

Prevalence, Antimicrobial Resistance, and Relatedness of Salmonella Isolated from Chicken, Pork and the Environment at Abattoirs and Supermarkets in Chongqing, China

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Abstract

Background: *Salmonella* is one of the most important foodborne pathogens, causing outbreaks of human salmonellosis worldwide. Owing to large scales of consumption markets, pork and poultry that contaminated by *Salmonella* could pose a tremendous threat to public health. The aim of this study was to investigate the contamination of *Salmonella* from chicken, pork and the environment in slaughtering and retailing segments in Chongqing, China. Results: A total of 115 *Salmonella* isolates were recovered from 1122 samples, with 10.76% (47/437) in slaughterhouses and 10.07% (68/675) in supermarkets. 30 different serotypes were identified, in which S. Derby, S. London and S. Rissen were mostly detected. Antimicrobial susceptibility testing and resistance genes verification were carried out to investigate the relationship between phenotypes and genotypes. Altogether, 75.65% isolates showed resistance to tetracycline, followed by 60.87% to doxycycline and 69.5% to ampicillin. More than half (50.43%) of the isolates were multidrug resistant, which were mostly from supermarkets ($P < 0.05$). According to antibiotic resistance genes detection results, a high correlation between antibiotic phenotypes and genotypes was presented by lactam-, tetracycline-, and sulfonamide-resistant isolates. Multilocus sequence typing results showed 24 out of 115 isolates were ST40, which was the most prevalent. Furthermore, isolates from supermarkets ($n=15$) had more sequence types than that in slaughterhouses ($n=3$). Conclusion: Our study highlighted the fact that *Salmonella* contamination were more severe in pork production chain than that of chicken. Isolation rates were similar in slaughterhouses and supermarkets for both pork and chicken, but isolates from supermarkets had more MDR profiles and represented a wide range of serotypes and sequence types, indicating that more diverse sources of contamination in retailing.

Background

Salmonella, a foodborne pathogen, causes diarrhoeal diseases even death in both humans and animals [1]; it can survive in a dry environment for several weeks or even in water for several months [2]. According to previous surveys, the aetiological agent of salmonellosis largely attributed to contaminated food, which mostly were poultry and pork [3, 4]. In China, pork is the mainstream of meat consumption. Meanwhile, the consumption of poultry is rising year by year. Slaughtering process of chicken and pork as well as their corresponding selling process, which were contaminated by *Salmonella*, could be a potential pathway to threat public health.

Although illness caused by *Salmonella* is always self-limiting, antimicrobial treatment in severe cases is still necessary [5]. Long-term medication with antibiotics has led to selection pressure to bacterial, which causes antimicrobial resistance, and even multidrug resistance (MDR). Extended-spectrum- β -lactams (ESBL) and fluoroquinolones are frequently used to treat *Salmonella* infections, so the increasing resistance to these antimicrobial agents is an emerging problem worldwide [6]. Up to now, some studies have reported some specific genes in *Salmonella* associated with antimicrobial resistance [7, 8]. TEM, CTX-M, OXA are β -lactamases that mediated the resistance to β -lactam antibiotics in *Salmonella* [9, 10].

Especially *bla*_{CTX-M} and *bla*_{OXA} code for cephalosporinases that hydrolyse most of β -lactams [11]. In addition, florfenicol exporter genes *cmlA9* and *floR* account for phenicol resistance in *Salmonella* [8]. Tetracycline efflux can occur through Tet major facilitator superfamily (MFS)-type pumps [12]. The most frequent types of *tet* genes are classes A, B, C, D, and G [13, 14]. Dihydropteroate synthase genes (*sul1*, *sul2*, *sul3*), which are responsible for sulfonamide resistance, has been detected in *Salmonella* [8].

Typing methods used to investigate the characterization of *Salmonella* can help to enrich our knowledge of its regularity of dissemination. Serotyping presents a well-established methodology for typing of *Salmonella* [15]. To date, approximately 2600 serotypes have been discovered. The traditional method for serotyping—the Kauffmann-White-Le Minor Scheme requires a series of antisera, consuming time and money. Hence, a various of typing methods were established to study the molecular epidemiologic characterization of *Salmonella* with its transmission dynamics, including pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), whole-genome sequencing (WGS), and multilocus sequence typing (MLST) [16-19]. Compared with other methods, MLST is a highly repeatable typing method that based on sequence analysis of selected housekeeping genes. Recently, approximately 224,516 *Salmonella* strains has been uploaded by users in the MLST database [20], which becomes a convenient tool for researchers.

