbiological safety of food (Newell et al., 2010). Successful prevention of foodborne salmonellosis originating from animal production comprise three lines of defence against *Salmonella*: a) controlling *Salmonella* in the food-producing animal at farms (pre-harvest control); b) improving hygiene during the slaughter and further processing of the meat (harvest control); c) the final food preparation by underlying good hygiene practices (post-harvest control) (Forshell and Wierup, 2006). Therefore, slaughter is the most appropriate stage of food chain for the evaluation on the carriage of zoonotic agents by farm animals, the level of *Salmonella* infection in animal carcasses and subsequently meat products in the finishing poultry population, and the proportions of self- and cross-contamination during slaughter and processing (Bonardi et al., 2013).

Most studies about the prevalence and antimicrobial resistance of *Salmonella* in either animals (Ahmed and Shimamoto, 2012; Lai et al., 2014; Mainali et al., 2014; Pan et al., 2010) at chicken farms or in retail poultry meats of marketplace (Chen et al., 2004; Kim et al., 2012; Yan et al., 2010; Yang et al., 2013; Yang et al., 2010; Yang et al., 2011), or in partial processing stages in the slaughtering line (Akbarmehr, 2012; Bai et al., 2015; Olsen et al., 2003; RIVERA-PÉREZ et al., 2014; Ziech et al., 2016) have been separately performed. Additionally, several surveys have been carried out at the molecular level to monitor the distribution of resistance genes in *Salmonella* from broiler chickens and chicken meat (Asai et al., 2006; Gong et al., 2014; Li et al., 2013). To date, some research on prevalence of *Salmonella* from animals to chicken

meat products along the slaughtering process (Choi et al., 2014; Cui et al., 2016; Li et al., 2013; Van der Fels-Klerx et al., 2008), and the potential role of the food production chain in the dissemination of antimicrobial resistance and resistance genes of *Salmonella* (Cui et al., 2016; Li et al., 2013) have been presented. However, the present studies have reported β-lactamase genes and class 1 integron of *Salmonella* isolates from the broiler chicken supply chain in China, while other kinds of resistance genes in *Salmonella* along the slaughtering process were still almost not reported. In addition, the poultry sector in China has experienced vigorous growth over the past two decades. Chicken production is the predominant subsector, accounting for 70 percent of poultry meat production. The poultry sector is no longer dominated by hundreds of millions of smallholders. Instead, the number of large producers in poultry and broiler chickens production increased substantially (Bingsheng and Yijun, 2007). Therefore, taking into account the important role of large-scale slaughterhouse and poultry farm, and the high consumption of chicken meat in China, more comprehensive investigations at the molecular level to monitor the distribution and dissemination of antimicrobial resistance during slaughter were needed.

Therefore, in this study, four processing points along the slaughtering process were selected to monitor the prevalence, antimicrobial resistance, and resistance gene dissemination of *Salmonella*. In detail, *Salmonella* isolates were collected at the four processing points in a broiler slaughter and processing chain during a three-year period of 2012-2014, in Sichuan province of China. We investigated the prevalence,

serotypes, antimicrobial resistance of *Salmonella* isolates to ten categories of common antimicrobial agents, the presence of several kinds of antimicrobial resistance genes associated with β-lactams, aminoglycosides, tetracycline, florfenicol, and sulfonamide, and class 1 integron, *gyrA* and *parC* mutations of quinolone-resistant isolates. On the basis of these results, we analyzed the correlation between the antibiotics-resistant phenotypes, serotypes, and resistance genes of *Salmonella* isolated from different stages of the poultry meat production chain, and identified possible routes of *Salmonella* transmission.

2. Material and methods

2.1. Sample collection

From March 2012 to October 2014, a total of 627 samples were collected from four processing points (cecal content of broiler chicken, n=196; chicken carcasses, n=181; chicken meat after cutting step, n=150; frozen chicken meat products, n=100) during slaughter and processing at a local broiler chicken slaughtering plant in Sichuan province of China where about 30,000,000 broiler chickens are processed each year. All these samples were stored on ice during transportation to our laboratory, and analyzed within 3 h.

The main slaughter process in this slaughterhouse and four sampling points were given in Fig. S1 in supplementary materials. Fresh broiler chicken cecal contents (point 1), representing samples of broiler at the farm level, were obtained after evisceration, and collected in sterile plastic stomacher bags in accordance with previously described method (Allen et al., 2007). Chicken carcasses (point 2) were

sampled after evisceration and before chilling by using a whole-carcass swab method (McEvoy et al., 2005). Each swab was placed inside a sterile stomacher bag and pre-moistened immediately before use with 25 mL of buffered peptone water (BPW). The swabs after sampling were returned to its original bag, and transported on ice to lab. For sampling points 3 (chicken meat after cutting step) and 4 (frozen chicken meat products), 25 g of each sample was incised, and collected in a sterile plastic stomacher bag according to the methods described in the ISO 17604:2003 standard (International Organization for Standardization, Geneva, Switzerland). For sampling point 4, chicken meat products in this slaughterhouse were packaged after quick-freezing, and then the packaged quicken-frozen chicken meat products were directly collected without being stored by using our sampling method.

2.2. Salmonella isolation and serotyping

All of the samples were subjected to *Salmonella* isolation in accordance with national food safety standard of China-Food microbiological inspection: *Salmonella* (GB 4789. 4-2010), with some modifications for samples obtained at point 2. For samples obtained at point 2, they were stomached, incubated at 37 °C for 18 h (bacteria pre-enrichment), and then subjected to centrifugation (12000 rpm, 4 °C, 10 min). Finally, the BPW supernatant was removed and discarded, and about 4 mL of the pre-enrichment culture containing bacterial precipitate was obtained. Then, aliquots of 1.0 mL were transferred into 10 mL of tetrathionate broth (TTB) and selenite cysteine (SC) broth, respectively. The subsequent procedure was carried out in accordance with GB 4789.4-2010.

