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Salmonella isolated from the slaughterhouses and correlation with pork contamination in free market

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ABSTRACT

This study surveyed the distribution, antimicrobial susceptibility profiles and serotype of *Salmonella* isolated from three slaughterhouses, and performed molecular typing on these isolates, to understand the relationship between strains of *Salmonella* obtained from the pork production chain in Yangzhou, China. Samples from slaughtered pigs and the slaughtering environment were collected from three slaughterhouses in Yangzhou, Jiangsu province, from October 2012 to July 2013. The positive identification rates of *Salmonella* in slaughtered pigs and the environmental samples were 46.6% and 48.8%, respectively. The prevalence of *Salmonella* in slaughterhouses were affected by seasonal factors and reached the peak in summer. Among the *Salmonella* serovars identified, *S.* Derby was most prevalent in slaughterhouses, but other serovars like *S.* Typhimurium, *S.* Meleagridis and *S.* Anatum were also widespread. Antimicrobial susceptibility testing revealed that 32 and 131 different MDR patterns were found among the strains from the environment and slaughtered pig samples, respectively. Fifty-six isolates of *S.* Derby and 16 strains of *S.* Typhimurium were characterized by the technique of pulsed-field gel electrophoresis (PFGE) using the restriction enzyme *Xba* I. 35 and 11 PFGE patterns were generated among the selected isolates. Four isolates of *S.* Derby isolates with the same pattern (PF26) were isolated from cooling water, evisceration and carcass, suggesting that cross contamination occurred between the environment and the slaughtered pigs. Six *S.* Typhimurium in cluster 1 with the same ST type (ST19) came from different parts of the slaughtered pig, which could have occurred because of horizontal transmissions along the slaughtering process. The same PFGE patterns of *Salmonella* were found in both samples from carcasses in the slaughterhouse and in the Yangzhou pork market, proving that *Salmonella* had spread from the slaughterhouse to the pork market. In conclusion, our study demonstrate that serious cross contamination occurred in Yangzhou slaughterhouses and can contribute *Salmonella* contamination in pork sold in the local public market.

1. Introduction

Salmonella is recognized as a major human food-borne pathogen and can cause a heavy economic burden for health care systems worldwide. Animals used as food play a role as a primary reservoir of non-typhoid *Salmonella*, and most human *Salmonella* infections are a consequence of eating foods of animal origin contaminated with *Salmonella* (EFSA, 2011). Contaminated pork and pork products are major sources of human *Salmonella* infections in many countries (Bonardi et al., 2013; De Busser et al., 2011; Delhalle et al., 2009).

Research has shown that *Salmonella* transmission may occur throughout the pork production chain (Lo Fo Wong et al., 2004), and many opportunities exist within the pig slaughterhouse for the contamination of pork carcasses with *Salmonella*. Based on an EFSA report (EFSA, 2011), the prevalence of *Salmonella*-positive swine-breeding holdings and swine-production holdings in the European Union was 28.7% and 33.3%, respectively, indicated a serious contamination in pig farms. The prevalence of shedding pigs may increase from farm to abattoir, mainly because of the stress of transportation, consequently increasing interinfection of pigs

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during lairage. *Salmonella* is spread to the carcass surface mainly from the carrier pig during the evisceration and slaughter operations can influence the bacterial contamination of pork carcasses in many ways, therefore, proper slaughter operation and good hygienic practices in the slaughterhouse can have a positive effect on the elimination of contamination in carcasses (Bonardi et al., 2003; De Busser et al., 2011). Slaughterhouses vary in their capability of dealing with *Salmonella*-positive pigs. Proper cleaning and control measures can reduce the risk of carcass contamination in slaughterhouses. In this sense, identifying the serotype and genotype of *Salmonella* isolates throughout the pig production chain were necessary to determine the cross-contamination and explore the relationship of the strains isolated from the environment and the pig carcass.