Contamination and antimicrobial resistance of *Salmonella* isolated from food-producing animals is severe worldwide [21], particularly in China [22-24]. Furthermore, several studies reported that *Salmonella* isolates could be recovered from farms, slaughterhouses and retail markets [25, 26] and a previous study indicated that *Salmonella* isolates could transmitted from slaughterhouses to retail markets in pig production chain [23]. However, few studies focused on the comparison of *Salmonella* contamination in pig and chicken as well as their slaughtering and retailing chain. Therefore, the intention of this study was to compare the prevalence, antimicrobial resistance, and genetic relationship of *Salmonella* isolates recovered from the environment, chicken and pork at abattoirs and supermarkets located in Chongqing, China.

Methods

Sample collection

From March to October in 2015, a total of 1122 samples were isolated from 7 slaughterhouses (chicken meat, n=150; slaughtering environment of chicken, n=109; pork, n=42; slaughtering environment of pork, n=136) and 5 supermarkets (chicken, n=92; retail environment of chicken, n=350; pork, n=58; retail environment of pork, n=175), in 12 districts of Chongqing, China. Meat mentioned above was from unpacked fresh meat; slaughtering environment including wash water, knives, floor, feces, apparatus, containers, tables, carcasses, and blood; retail environment including chopping boards, ice, knives, floor,

containers, wash water, and tables. All collected samples were stored in an icebox and transported to a laboratory within 2 hours of collection for immediate processing and then held in a refrigerator at 4°C.

Isolation and serotyping

After a pre-enrichment step of each samples in 10 mL sterile buffered peptone water (BPW) and incubated overnight at 37°C. 0.2mL of each pre-enriched suspensions were added into 2 ml of Rappaport-Vassiliadis enrichment Broth (RVB) and 2 ml of Tetrathionate broth (TTB) respectively, then incubated at 42°C for 24 h. One loopful of each RVB and TTB culture was then streaked onto Xylose Lysine Tergitol 4 (XLT-4) agar plates, which were incubated at 37°C for 24 to 48 h. Among suspected colonies, only one was picked up from a plate and confirmed by specific gene through Polymerase Chain Reaction (PCR) of *Salmonella* using assays. Each isolate was serotyped by slide agglutination based on the Kauffmann-White-Le Minor Scheme [43].

Antimicrobial susceptibility testing

The standard Kirby-Bauer disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010) was carried out to test antimicrobial susceptibility of the *Salmonella* isolates to 13 categories of antimicrobials (Hangzhou Microbial Reagent., Ltd.): ampicillin (AMP 10 µg), cefoperazone (CFP 75 µg), piperacillin (PRL 100 µg), tetracycline (TE 30 µg), ceftazidime (CAZ 30 µg), doxycycline (DOX 30 µg), ceftriaxone (CRO 30 µg), minocycline (MH 30 µg), norfloxacin (NOR 10 µg), sulfamethoxazole (SXT 1.25 µg), ofloxacin (OFX 5 µg), chloramphenicol (C 30 µg) and ciprofloxacin (CIP 5 µg). *Escherichia coli* ATCC 25922 was invoked as the control organism. According to the CLSI, the isolates were considered to be susceptible, intermediate, or resistant. *Salmonella* isolates resistant to three or more antimicrobial classes were defined as MDR isolates.

Antimicrobial resistance genes

All of the drug-resistant *Salmonella* isolates were examined for the presence of resistance genes, including β-lactams (*bla*_{TEM}, *bla*_{CTX-M} and *bla*_{OXA}), tetracycline (*tet*(A), *tet*(B) and *tet*(C)), florfenicol (*florR*), and sulfonamide (*sul1*, *sul2* and *sul3*). All of the genes were identified by PCR amplification. The primer sequences and predicted sizes for PCR amplification of different resistance genes from *Salmonella* were listed in Table 1. PCR amplification was performed using a DNA Thermal Cycler (Life Technologies, USA) with the following conditions: 95°C for 10 min; 30 cycles of 94°C for 45 s, 55-70°C for 50 s, 72°C for 50 s; and 72°C for 10 min. 2% (w/v) agarose (Biowest, Spain) gel electrophoresis was used to analyze the PCR products.