Suspected *Salmonella* colonies from each sample were further identified on the basis of biochemical characterization and specific genes of *Salmonella* using duplex PCR assays (Cohen et al., 1993; Rahn et al., 1992). A single confirmed *Salmonella* isolate from each positive sample was serotyped by slide agglutination test for O and H antigens using commercially available antiserum (Tianrun Bio-Pharmaceutical, Ningbo, China) following manufacturer's instructions.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the *Salmonella* isolates to 10 categories of antimicrobials was carried out in accordance with the standard Kirby–Bauer disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010) (Cockerill, 2011). All the antimicrobial disks were obtained from Oxoid (Thermo Fisher Scientific, Basingstoke, England). *Escherichia. coli* ATCC 25922 was used as the control organism. The concentrations of the antimicrobials and abbreviation of these antimicrobial agents are: ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), ceftriaxone (CRO, 30 μg), gentamycin (GEN, 10 μg), spectinomycin (SPE, 100 μg), tetracycline (TET, 30 μg), florfenicol (FLO, 75 μg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg), nalidixic acid (NAL, 30 μg), and ciprofloxacin (CIP, 5 μg). The isolates were classified as susceptible, intermediate, or resistant according to the CLSI (2010) guidelines. *Salmonella* isolates resistant to three or more antimicrobials were defined as MDR isolates (Li et al., 2013; Pokharel et al., 2006).

2.4. PCR amplification of antimicrobial resistance genes

Template DNA of *Salmonella* isolates for PCR was prepared by the heat lysis method (Pitout et al., 1998), except that bacteria were directly inoculated into 3.0 mL of nutrient broth in Eppendorf tubes for overnight culture. Finally, the template for amplification was obtained, and stored at −20 °C for use.

Salmonella isolates, which showed resistance to each category of antimicrobial agent, were examined for the presence of resistance genes. The presence of genes associated with beta-lactams (*bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$), aminoglycosides (*aac(3)-II*, *aac(3)-IV*, and *ant(2")-I*), tetracycline (*tet*(A), *tet*(B), *tet*(C), and *tet*(G)), florfenicol (*floR*), and sulfonamide (*sul1*, *sul2*, and *sul3*) was detected by PCR amplification. The primer sequences and predicted sizes for PCR amplification of different resistance genes from *Salmonella* are listed in Table 1. PCR amplification was performed using a DNA thermal cycler (Bio-Rad, CA, USA) as (1) 95 °C for 10 min; (2) 35 cycles of 94 °C for 45 s, 55-70 °C for 50 s, 72 °C for 50 s; (3) 72 °C for 10 min. The PCR products were analyzed through 1.5% (w/v) agarose gel electrophoresis and sequenced by Takara biotechnology Co., Ltd. (Dalian, China). Resulting DNA sequence data were compared with data in the GenBank database using the BLAST tool available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

2.5. Detection and characterization of class 1 integron

Multidrug-resistant isolates (n=117) were examined for the presence of the class 1 integron using specific primers for *intI* 1 gene (encoding the specific integrase) by PCR. The variable region of class 1 integron in all *intI* 1-postive isolates was also

characterized by PCR and sequencing. The size of inserted resistance gene cassettes of the integrase-positive isolates was detected by PCR using primers for the conserved segment regions (CS-PCR) (Table 1). The PCR system (25 μL) contained 2 μL of template DNA, 0.5 μL of each primer (10 μmol/L), 12.5 μL of 2× PCR Mix, 5.5 μL of deionized water. PCR amplification was performed using a DNA thermal cycler (Bio-Rad, CA, USA) as (1) 95 °C for 5min; (2) 35 cycles of 95 °C for 45 s, 70 °C for 50 s (*intI* 1) (*intI* 1 variable region, 55 °C for 50 s), 72 °C for 50 s; (3) 72 °C for 10 min. The PCR products were analyzed through 1.5% (w/v) agarose gel electrophoresis and sent for sequencing by Takara biotechnology Co., Ltd. (Dalian, China). The DNA sequence data were compared with data in the GenBank database using the BLAST tool available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

2.6. Detection of the gyrA and parC mutations by MAMA-PCR of quinolone-resistant isolates of Salmonella

The most common mechanisms of resistance to quinolones are mutations in DNA gyrase (topoisomerase II, in *gyrA* and *gyrB* genes) and topoisomerase IV (in *parC* and *parE*). These mutations are primarily located in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and its homologous region of the *parC* gene (Qiang et al., 2002). Four amino acid substitutions, two in *gyrA* and two in *parC* subunits of DNA gyrase and topoisomerase IV, are commonly responsible for fluoroquinolone resistance.

Mismatch amplification mutation (MAMA) PCR assay was used to detect

mutations within both DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) gene using primers listed in Table 2. PCR was carried out according to the reference (Qiang et al., 2002). The PCR products were analyzed through 1.5% (w/v) agarose gel electrophoresis and sequenced by Takara biotechnology Co., Ltd. (Dalian, China). The nucleotide sequences were analyzed using the BLAST tool available from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

2.7. Statistical data analysis

Confidence intervals of proportions were calculated with EPi tools (http://epitools.ausvet.com.au) using the binomial exact method. Statistical significance of differences between proportions was evaluated by Chi-square (χ^2) test.

3. Results and discussion

3.1. Prevalence and serotyping of Salmonella isolates

A total of 189 *Salmonella* isolates (2012, n=84; 2013, n=72; 2014, n=33) were recovered from 627 samples collected from four processing points along the slaughtering process. All the isolates were identified by biochemical characterization and further confirmed by detecting one genus-specific gene and one virulence gene (*invA*) using duplex PCR amplification. The gel image of PCR products of partial strains was shown in Figure S2 in the supplementary materials. The overall prevalence of *Salmonella* was 30.14% (95% C.I. 26.57%–33.90%). The isolation rate of *Salmonella* spp. was 47.96% (95% C.I. 40.79%–55.19%) for broiler chicken cecal samples (point 1). 18.78% (95% C.I. 13.37%– 25.25%) of samples from carcasses

surface after evisceration (point 2) were positive. The occurrence of *Salmonella* at sampling points 3 and 4 were 31.33% (95% C.I. 24.02%– 39.41%) and 14.00% (95% C.I. 7.87%– 22.37%) respectively (Table 3).