Salmonella enterica serotypes Derby and Typhimurium were the two most commonly detected serotypes in carcasses and in both clinical and nonclinical veterinary swine samples (Foley, Lynne, & Nayak, 2008). *S.* Typhimurium was also the most frequently isolated serotype from all human invasive infections reported. Similar results were reported by EFSA, where the most frequently reported serovars in human salmonellosis were in agreement with those isolated in slaughter pigs (EFSA, 2011). These findings support the notion that pigs and pork contribute to *Salmonella* infection in humans, although it is acknowledged that foods from other animal species also play a role as a source of these infections in humans (Hugas et al., 2014).

China is the largest producer of pork with the fastest growth rate in the world and this growth will continue in 2014: swine production is expected to reach 723 million head, pork production is expected to increase by two percent to 54.7 million tons, and consumption expected to rise to 55 million tons (Schneider et al., 2014). However, few reports have been published on *Salmonella* contamination throughout the slaughter process in China. Therefore, the aim of this study was to investigate the prevalence and antimicrobial resistance patterns of *Salmonella* contamination along the slaughter line through the detection of *Salmonella* in slaughtered pigs and their slaughterhouse environmental samples. Furthermore, sero- and genotyping were performed to define clonal relationships between isolates, assessing the dispersion of recovered strains and their involvement in cross contamination. Some of the strains isolated from pork in our previous study were also involved in our analysis to reveal the correlation between these two studies.

2. Materials and methods

2.1. Slaughterhouse description and sample plan

The study was carried out from October 2012 to July 2013 in three slaughterhouses (A, B and C) in Yangzhou, which were distributed in different areas of Yangzhou and represented 80% of the annual number of pigs slaughtered in Yangzhou. Table 1 describes the situations of the three slaughterhouses. Each visit was performed on a Tuesday during the nine sampling visits: V1 (slaughterhouse A, December 2012; slaughterhouse B, November 2012; slaughterhouse C, October 2012), V2 (slaughterhouse A, April 2013; slaughterhouse B, March 2013; slaughterhouse C, February 2013), V3 (slaughterhouse A, July 2013; slaughterhouse B, June 2013; slaughterhouse C, May 2013) and sampling started with the first batch of pigs slaughtered that day.

2.2. Slaughterhouse samples

A total of 684 samples were collected from the three slaughterhouses, of which 522 were slaughtered pig samples and 162 were slaughterhouse environmental samples. The description of the number and type of samples are shown in Table 2.

2.2.1. Slaughtered pig samples

From each chosen slaughtered pig, samples were collected as follows:

Mesenteric lymph nodes (MLNs): At least 5 lymph nodes in the ileocaecal regions were cut out of the intestine packet with a sterile, disposable scalpel.

Carcass swabs: Carcass swabs were selected at three high-risk cross-contamination points along the slaughter line (submitting, evisceration, and after washing carcass). Carcasses were swabbed on the external and internal surfaces at four different points: cheek, ham (right and left), rib cage and neck-upper-shoulder. Sampling was done using a sterile moistened (0.1% peptone water) cotton ball to swab a square of 15 × 15 cm within each point (approx. 1350 cm^2 in total). The five cotton balls collected from each carcass were pooled and put into a sterile stomacher bag.

2.2.2. Environmental samples

During the slaughter process, environmental samples were also collected as follows:

Scalding and cooling water: During slaughtering activities, 10 ml of water samples were taken from the scalding and cooling tank (The temperature range were kept between 5 and 10 °C) once every hour. The water samples were collected using a sterile collection tube and the temperature was measured.

Lairage: At the beginning of the sampling, pens in the lairage were already filled with pigs to be slaughtered that day. For every visit, five pens were sampled using the overshoes method which was carried out by walking around in an "8"-shaped track in the pen using one pair of disposable, liquid absorbing overshoes (Cobbaut, Houf, Douidah, Van Hende, & De Zutter, 2008).

Floor: The slaughterhouses were divided into five parts, according to the overall layout of the slaughterhouses and each part covered a 1 × 1 m^2 area. Sampling was done using four sterile moistened (0.1% peptone water) cotton balls to swab each part. The four cotton balls collected from each part were pooled and put into a sterile stomacher bag.