Multilocus sequence typing

Protocols used for MLST of *Salmonella* were described online [51]. Seven housekeeping genes were amplified by PCR, including *thrA*, *purE*, *sucA*, *hisD*, *aroC*, *hemD*, and *dnaN*. PCR products were purified and sequenced by Sanger method, and the alleles and STs were assigned according to the MLST scheme [52]. The unweighted pair group method with arithmetic means analysis (UPGMA) was utilized to infer relationships among the isolates through MEGA7 software [53].

Statistical analysis

All statistical analyses were done using SPSS 20.0 (SPSS Inc., Chicago, IL), and the chi-squared test was applied to assess any statistically significant ($P < 0.05$) differences in this study.

Results

Prevalence and serotyping of *Salmonella* isolates

A total of 115 (10.25%) *Salmonella* isolates were recovered from 1122 samples, which were collected from chicken (9.50%, 23/242), pork (44.00%, 44/100), and the environment (6.23%, 48/770) at slaughterhouses (10.76%, 47/437) and supermarkets (10.07%, 68/675) (Table 2). In slaughterhouses, the isolation rates of pork, chicken and the environment were 42.86%, 9.33%, and 6.12%, respectively. In supermarkets, the isolation rates of pork, chicken and the environment were 44.82%, 9.78%, and 6.29%, respectively.

30 distinct serovars were identified, including 10 in slaughterhouses (Fig. 1A) and 26 in supermarkets (Fig. 1B). In addition, seven isolates were uncertain serotypes, which were not included in the 30 identified serotypes. *Salmonella* Derby (n=26), London (n=15), and Rissen (n=12) were the most commonly observed serotypes in this study. Six serotypes (Derby, Typhimurium, London, Give, Rissen, and Jerusalem) were shared both in supermarkets and slaughterhouses. *Salmonella* Rideau, Seegefeld, Weltevreden, and Kiel were only isolated in slaughterhouses. Twenty serotypes, including Entertidis, were only detected in supermarkets (Fig. 1A and B).

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Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of 115 *Salmonella* isolates to 13 antimicrobials was also performed. Resistance to TE was the most commonly observed in both the supermarkets (75.00%) and the slaughterhouses isolates (70.21%). High rates of resistance were also noted for AMP (70.58% and 61.70%) and DOX (51.47%, 70.21%) (Fig. 2A) in supermarkets and slaughterhouses, respectively. Overall, 85.22% isolates were resistant to at least one antibiotic; 50.43% were MDR (Fig. 2E), including 13.04% from slaughterhouses and 37.39% from supermarkets. Specifically, in slaughterhouses and supermarkets, the rates of one-class-resistant isolates in chicken were 28.87% and 11.11%, in pork were 16.67% and 11.54%, and in the environment were 13.33% and 9.09% (Fig. 2C); the rates of two-class-resistant isolates in chicken were 28.57% and 11.11%, in pork were 27.78% and 15.38%, and in the environment were 53.33% and 6.06% (Fig. 2D).

Prevalence of antimicrobial resistance genes

Among 82 beta-lactam-resistant isolates, 87.80% (n=72) had at least one resistance gene of *bla*_{TEM}, *bla*_{CTX-M} or *bla*_{OXA}. Tetracycline resistance genes existed in 95.35% (n=82) tetracycline-resistant isolates (n=86). 92.86% (n=52) sulfonamide-resistant isolates harbored at least one resistance *sul* gene. Unlike other categories of antibiotic-resistance isolates, the *floR* gene was only identified in 41.07% (n=23) florfenicol-resistant *Salmonella* isolates (n=56) (Table 3).

Multilocus sequence typing

Multilocus sequence typing was used to identify the relatedness of *Salmonella* in pork, chicken and the environment from slaughterhouses and supermarkets. 108 of 115 isolates belonged to 23 different STs (Fig. 3), including 15 from supermarkets, 3 (ST543, ST365 and ST516) from slaughterhouses and 5 (ST19, ST34, ST40, ST155 and ST469) from both sites. Most of the serotypes were detected in less than 10 isolates, except for ST40, ST155 and ST469. The largest population of isolates were ST40 (n=24), including 18 from slaughterhouses and 6 from supermarkets. ST155 (n=19) and ST469 (n=17) also occupied a large proportion. ST155 isolates uniformly distributed, whereas ST469 isolates were mostly from the environment (n=9). Some *Salmonella* isolates presented similar sequence types that belonged to the same serovar. For example, all of ST40 isolates were S. Derby, 17 out of 19 S. London isolates were ST155 and 12 out of 17 Rissen isolates belonged to a single cluster (ST469). Similarly, the antibiotic susceptibility of the same cluster resembled such as ST17, of which 5 out of 6 isolates showed resistance to 8 to 12 categories of antibiotics (Fig. 3).