In the present study, the prevalence of *Salmonella* isolates from samples collected along the slaughter process (30.14%) is higher than 14.98% in Qingdao City, China (Cui et al., 2016), and 16.06% (195/1214) in Korea (Choi et al., 2014) in one vertically-integrated commercial broiler chicken supply chain. The isolation rate of *Salmonella* spp. was 47.96% for broiler chicken cecal samples (point 1), which represented the contamination level of food-producing animals. The result was also significantly higher than previously reported 19.84% of positive samples for chickens in Shandong province of China, 2009-2012 (Lai et al., 2014). Broilers are widely considered to be an important reservoir for *Salmonella* transmission, due to the ability of *Salmonella* to proliferate in the gastrointestinal tract of the chickens and subsequently contaminate processed broiler carcasses in the slaughter and processing chain (Todd, 1980). Therefore, high prevalence in food-producing animals in this commercial processing plant increases the risk of carcass contamination and the human salmonellosis.

Compared with the occurrence at point 1 (47.96%), the contamination level of chicken carcasses decreased significantly at point 2 (18.78%) ($P<0.01$), which may be attributed to hygiene management in the slaughterhouse and rinse treatment on the chicken carcasses after evisceration. However, the broiler chicken meat at point 3 presented much higher prevalence rate (31.33%) of *Salmonella* spp. than 18.78% at

point 2 ($P<0.01$). It could be explained by cross-contamination occurring at the several stages between points 2 and 3 during slaughter. Between the points 2 and 3, there are several main stages including chilling of chicken carcasses, carcasses hanging, and segmentation. In this processing plant, immersion chilling of chicken carcasses is performed in pre-chillers with two tanks in series containing sodium hypochlorite. By chilling, the carcasses would be reduced to a temperature of 7-10 °C that is generally regarded as the maximum temperature for preventing the proliferation of mesophilic, enteric pathogens (Sofos, 2005). However, several studies have been conducted to evaluate the effect of chilling on numbers and prevalence of *Salmonella*. Immersion chilling has a minimal effect on the prevalence of *Salmonella* on poultry carcasses. Other researchers also have suggested that the washing effect during immersion chilling physically removes bacterial cells and thus reduces bacterial recovery, but this effect is likely offset by carcass cross-contamination (THOMSON et al., 1979). In addition, other processing stages like carcasses hanging and segmentation may also lead to the cross-contamination via external animal surfaces, workers' clothes, hands or equipment, other carcasses, plant equipment, and plant environment, etc. Therefore, all these factors might lead to the increasing prevalence of *Salmonella* from samples at sampling point 3. A significant decrease in the prevalence of *Salmonella* was detected at sampling point 4 (14.00%), which could be explained by that the quick-freezing procedure makes *Salmonella* inactivation, thus reducing the risk of human infection.

Apart from 28 untypable strains, the remaining 85.18% of the *Salmonella* strains

were identified into 2 serovar groups, including *S*. Enteritidis (n=132, 69.84%) and *S*. Typhimurium (n=29, 15.34%) (Table 3). The serotyping results indicated that *S*. Enteritidis and *S*. Typhimurium were the dominant serovars in this slaughter and processing chain. *S.* Enteritidis isolates were detected in all sampling points, from which 79 isolates at point 1 (40.31%) (95% C.I. 33.38%– 47.53%), 25 at point 2 (13.81%) (95% C.I. 9.14%– 19.71%), 21 at point 3 (14.00%) (95% C.I. 8.88%– 20.60%) and 7 at point 4 (7.00%) (95% C.I. 2.86%– 13.89%). In addition, 29 *S.* Typhimurium isolates were from the latter three processing stages, and *S.* Typhimurium was not detected from fresh broiler cecal samples (point 1), which indicated that there might be cross-contamination between chicken carcasses and processing environment or workers during slaughter. The serotypes of *S*. Enteritidis and *S*. Typhimurium are two common types isolated from broiler carcasses in slaughter and humans infected by food borne diseases (de Oliveira et al., 2005; Rabsch et al., 2001), which could cause a range of clinical syndromes, including diarrhoeal disease. A survey conducted by Wang et al. (2013) in China isolated and identified 23 isolates from the slaughterhouse, and these isolates belonged to six different serotypes, including *S*. Indiana, *S*. Infantis, *S*. Derby, *S*. Heidelberg, *S*. Agona and *S*. Typhimurium. Li et al. (2013) identified fourteen serotypes among 129 *Salmonella* isolates from pigs, ducks and chickens in one food production chain in Sichuan province, China, and the top three serotypes were *S*. Derby (n=76), *S*. Typhimurim (n=16), and *S*. Potsdam (n=9). By contrast, *Salmonella* isolates from the present slaughterhouse showed less variety in serotype. Furthermore, it could be

found that although samples were collected from the same region, there also existed considerable difference in serotypes of *Salmonella* isolates in different slaughterhouses.

3.2. Antimicrobial resistance of Salmonella isolates

Figure 1a shows the antimicrobial resistance of 189 *Salmonella* isolates to ten categories of antimicrobial agents, which showed that all *Salmonella* isolates were resistant to at least one antimicrobial agent. High resistance rates were observed against nalidixic acid (99.5%), ampicillin (87.8%), tetracycline (51.9%), ciprofloxacin (48.7%), trimethoprim/sulfamethoxazole (48.1%), and spectinomycin (34.4%). Lower levels of resistance were found for florfenicol (25.4%), gentamycin (10.3%), amoxicillin/clavulanic acid (8.5%). Most of the isolates were susceptible to ceftriaxone.