Visceral processing countertop and waste water: At the end of slaughtering, four areas of the countertop were sampled according to its size and each part covered a 1 × 1 m^2 area. The sample method was the same as with the slaughterhouse floor. During slaughtering activities, water samples were taken from the sewerage tank in the visceral processing room or the drain in the countertop (slaughterhouse B without the visceral processing room).

Table 1
Description of the three slaughterhouses included in the study.

	Capacity of slaughterhouse (pigs/night)	Usage times of equipment (years)	Floor type of Lairage	Cleaning of slaughterhouse	Additional information
A	500	4	Solid	Daily	Visceral processing room
B	600	10	Solid	2 to 3 days	
C	400	8	Solid	Weekly	Visceral processing room

Table 2
The number of the samples from different slaughterhouse in every visit.

Slaughterhouse	Enviromental samples	Lairage	Water in			Floor	Visceral processing countertop	Slaughtering pig samples	Lymph node	Carcass swab		
			Scalding tank	Cooling tank	Sewer tank					Submitting	Evisceration	After washing carcass
A	18	5	3	3	1	5	1	53	8	15	15	15
B	18	5	3	3	1	5	1	53	8	15	15	15
C	18	5	3	3	1	5	1	68	8	20	20	20

Each slaughterhouse had been visited 3 times.

Sterile gloves were used during the sampling procedures and were changed between each sample. Samples were sent to the laboratory in cooled containers (5–10 °C) within the same day for immediate analysis.

2.3. Sample preparation

2.3.1. Slaughtered pig samples preparation

Mesenteric lymph nodes (MLNs): Once in the lab, the MLNs were processed as previously described (Regulation EC 668/2006) by removing the fat and the capsula followed by immersion in alcohol 70% (v/v) and flaming to sterilize its surface. Finally, the MLNs were cut into small pieces using sterile scissors, weighed and transferred into 225 ml of buffered peptone water (BPW) (Difco, BD, USA) for 18 h at 37 °C.

Carcass swabs: All swabs of one carcass and according to one sampling place were pooled and immediately immersed in 50 ml of BPW and incubated directly by shaking.

2.3.2. Environmental samples preparation

Waste water, Scalding and cooling water: All the water samples were carried pre-enrichment directly without the need to process.

Lairage, Floor and Visceral processing countertop samples: The carcass swabs samples were immediately immersed in 50 ml of BPW and incubated directly by shaking.

2.4. Salmonella isolation and identification

Pre-enrichment was performed by 1:10 dilution of scalding tank, cooling tank and waste water in BPW followed by incubation at 37 °C for 18–24 h. The swab samples such as carcass swabs, lairage and floor samples that were already immersed in 50 ml of BPW were shaken and directly incubated. After stomaching, the buffered peptone water suspensions were incubated at 37 °C for 24 h, and 0.1 ml of this broth culture was subcultured in 10 ml of rappaport-Vassiliadis enrichment broth (RV) broth (Difco, BD, USA) at 42 °C for a further 24 h. Following incubation, one loopful of each RV broth was streaked onto xylose lysine tergitol 4 (XLT4) (Difco, BD, USA), which was then incubated at 37 °C for 24–48 h. The presumptive *Salmonella* colony was picked from each plate and streaked onto biochemical test triple sugar iron (TSI) (slants) and lysine iron (LIA) agars (Hangzhou Microbial Reagent Co.), and incubated for 24 h at 35 °C. Isolates with typical color reactions were confirmed as *Salmonella* based on results of the API-20E test kit (bioMerieux, Marcy l'Etoile, France). All strains were serotyped according to the Kauffman-White scheme (Grimont et al., 2007).