Discussion

For the purpose of this study, *Salmonella* isolates were recovered from scores of sites, including chicken, pork and the environment at abattoirs and supermarkets. All isolates were used to assess the relationship

between antimicrobial-resistant phenotypes and genotypes, then analyzed by serotyping and MLST to determine their genetic relationships. The results showed, compared with chicken, pork was a more severe contaminated reservoir. Again, supermarkets exhibited a higher MDR *Salmonella* isolation rate and more diversity in serotypes and sequence types than slaughterhouses, becoming a dangerous segment to public health.

The overall prevalence of *Salmonella* in our study was 10.25%, which was lower than previous studies conducted in Sichuan province [17] and Yangzhou city [23], but close to surveys in three provinces of central China [27] and Germany [28]. It should be noted that although isolation rate in slaughterhouses (10.76%) was similar to that in supermarkets (10.07%), the contamination of pork (44%) from the two sites was much more severe than chicken (9.50%). Other studies also showed that *Salmonella* contamination rates in pork varies from 30% to 70% at retail markets [29, 30] and from 10% to 50% at slaughterhouses [31, 32], indicating that poor control measures were performed in slaughtering and retailing chain of pork. For example, poor general hygiene and unsuitable storage conditions were commonly detected in pig slaughterhouses. Also, lacking of appropriate storage methods and regular disinfection increased the risk of *Salmonella*-colonizing activity at retail markets. In general, all of the differences attributed to collection seasons, amounts of samples and types, isolation methods and management.

As for serotyping, all isolates belonged to 30 distinct serotypes. *S. Derby* was the dominant serotype, which was the same with other studies [17, 23]. *S. Derby*, generally detected in pork, could cause salmonellosis in many countries [33]. But in our study, *S. Derby* were mostly detected in the environment. *S. Rissen* was generally considered to be transported through pig products in European countries [34]. In contrast, we isolated it from all kinds of sources, which indicated that the two serovars could not only exist in pig and pork productions. To our knowledge, *S. Typhimurium* and *S. Enteritidis* are the main pathogens causing acute human infection [35]. This study detected *S. Enteritidis* from supermarkets and *S. Typhimurium* from both sites, which had potential threats to public health. In addition, more categories of serotypes were detected in supermarkets than slaughterhouses, demonstrating the various and abundant sources of contamination in retail segments.

Irrational use of drugs will lead to the generation of antimicrobial resistant isolates, making it more difficult to cure salmonellosis. So, it is important to investigate the situation of antibacterial resistance and provide a guidance for clinical medication. In this study, most of the isolates showed resistance to tetracycline and ampicillin, which was similar to a previous study [36]. The high prevalence of ampicillin- and tetracycline-resistance indicated high doses of these two antibiotics consumption in this area. It was noteworthy that more than half of the isolates exhibited MDR profiles, which were mostly from

supermarkets ($P < 0.05$). The results showed that, compared with slaughterhouses, *Salmonella* contamination occurred in supermarkets was much more severe. The sanitation control of supermarkets was often neglected. When the most direct place that contact with consumer—supermarkets were contaminated by MDR *Salmonella*, the damage to public health is unimaginable. So it is necessary to establish a standard environmental control method in supermarkets.

More than half of the antibiotic resistant isolates harbored genotypes, especially 95.35% of tetracycline-resistant isolates had the same kind of antibiotic genes, indicating a close relationship between antimicrobial resistance genes and phenotypes. Another antibiotic—florfenicol, used as a replacement for chloramphenicol in animals' salmonellosis treatment, appeared to have more and more drug-resistant isolates in recent years [37]. The *floR* gene is a florfenicol-chloramphenicol resistance gene in *Salmonella enterica* that belongs to the major facilitator (MF) superfamily of drug exporters [38]. Some studies reported the appearance of florfenicol-resistant phenotype had a high correlation with *floR* [39, 40]. However, in the present study it was approximately 41.07%. The possible reason was that florfenicol resistance may cause by other genes, such as *optrA* and *cfr* [41].