When antimicrobial resistance was analyzed by sampling points (Fig. 1b), higher resistance rates to CRO, TET, SXT, AMC, CIP, GEN, and SPE were observed in isolates from points 3 and 4 in comparison with points 1 and 2, with the exception of FLO, AMP and NAL, which showed roughly consistent resistance rate in these four sampling points. Overall, higher resistance rates were found in the latter stages of slaughter.

Salmonella isolates with different serotypes showed difference in the resistance to 10 antimicrobial agents (Fig. 1c). Resistance of *S*. Enteritidis to FLO, SXT, NAL, and GEN was slightly higher than, or almost equal to those of *S*. Typhimurium, while *S*. Typhimurium showed higher resistance to CRO, TET, AMP, AMC, CIP, and SPE than

S. Enteritidis.

The antimicrobial resistance of *Salmonella* isolates from samples in different years also showed the difference (Fig. 1d). It could be seen that antimicrobial resistance of *Salmonella* isolates to CRO, TET, SXT and SPE increased gradually from the year 2012 to 2014. Additionally, the resistance of *Salmonella* strains in 2014 to FLO, AMC and CIP was much higher than those in 2012. There was almost no difference in resistance to AMP, NAL, and GEN in the three-year period. Overall, there was an increasing trend in antimicrobial resistance with time.

Totally, 45 resistance patterns of these isolates to 10 categories of antimicrobials were found (Table 4). 60.8% (n=115) of isolates were MDR strains. 19.6% (n=37) of isolates were resistant to seven or more antimicrobials and 3.2% (n=6) of isolates were resistant to up to nine antimicrobials. The dominant resistance pattern was NAL-AMP (28.6%, 54/189), followed by NAL-SXT (8.5%, 16/189), AMP-CIP-TET-SPE-NAL (7.9%, 15/189) and AMP-CIP-SXT-TET-SH-NAL-FLO (6.3%, 12/189). Along the slaughtering process, there was a descending trend for the numbers of resistance patterns (point 1, n=25; point 2, n=16; point 3, n=17; point 4, n=9).

The results showed that 66.7% (n=88) of *S*. Enteritidis isolates were resistant to one to three categories of antimicrobial agents, while *S*. Typhimurium isolates were mainly resistant to four to six categories of antimicrobials (89.7%, n=26), which indicated that MDR phenotypes in *Salmonella* are strongly associated with serotype (Cui et al., 2016). *Salmonella* isolates from different sampling points also showed

different multidrug resistance (Table 5). Isolates from point 1 (55.4%) and point 2 (50.0%) were mainly resistant to one to three classes of antimicrobials, while isolates from point 3 (51.1%) and point 4 (50.0%) were mainly resistant to four to six kinds of antimicrobials. Higher resistance rates to up to 9 categories of antimicrobials were found in isolates from point 4 (21.4%). Therefore, it could be concluded that the multidrug resistance became more serious along the slaughtering process. *S.* Typhimurium isolates were mainly resistant to four to six categories of antimicrobials (89.7%, n=26), including 6 isolates from point 2, 16 isolates from point 3, 4 isolates from point 4; in addition, two *S.* Typhimurium isolates from point 4 were resistant to up to nine categories of antimicrobials. Additionally, as described above, 29 of *S.* Typhimurium strains were isolated from the latter three processing stages, and not detected in fresh broiler cecal samples (point 1). Therefore, it could be concluded that there might be cross-contamination between chicken carcasses and processing environment or workers in this processing plant, especially locating between the points 2 and 3, which leads to the increase of percentage of MDR strains in the latter processing stages, and facilitates the dissemination of the multi-drug resistance to consumers.

In addition, we can see from the Table 5, there was a decreasing trend in multi-drug resistance in *S.* Enteritidis from point 1 (34.2%) to point 3 (23.8%), while 57.2% of *S.* Enteritidis isolates at point 4 were MDR strains. Therefore, it might be concluded that the *Salmonella* isolates due to cross-contamination between point 3 and point 4, instead of raw broiler chicken, increase the risk of MDR strains in

finishing chicken meat products. For untyped serotypes, all strains isolated from point 1 (n=15), point 2 (n=3), and point 3 (n=10) were MDR strains, which indicated that due to rinse treatment on chicken carcasses, the prevalence of *Salmonella* and MDR strains decreased significantly; there might exist cross-contamination between points 2 and 3. However, there were no isolates from finishing frozen chicken meat products (point 4), which terminates the transmission of MDR strains to consumers.

In terms of sampling time, isolates recovered in 2012 were mainly resistant to one to three categories of antimicrobials (60.7%), while 51.4% and 43.0% of isolates from 2013 were resistant to one to three antimicrobials and four to six categories of antimicrobials respectively. By contrast, more serious multi-drug resistance occurred in 2014, when all the isolates were resistant to at least four to six categories of antimicrobials, which indicated that multi-drug resistance also exhibited an increasing trend over the three-year period. Antibiotics are commonly used in concentrated animal feeding operations worldwide to treat animal diseases and promote animal growth (Sarmah et al., 2006) . In the slaughterhouse we chose, these broiler chickens are from one poultry farm where the workers at the farm also continuously used the common antibiotics categories in alternation in the long term for treating and preventing bacterial infections, thus improving growth and production, which necessarily results in the increasing antibiotic resistance. Veterinary drug residues including tetracyclines, beta-lactam antibiotics and quinolones, etc. have been detected in poultry meat and animal feedingstuffs (Al-Ghamdi et al., 2000; De Wasch et al., 1998; Kabir et al., 2004; McEvoy, 2002; Okerman et al., 2001). Figuring out the

correlation between the antibiotics used in the animal feed and the antibiotic resistance profile presented in this slaughterhouse is quite necessary, and requires further investigation in future.

Therefore, in this slaughterhouse, efficient measures to facilitate the reasonable use of antimicrobials in animal husbandry must be taken (McEwen and Fedorka-Cray, 2002). Also, more importantly, strict hygiene method and HACCP (Hazard Analysis and Critical Control Points) management during slaughter are vital for preventing food infection caused by *Salmonella* (Harris et al., 1995).