2.5. Antimicrobial susceptibility testing

Antimicrobial resistance was determined by agar disk diffusion tests on Müeller-Hinton agar plates according to the guidelines of the Clinical and Laboratory Standards Institute CLIS (Clinical Laboratory Institute Standards, 2009). The isolates were tested against a total of 16 antimicrobials as follows: ampicillin (Amp, 10 μg), mezlocillin (Mez, 75 μg), cefazolin (Cfz, 30 μg), ceftriaxone (Cro, 30 μg), cefotaxime (Ctx, 30 μg), gentamicin (Gen, 10 μg), kanamycin (Kan, 20 μg), streptomycin (Str, 10 μg), tetracycline (Tet, 30 μg), chloramphenicol (Chl, 30 μg), ofloxacin (Ofx, 5 μg), enrofloxacin (Enr, 5 μg), norfloxacin (Nor, 5 μg), ciprofloxacin (Cip, 5 μg), nalidixic acid (Nal, 30 μg) and trimethoprim/sulfamethoxazole (Sxt, 1.25–23.75 μg). Pure cultures were grown overnight in Luria–Bertani Broth (LB) (Merck, Germany) at 37 °C and the concentration was adjusted using sterile saline solution until a 0.5 McFarland turbidity was attained. One hundred microliters of the culture was then swabbed onto Mueller Hinton agar using a sterile cotton swab. Antimicrobial disks were placed on the surface of the agar plate with enough distance to avoid overlapping of inhibition zones. The plates were incubated at 37 °C for 16–18 h and the results were interpreted as sensitive, intermediate, or resistant according to Clinical and Laboratory Standards Institute guidelines (Clinical Laboratory Institute Standards, 2012). *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains. An isolate was defined as 'resistant' after confirmation of resistance to at least one agent tested, while 'multidrug-resistances' (MDR) was defined as resistance to two or more agents.

2.6. Multilocus sequence typing (MLST) and sequence data analysis

Strains were grown aerobically in Luria–Bertani broth with shaking at 37 °C for 16–18 h. Genomic DNA isolation was carried out using the TIANamp Bacteria DNA Kit (TIANGEN Biotech Co., Ltd, China) according to the manufacturer's protocol. MLST was carried out based on the scheme published on the Multilocus Sequence Typing home page (http://mlst.ucc.ie/mlst/dbs/Senterica, accessed December 30, 2012). MLST results were analyzed using BioNumerics 5.0 software (Applied-Maths, Kortrijk, Belgium) and a Minimum Spanning Tree (Le Hello et al., 2011) was made to study the relatedness of sequence types (STs) from the three slaughterhouses.

2.7. PFGE

The isolates that were chosen for PFGE are shown in Table 3. Besides these, isolates with the same serotypes from pork, which were previously isolated in our routine monitoring (Li et al., 2014), were also used (Table 3). PFGE was performed according to the

Table 3
72 Strains were used in PFGE analysis.

Source	Region	Derby	Typhimurium	Number isolates
Pork[a]	Yangzhou	11	6	17
Slaughterhouse A	Yangzhou	16	5	21
Slaughterhouse C	Yangzhou	29	5	34
Total		56	16	72

[a] The isolates from pork were identified from major free markets in Yangzhou city during the same period.

protocol developed by the Centers for Disease Control and Prevention (CDC) (Ribot et al., 2006). Briefly, DNA embedded in agarose plugs was digested with 50 U of *Xba* I (TaKaRa, Biotechnology Co. Ltd., Dalian, China) for 3 h in a water bath at 37 °C. The digested DNA was separated by electrophoresis in 0.5 × TBE buffer at 14 °C for 22 h using a CHEF Mapper electrophoresis system (Bio-Rad, Hercules, CA, USA) with pulse times of 2–63.8 s. The PulseNet "universal" standard strain, *S. enterica* serovar Braenderup H9812, was used as the control strain. PFGE agarose gels were stained with ethidium bromide and the DNA band images were visualized on a UV trans-illuminator (Bio-Rad). PFGE patterns were interpreted with the aid of BioNumerics 5.0 software (Applied-Maths, Kortrijk, Belgium) and compared by cluster analysis using Dice coefficient and the unweighted-pair group method with arithmetic averages (UPGMA dendrogram type) with a position tolerance of 1.5% and optimization of 0.5%.

2.8. Statistical analysis

Statistical comparison of *Salmonella* positive rates between different slaughterhouses and different points in the same slaughterhouse as well as the isolates resistance rate to the 16 antimicrobials were analyzed with the Chi-square (χ^2)test, which was performed using the Statistical Package for the Social Sciences (version 15.0, SPSS, Chicago, IL) with $p \leq 0.05$ considered as statistically significant.