A total of 108 isolates belonged to 23 different STs. More STs detected in supermarkets indicated that, compared with slaughterhouses, supermarkets had a more diversity of contamination sources. Some STs in this study related to specific serovars, for instance S. London with ST155, ST469 with S. Rissen, and ST40 with S. Derby. The results confirm the conjecture that multilocus sequence typing could be an alternative method for serotyping in the future [42]. As for the relationship between MLST and antibiotic resistance, most of ST17 isolates showed resistance to a wide range of antibiotics, which was a dangerous sequence type in this study.

Abbreviations

MDR: multidrug resistance; **ESBL:** Extended-spectrum- β -lactams; **CLSI:** Clinical and Laboratory Standards Institute; **MLST:** multilocus sequence typing; **ST:** sequence type; **AMP:** ampicillin; **CFP:** cefoperazone; **PRL:** piperacillin; **TE:** tetracycline; **CAZ:** ceftazidime; **DOX:** doxycycline; **CRO:** ceftriaxone; **MH:** minocycline; **NOR:** norfloxacin; **SXT:** sulfamethoxazole; **OFX:** ofloxacin; **C:** chloramphenicol; **CIP:** ciprofloxacin; **UPGMA:** the unweighted pair group method with arithmetic means analysis.

Declarations

Ethics approval and consent to participate

All animal procedures were reviewed and approved by the Ethics Committee of Southwest University.

Consent for publication

Not applicable.

Availability of data and material

All data are fully available without restriction.

Competing interests

The authors declare that they have no competing interests.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Authors' contributions

T.C., J.J., and D.X. performed the experiments and analyzed the data. Z.Z., J.X., and X.C. collected samples. C.Y., D.H., and Y.P. helped in analyzing the data and designing the experiments. R.F., supervised the study, R.F., T.C., and J.J., drafted the manuscript. All authors read and approved the final manuscript.

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Tables

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

Antimicrobials	Target genes	Nucleotide sequences	Size (bp)	References
β-Lactams	<i>bla_{TEM}</i>	F: GCGGAACCCCTATTT	964	[44]
		R:TCTAAAGTATATATGAGTAAACTTGGTCT		
	<i>bla_{CTXM}</i>	F: TTTGCGATGTGCAGTACCAGTAA	544	[45]
Florfenicol	<i>bla_{OXA}</i>	R:CGATATCGTTGGTGGTGCCATA	590	[46]
		F: ACCAGATTCAACTTTCAA		
		R: TCTTGGCTTTTATGCTTG		
Sulfonamide	<i>floR</i>	F: AACCCGCCCTCTGGATCAAGTCAA	549	[47]
		R: CAAATCACGGGCCACGCTGTATC		
		F: CTTCGATGAGAGCCGGCGGC		
Sulfonamide	<i>sul1</i>	R: GCAAGGCGGAAACCCGCGCC	437	[48]
		F: GCGCTCAAGGCAGATGGCATT		
		R: GCGTTTGATACCGGCACCCGT		
Sulfonamide	<i>sul2</i>	F: AGATGTGATTGATTTGGGAGC	443	[49]
		R: GCGTTTGATACCGGCACCCGT		
		F: AGATGTGATTGATTTGGGAGC		
Tetracycline	<i>sul3</i>	R: TAGTTGTTTCTGGATTAGAGCCT	658	[48]
		F: GCTGTCGGATCGTTTCGG		
		R: CATTCCGAGCATGAGTGCC		
Tetracycline	<i>tet(A)</i>	F: CTGTCGCGGCATCGGTCAT	615	[50]
		R: CAGGTAAAGCGATCCCACC		
		F: CTTGAGAGCCTTCAACCCAG		
Tetracycline	<i>tet(B)</i>	R: ATGGTCGTCATCTACCTGCC	418	[49]
		F: CTTGAGAGCCTTCAACCCAG		
		R: ATGGTCGTCATCTACCTGCC		

Table 2. Positive isolation rates of *Salmonella* from different sampling sources

Sampling Site	Sampling Sources	No. of Samples	Positive No. of Isolates	Isolation Information
Slaughterhouse	Chicken	150	14	9.33%
	Pork	42	18	42.86%
	Environment	245	15	6.12%
	Total	437	47	10.76%
Supermarket	Chicken	92	9	9.78%
	Pork	58	26	44.82%
	Environment	525	33	6.29%
	Total	675	68	10.07%