3.3. Antimicrobial resistance genotypes of Salmonella isolates

Among 166 beta-lactam-resistant isolates, 94.6% (n=157) harbored at least one resistance gene of bla_{TEM} or $bla_{\text{CTX-M}}$. The bla_{SHV} gene was not detected in any of the isolates. The PCR results were in accordance with those of antimicrobial susceptibility tests. The bla_{TEM} gene (93.4%, n=155) was the most prevalent in beta-lactam-resistant isolates, followed by $bla_{\text{CTX-M}}$ gene (12.7%, n=21). Both bla_{TEM} and $bla_{\text{CTX-M}}$ genes were simultaneously detected in 11.4% (n=19) of the isolates.

Higher frequency of $bla_{\text{CTX-M}}$ gene was found in isolates from point 4 (16.7%) compared with isolates from other points. In terms of serovars, the prevalence of bla_{TEM} for *S.* Enteritidis and *S.* Typhimurium was 97.3% and 93.1%, respectively. The $bla_{\text{CTX-M}}$ gene was found in 46.9% (n=15) of isolates in 2014, compared with only 6.0% (n=3) and only 3.4% (n=3) of isolates in 2013 and 2012, respectively.

Among 91 sulfonamide-resistant isolates, 97.8% (n=89) harbored at least one gene of *sul1*, *sul2* or *sul3*. The *sul2* gene had the highest occurrence (97.8%, n=89),

followed by *sul3* (50.5%, n=46) and *sul1* (50.5%, n=46). The co-occurrence of *sul1-sul2-sul3* was most prevalent (28.6%, n = 26), followed by *sul2-sul3* (22.0%, n=20) and *sul1-sul2* (22.0%, n=20).

Among 98 tetracycline-resistant isolates, 85.7% (n=84) harbored at least one *tet* gene. The *tet*(C) gene (71.4%, n=70) was the most prevalent, followed by *tet*(B) (50%, n=49) and *tet*(A) (23.5%, n=23). The most popular co-occurrence was *tet*(B)-*tet*(C) (20.4%, n=20), followed by *tet*(A)-*tet*(B)-*tet*(C) (17.3%, n=17), *tet*(A)-*tet*(C) (5.1%, n=5) and *tet*(A)-*tet*(B) (1.0%, n=1). None of the isolates was positive for *tet*(G). Isolates from point 1 (85.7%, n=36) and point 2 (85.7%, n=12) carried the *tet*(C) gene, while the *tet*(B) gene was the main tetracycline-resistant gene among isolates from point 3 (58.1%, n=18) and point 4 (81.8%, n=9).

Except for one *S.* Typhimurium strain, the *floR* gene was identified in 97.8% (n=44) of florfenicol-resistant *Salmonella* strains (n=45). The antimicrobial resistance genotypes were highly in accordance with the phenotypes.

For 74 aminoglycosides-resistant isolates, 49 *Salmonella* strains (66.2%) carried *aac(3)-II* or *aac(3)-IV*, and none of the isolates harbored *ant(2'')-I* gene. The occurrence of *aac(3)-II* and *aac(3)-IV* was 35.4% and 53.2%, respectively. Additionally, 21 strains carried both *aac(3)-II* and *aac(3)-IV* genes. We found that 21 of 22 gentamicin-resistant isolates gave a positive signal for *aac(3)-II* (72.7%) or *aac(3)-IV* gene (81.8%). 53.8% (35/65) of spectinomycin-resistant isolates carried *aac(3)-II*, *aac(3)-IV*, or *ant(2'')-I* gene, which indicated the relatively low consistency between antimicrobial resistance phenotype and genotypes. This might be explained

by other resistance mechanisms or antimicrobial resistance genes which mediate the resistance to spectinomycin.

3.4. Prevalence and characteristics of class 1 integron

Of the 115 MDR *Salmonella* strains which were screened for the presence of class 1 integron, 43 isolates (37.4%, 43/115) harbored class 1 integron. Among them, 21 strains were positive for resistant gene cassettes containing two distinct patterns, including $bla_{\text{OXA-30}}$-*aadA1* (19 isolates), $bla_{\text{OXA-30}}$-*aadA1*/*drfA1-orfC* (2 isolates). In the present study, 72.4% (n=21) of *S. Typhimurium* and 31.7% (n=19) of *S. Enteritidis* harbored the *intI* 1 gene respectively. The highest prevalence of class 1 integron was found in isolates from point 4 (72.7%, n=8), followed by isolates recovered from point 2 (38.9%, n=7). The results indicated that the carriage of class 1 integron was related to serotype, source, and other factors (Hur et al., 2011; Wannaprasat et al., 2011). In addition, 48.8% of *intI* 1-positive strains were found to carry gene cassettes in our study, which is similar to the prevalence of 61.5% (Molla et al., 2007) and 61.1% (Khemtong and Chuanchuen, 2008). In 2013, 57.8% of isolates harbored class 1 integron in contrast to 44.7% in 2012 and 0 in 2014, respectively.

Twenty-one of *S.* Typhimurium isolates were positive for gene cassettes, while no gene cassette was detected in *S.* Enteritidis and untypable strains. Higher prevalence of resistant gene cassettes was found in isolates from point 2 (n=6), point 3 (n=8) and point 4 (n=7), compared with isolates from point 1 (n=0). The results indicated that cross-contamination between chicken carcasses and processing environment or workers during slaughter might play an important role in transmission of multi-drug

resistance and resistance genes to consumers. All *intI* 1-positive *S.* Typhimurium harbored gene cassettes in our study, which is consistent with previous studies that 78% to 100% of *S.* Typhimurium strains were positive for the *intI* 1 gene harboring gene cassettes (Antunes et al., 2006). In the present study, the gene cassettes $bla_{\text{OXA-30}}$-*aadA1*and *drfA1-orfC* were detected in *S.* Typhimurium, which have been identified previously (Khan et al., 2009; Weill et al., 2006). The presence of class 1 integrons on plasmids is considered to be the main mechanism for the rapid spread of multidrug-resistant phenotypes among gram-negative bacteria (Rowe‐Magnus et al., 2002). Other gene cassettes, such as *dhfr7*, *aacA4*, *dfrA5*, *addA2*-$bla_{\text{PSE-1}}$, and $bla_{\text{OXA-1}}$ in *S.* Typhimurium, have also been reported in previous studies (Eguale et al., 2014; Lee et al., 2004).