Table 3. Prevalence of antibiotic-resistant genotypes in antibiotic-resistant *Salmonella* isolates

Categories of genes	Chicken	Pork	Environment	Total
β -Lactams	17 ^a /18 ^b	25/30	30/34	72/82(87.80%)
Tetracycline	18/19	34/35	30/32	82/86(95.35%)
Sulfonamide	12/13	17/19	23/24	52/56(92.86%)
Florfenicol	6/10	10/30	7/16	23/56(41.07%)

^aNumber of positive *Salmonella* isolates

^bNumber of antibiotic-resistant *Salmonella* isolates

Figures

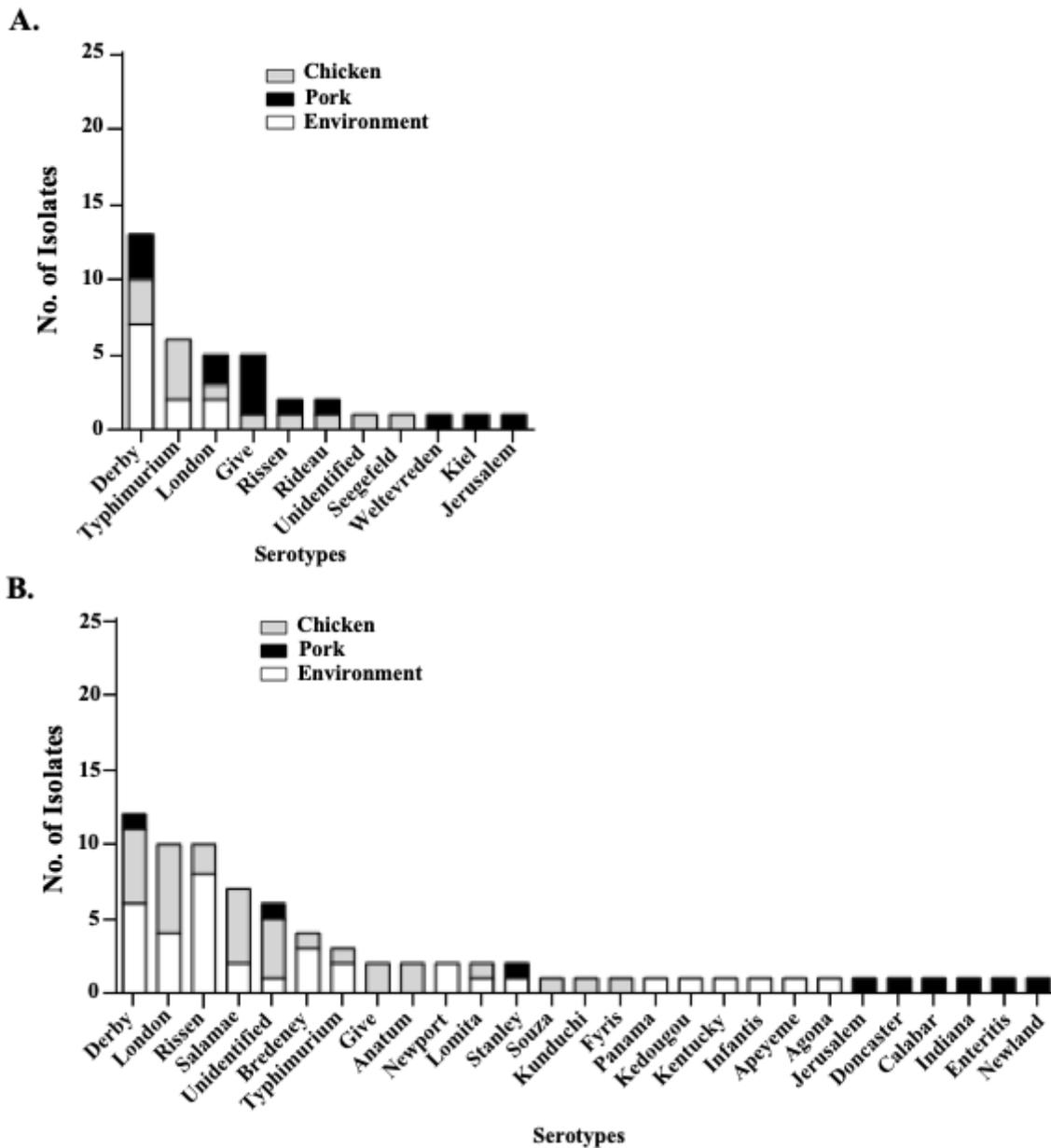


Figure 1

Serotype results of Salmonella isolates (A) Serotype results of isolates from chicken, pork and the environment in slaughterhouses. (B) Serotype results of isolates from chicken, pork and the environment in supermarkets.

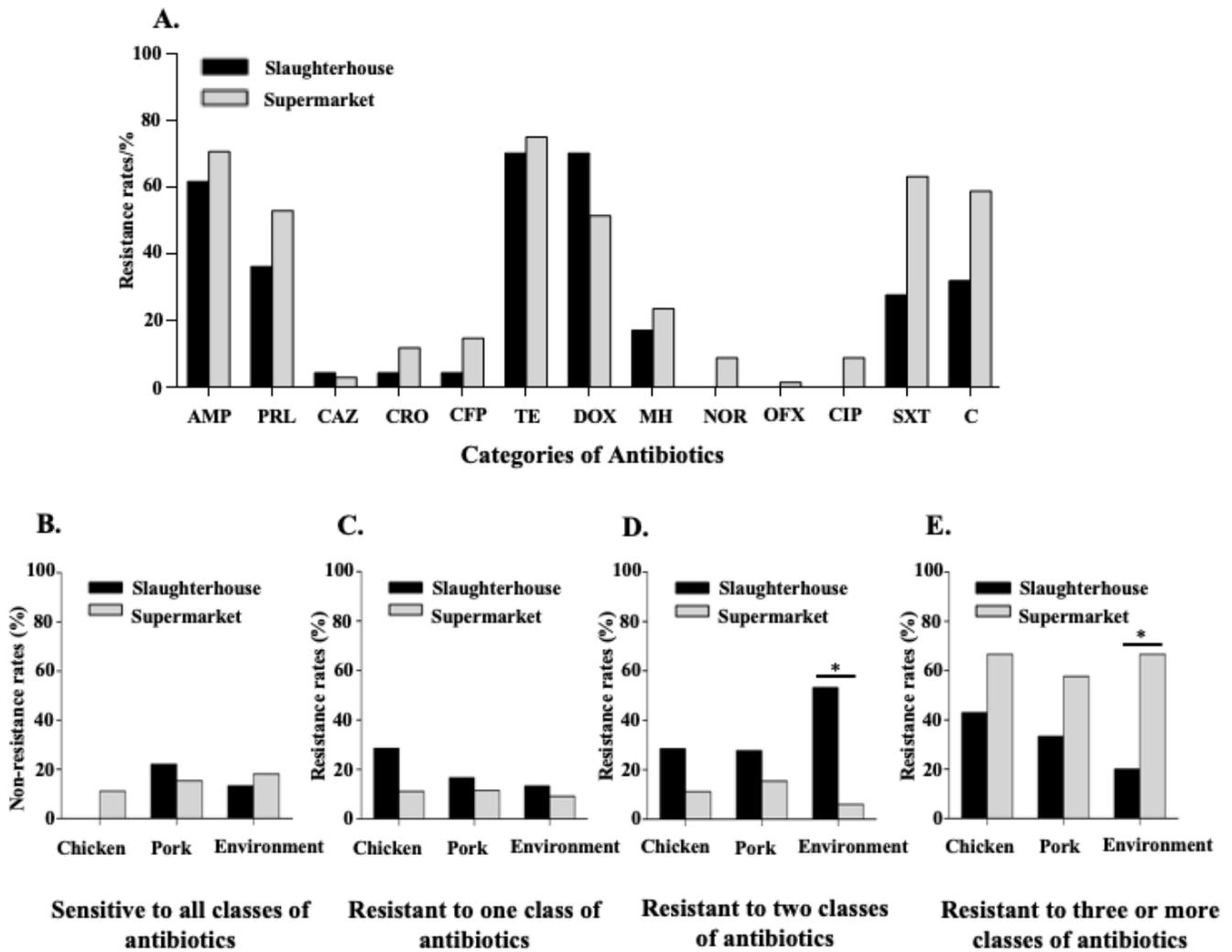


Figure 2

Antimicrobial resistance results of Salmonella isolates (A) The resistance rates of Salmonella from different sampling sources. (B) The rates of sensitivity Salmonella isolates to all classes of antibiotics. (C) The rates of resistant Salmonella isolates to one class antibiotics. (D) The rates of resistant Salmonella isolates to two classes antibiotics. (E) The rates of MDR Salmonella isolates. Statistical significance was determined by chi-squared test (*P<0.05).