3.5. Mutations within gyrA and parC

One hundred and eighty-eight quinolone-resistant isolates were analyzed by MAMA PCR. The results defined fifteen groups according to mutations in *gyrA* and *parC* (Table 6). Except for four strains which had no mutations in *gyrA* and *parC*, the remaining 184 quinolone-resistant isolates had at least one mutation within *gyrA* or *parC* (alone or in combination with other mutations in *gyrA* or *parC*), which indicated that resistance phenotype was considerably associated with the occurrence of resistance gene.

Mutations were found in *gyrA* (Ser-83, n=144; Asp87, n=152) and *parC* (Ser-80, n=140; Glu-84, n=145). A total of 64 isolates had a single mutation in either Ser-83 or Asp-87 of *gyrA*, while 55 isolates had one mutation either in Ser-80 or Glu-84 of

parC. Moreover, 32 isolates had double mutations in *gyrA*, one mutation in *parC* (Glu-84 or Ser-80), and 34 isolates had a single mutation in *gyrA* (Ser-83 or Asp-87) and double mutations in *parC*. Additionally, 79 isolates had double mutations in both *gyrA* and *parC*. High prevalence of mutations in Ser-83 (100%, n=14) and Asp-87 (100%, n=14) of *gyrA* and Glu-84 (85.7%, n=12) of *parC* was found from frozen meat samples. Lower frequency of mutations in *gyrA* and *parC* was found among isolates from 2014 (Ser-83, 21.9%; Asp-87, 65.5%; Ser-80, 40.6%; Glu-84, 46.9%).

In conclusion, overall, antimicrobial resistance presented an increasing trend along the slaughtering process. The result suggests that raw broiler chicken might act as the reservoir for MDR *Salmonella* to some extent, and cross-contamination between chicken carcasses and processing environment or workers in this processing plant occurs, which leads to the increase of percentage of MDR strains in the latter processing stages and may facilitate the dissemination of the resistance genes to consumers along the slaughter and processing chain. Therefore, efficient measures to facilitate the reasonable use of antimicrobials in animal husbandry must be taken. Also, more importantly, strict hygiene method and HACCP management during slaughter are vital for preventing food infection caused by *Salmonella*.

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Figure Captions

Fig. 1. Antimicrobial resistance patterns of *Salmonella* isolates from slaughter line**.** (a) Antimicrobial resistance patterns of *Salmonella* isolates to 10 antimicrobial agents. (b) resistance patterns of *Salmonella* from different sampling points, (c) resistance patterns of *Salmonella* with different serotypes, (d) resistance patterns of *Salmonella* isolates from the year 2012 to 2014.

Table 1 Primers used for detection of genes encoding resistance to different antimicrobials

Antimicrobial(s)	Target gene	Nucleotide sequences	Size (bp)	Reference
β-lactams	*bla*$_{TEM}$	F: GAGTACTCACCA TCACAGAA AAGC R: GACTTCCCGTCGTGTAGATAAC	489	(Lu et al., 2010)
	bla$_{SHV}$	F: TTA TCT CCC TGT TAG CCA CC R: GATTTGCTGATTTCGCGCCG	797	(Poirel et al., 1999)
	bla$_{CTX-M}$	F: TTTGCGATGTGCAGTACCAGTAA R: CGATATCGTTGGTGGTGCCATA	544	(Edelstein et al., 2003)
aminoglycosides	*aac(3)-II*	F: TGAAACGCTGACGGAGCCTC R: GTCGAACAGGTAGCACTGAG	369	(Jensen et al., 2006)
	aac(3)-IV	F: GTGTGCTGCTGGTCCACAGC R: AGTTGACCCAGGGCTGTCGC	627	(Jensen et al., 2006)
	ant(2'')-I	F: GGGCGCGTCATGGAGGAGTT R: TATCGCGACCTGAAAGCGGC	328	(Jensen et al., 2006)
tetracycline	*Tet*(A)	F: GTAATTCTGAGCACTGTCGC R: GAGACGCAATCGAATTCGG	956	(Aarestrup et al., 2003)
	Tet(B)	F: GAGACGCAATCGAATTCGG R: TTTAGTGGCTATTCTTCCTGCC	228	(Jiang and Shi, 2013)
	Tet(C)	F: CTTGAGAGCCTTCAACCCAG R: ATGGTCGTCATCTACCTGCC	418	(Fan et al., 2007)
	tet(G)	F: GCTCGGTGGTATCTCTGCTC R: AGCAACAGAATCGGGAACAC	468	(Fan et al., 2007)
florfenicol	*floR*	F: AACCCGCCCTCTGGATCAAGTCAA R: CAAATCACGGGCCACGCTGTATC	549	(Ghoddusi et al., 2015)
sulfonamide	*sul1*	F: CTTCGATGAGAGCCGGCGGC R: GCAAGGCGGAAACCCGCGCC	437	(Aarestrup et al., 2003)
	sul2	F: GCGCTCAAGGCAGATGGCATT R: GCGTTTGATACCGGCACCCGT	285	(Aarestrup et al., 2003)
	sul3	F: AGATGTGATTGATTTGGGAGC	443	(Zhang et al., 2009)

	R: TAGTTGTTTCTGGATTAGAGCCT		
intI 1	F: TCTCGGGTA ACATCA AGG R: AGGAGATCCGAAGACCTC	242	(Vo et al., 2006)
Conserved segment	CS-F: GGCATCCAAGCATCCTCG	a	
Conserved segment	CS-R: GGCATCCAAGCAGCAAG	a	

a: size depends on the gene cassette(s) inserted.