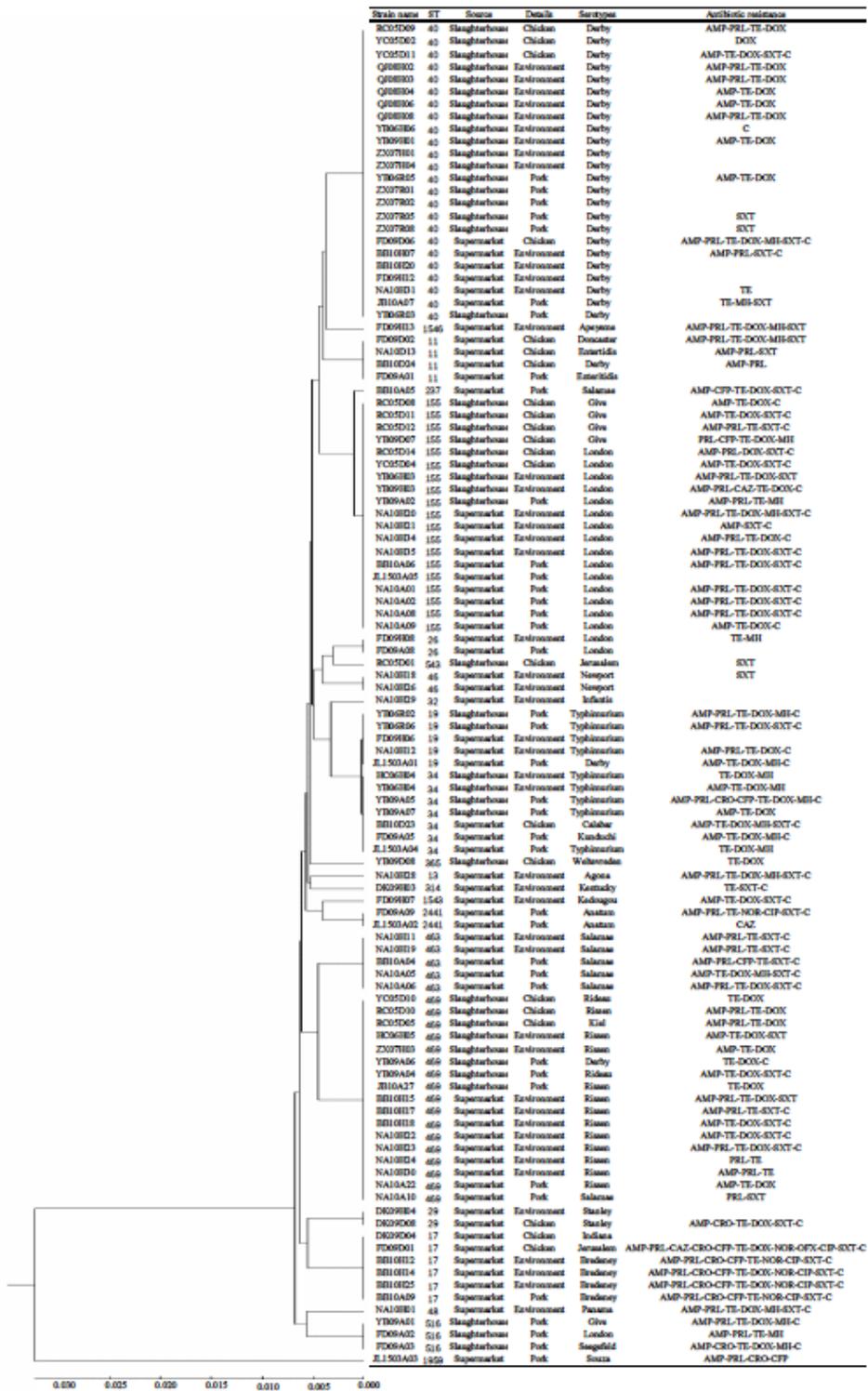


Figure 3

Unweighted pair group method with arithmetic means (UPGMA) dendrogram based on multilocus sequence typing (MLST) profiles of the 108 Salmonella isolates from slaughterhouses and supermarkets.