Table 2 Primer sequences of *gyrA* and *parC* genes by MAMA PCR (Qiang et al., 2002)

Primers	DNA sequence (5′→3′)	Size (bp)	Amino acid positions
WP*gyrA*	GACCTTGCGAGAGAAATTACAC	540	7-28
Control*gyrA*	GATGTTGGTTGCCATACCTACG	540	546-525
MAMA*gyrA*83	TCGTGTCATAGACCGGGC	259	Ser-83
MAMA*gyrA*87	GCGCCATGCGGACGATCGTTTC	274	Asp-87
WP*parC*	CGGAAAACGCCTACTTAAACTA	446	41-62
Control*parC*	GTGCCGTTAAGCAAAATGT	446	506-488
MAMA*par*C80	ATCGCTTCATAACAGGCTCT	217	Ser-80
MAMA*par*C84	CCATCAGGACCATCGCCTC	238	Glu-84

Table 3 Occurrence and serotypes of *Salmonella* isolates from four points in the slaughter chain

Sources	% (No.) of isolates			
	Total	*S. enterica* Ser. Enteritidis	*S. enterica* Ser. Typhimurium	Untypable
Point 1	47.96% (94/196)[a]	40.31% (79/196)[b]	0[b]	7.65% (15/196)[b]
Point 2	17.98% (34/181)[a]	13.81% (25/181)[b]	3.31% (6/181)[b]	1.66% (3/181)[b]
Point 3	31.33% (47/150)[a]	14.00% (21/150)[b]	10.67% (16/150)[b]	6.67% (10/150)[b]
Point 4	14.00% (14/100)[a]	7.00% (7/100)[b]	7.00% (7/100)[b]	0[b]
Total	30.14% (189/627)	21.05% (132/627)[c]	4.62% (29/627)[c]	4.46% (28/627)[c]

Note: 'a' represents the prevalence rate of *Salmonella* isolates from different sampling points; 'b' represents the prevalence rate of *Salmonella* with different serotypes from different sampling points; 'c' represents the overall percentage of *Salmonella* with different serotypes.

Table 4 Antimicrobial resistance profiles of *Salmonella* isolates from four sampling points

Resistant phenotypes	Point 1	Point 2	Point 3	Point 4	Total
NAL		3			3
NAL-AMP	33	10	10	1	54
SXT-NAL	7	3	4	2	16
AMP-NAL-CIP	4				4
NAL-AMP-TET	4		2		6
NAL-AMP-SPE	2	1			3
SPE-SXT-NAL	1				1
NAL-AMP-AMC	1				1
AMP-NAL-GEN-SPE			1		1
TET-SXT-AMP-NAL	3	4		1	8
TET-SXT-CIP-NAL	1				1
NAL-CIP-AMP-AMC		1			1
NAL-CIP-AMP-SPE		1			1
NAL-CIP-AMP-FLO-SXT	1				1
NAL-CIP-AMP-SPE-SXT	1				1
FLO-TET-AMP-NAL-CIP			1		1
TET-SXT-AMP-NAL-CIP	1	2		2	5
FLO-TET-SXT-CIP-NAL	1				1
AMP-AMC-SXT-TET-NAL			1		1

AMP-AMC-CIP-TET-NAL			4		4
AMP-CIP-TET-SPE-NAL			12	3	15
NAL-CIP-AMP-FLO-SPE-SXT	1	1			2
NAL-CIP-AMP-FLO-SPE-TET	1				1
NAL-CIP-AMP-FLO-TET-SXT	6				6
NAL-CIP-AMP-SPE-TET-SXT	4	1	2		7
NAL-CIP-AMP-GEN-TET-SXT		1			1
AMP-CRO-TET-SXT-SPE-NAL				1	1
TET-SXT-AMP-AMC-NAL-CIP			1		1
AMP-AMC- CIP-TET-SPE-NAL			2		2
AMP-SXT-TET-SPE-NAL-FLO	1				1
NAL-CIP-AMP-AMC-FLO-TET-SXT			1		1
NAL-CIP-AMP-FLO-GEN-TET-SXT	5	1			6
TET-SXT-AMP-NAL-GEN-CIP-SPE		1			1
TET-SXT-AMP-NAL-CIP-SPE-AMC	1				1
AMP-SXT-TET-CRO-SPE-NAL-FLO-CIP	1		1		2
AMP-CIP-SXT-TET-GEN-CRO-NAL			1		1
AMP-AMC-CIP-SXT-SPE-NAL-FLO			1		1
AMP-CIP-SXT-TET-SPE-NAL-FLO	10	2			12
NAL-CIP-AMP-AMC-SPE-GEN-TET-SXT	1				1
NAL-CIP-AMP-FLO-SPE-GEN-TET-SXT	2	1		1	4
AMP-AMC-CIP-SXT-TET-SPE-NAL-FLO	1				1

NAL-CIP-AMP-AMC-FLO-SPE-GEN-TET-SXT		1			1
NAL-CIP-AMP-CRO-FLO-SPE-GEN-TET-SXT			1	2	3
AMP-AMC-CIP-SXT-TET-GEN-CRO-SPE-NAL			1		1
FLO-TET-SXT-AMP-AMC-NAL-GEN-CIP-SPE			1	1	2
Total strains/sample groups	94	34	47	14	189

Table 5 Multi-drug resistance of *Salmonella* with different serovars from different sampling points in three years

Serovars	Numbers of antimicrobials / No. (%)			
	1-3	4-6	7-8	9
S. Enteritidis(n=132)	88 (66.7%)	26 (19.7%)	16 (12.1%)	2 (1.5%)
S. Typhimurium(n=29)	0	26 (89.7%)	1(3.6%)	2 (6.9%)
Untyped (n=28)	0	11 (39.3%)	15 (53.6%)	2 (7.1%)
Point 1 (n=94)	52 (55.4%)	21 (22.3%)	21 (22.3%)	0
Point 1 (*S*. Enteritidis, n=79)	52 (65.8%)	14 (17.7%)	13 (16.5%)	0
Point 1 (*S*. Typhimurium, n=0)	0	0	0	0
Point 1 (Untyped, n=15)	0	7 (46.7%)	8 (53.3%)	0
Point 2 (n=34)	17 (50.0%)	14 (41.2%)	2 (5.9%)	1 (2.9%)
Point 2 (*S*. Enteritidis, n=25)	17 (68.0%)	5 (20.0%)	2 (8.0%)	1 (4.0%)
Point 2 (*S*. Typhimurium, n=6)	0	6 (100%)	0	0
Point 2 (Untyped, n=3)	0	3 (100%)	0	0
Point 3 (n=47)	16 (34.0%)	24 (51.1%)	4 (8.5%)	3 (6.4%)
Point 3 (*S*. Enteritidis, n=21)	16 (76.2%)	4 (19.0%)	1 (4.8%)	0
Point 3 (*S*. Typhimurium, n=16)	0	16 (100%)	0	0
Point 3 (Untyped, n=10)	0	4 (40.0%)	3 (30.0%)	3 (30.0%)

Point 4 (n=14)	3 (21.4%)	7 (50.0%)	1 (7.1%)	3 (21.4%)
Point 4 (*S*. Enteritidis, n=7)	3 (42.8%)	3 (42.8%)	0	1 (14.3%)
Point 4 (*S*. Typhimurium, n=7)	0	4 (57.1%)	1 (14.3%)	2 (28.6%)
Point 4 (Untyped, n=0)	0	0	0	0
2012(n=84)	51 (60.7%)	15 (17.8%)	16 (19.0%)	2 (2.4%)
2013(n=72)	37 (51.4%)	31 (43.0%)	2 (2.8%)	2 (2.8%)
2014(n=33)	0	17 (51.5%)	13 (39.4%)	3 (9.1%)

Table 6 Substitution mutations within *gyr*A and *par*C genes

Mutation groups	No. of isolates	*gyr*A87	*gyr*A83	*par*C80	*par*C84
I	8	Asp-87			
II	1		Ser-83		
III	1			Ser-80	
IV	1				Glu-84
V	8	Asp-87		Ser-80	
VI	5	Asp-87	Ser-83		
VII	5	Asp-87			Glu-84
VIII	6		Ser-83	Ser-80	
IX	2			Ser-80	Glu-84
X	2		Ser-83		Glu-84
XI	10	Asp-87	Ser-83	Ser-80	
XII	15	Asp-87		Ser-80	Glu-84
XIII	22	Asp-87	Ser-83		Glu-84
XIV	19		Ser-83	Ser-80	Glu-84
XV	79	Asp-87	Ser-83	Ser-80	Glu-84

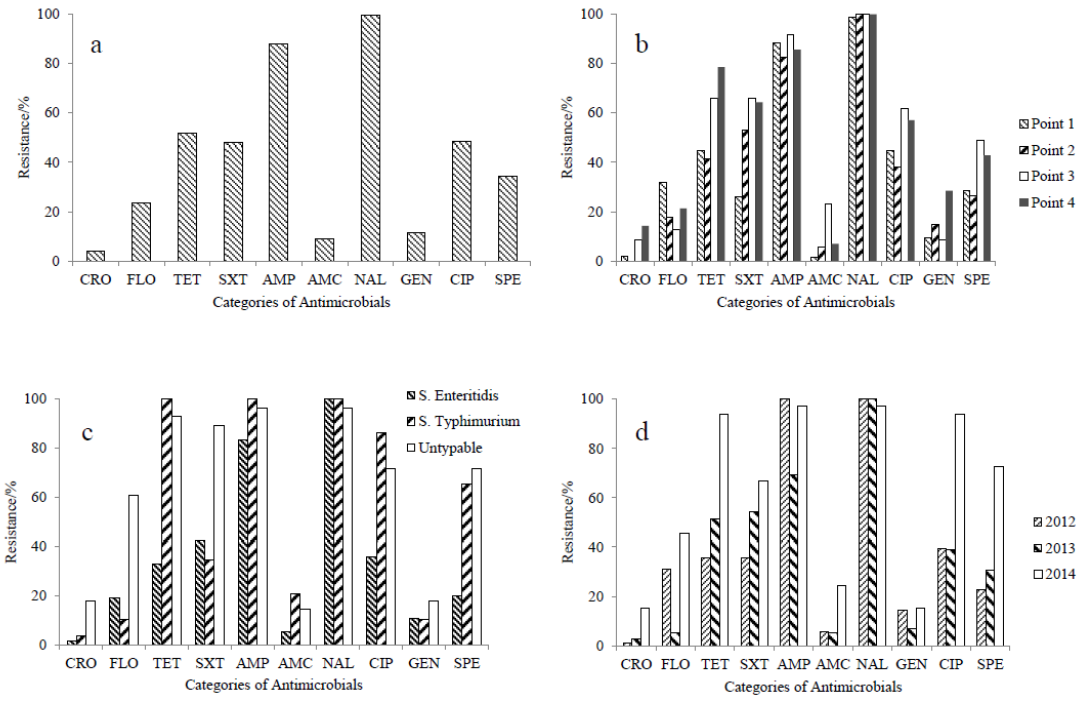

Fig. 1

Highlights

Prevalence and antibiotic resistance of *Salmonella* during slaughter were monitored.

Resistance genes, class 1 integron, *gyrA* and *parC* mutations were investigated.

Multidrug resistance rate was 60.8% (from 44.6% to 78.6% along the slaughter line).

Two gene cassettes were identified in class 1 integron-positive isolates.