3. Results

3.1. Salmonella prevalence and serotypes

Among the total of 684 samples analyzed, 322 (47.1%) were positive for *Salmonella.* The prevalence of positive samples was 51.6%, 47.9% and 43.1% in slaughterhouses A, B and C, respectively (Table 4). No significant differences were found amongst the different slaughterhouses ($\chi^2 = 2.6$, $p = 0.27$). However, high variations were observed amongst the different sampling occasions at the same slaughterhouse. On different sampling occasions, the positive rates of *Salmonella* in slaughterhouse A ranged from 39.4% to 59.3% ($p < 0.05$), from 36.6% to 64.8% in slaughterhouse B ($p < 0.05$) and from 39.5% to 45.3% ($p > 0.05$) in slaughterhouse C (Table 4).

On the three different sampling occasions (V1, V2 and V3), the prevalence of *Salmonella*-positive slaughtered pigs samples ranged from 38.5% to 59.2%. There were extremely distinct differences in the *Salmonella*-positive rate for the different sampling occasions ($\chi 2 = 0.8$, $p < 0.01$), and the risk of slaughtered pigs samples being contaminated by *Salmonella* was two times higher at V3 than compared with V1 (OR = 2, 95% CI:1.3–3.1) and V2 (OR = 2.3, 95% CI:1.5–3.6) but there was no significant difference between V1 and V2 ($p > 0.05$).

With regards to slaughtered pigs, *Salmonella* was found most frequently on carcass swab samples after evisceration (68.0%) and after washing carcass samples (58.0%) which were much higher than the submitting samples (32.0%) and lymph node samples (7.0%). On carcass swab samples after eviceration especially, for almost all the visits the prevalence rate of *Salmonella* was higher than 50.0%, and in B3 the rate even reached 100.0% (Table 5).

As with the trend found for the slaughtered pig samples, the risk of environmental samples positively contaminated with *Salmonella* was also two times higher in V3 as compared with V1 (OR = 2, 95% CI:0.98–4.6) and V2 (OR = 2.7, 95% CI:1.2–5.8) and there were significant differences between *Salmonella* prevalence in the environmental samples. In addition, overshoes taken in the lairage were highly contaminated with a small variation (ranging from 33.3 to 46.7%) between the three slaughterhouses.

Thirteen different serotypes were identified among the 322 *Salmonella* isolates (Table 6). *S.* Derby was the most prevalent serotype accounting for 52.8% of all isolates, followed by *S.* Meleagridis (13.4%) and *S.* Typhimurium (9.3%). Table 7 shows the details of the *Salmonella* serotypes isolated from the slaughtered pigs and environmental samples per slaughterhouse and per visit.

S. Derby was widely present in both slaughtered pigs and the environmental samples collected from all three slaughterhouses. Differences in serotypes were observed between the different slaughterhouses and the different visits. *S.* Saintpaul, *S.* Agona, *S.* Give and *S.* Corvallis were only found in slaughterhouse C, whereas *S.* Leeson and *S.* Worthington were only detected in slaughterhouse B. There were only four serotypes found in all slaughtered pigs and the environmental samples sampled from slaughterhouse A, where all isolates were identified as *S.* Derby, however one *S. Infantis* strain was found during visit 2 to slaughterhouse A. In addition, all the serotypes detected from the cooling water and floor were also found in strains isolated from slaughtered pig samples, similar to the serotypes identified from slaughtered pigs in the lairage and during submitting.

3.2. Antimicrobial susceptibility testing

All of the *Salmonella* isolated from the environmental samples were susceptible to norfloxacin, ceftriaxone, cefotaxime and ciprofloxacin. Resistance to tetracycline was the most common (55.7% of isolates) followed by resistance to trimethoprim-sulfamethoxazole (22.8%), ampicillin (20.3%) and nalidixic acid (12.7%) (Table 8). Among the β-lactams studied, a high degree of resistance to ampicillin (20.3% of isolates) was found, which was higher than the percentage of resistance to mezlocillin (3.8%) and cefazolin (1.3%) ($p < 0.01$). The aminoglycosides studied showed different levels of effectiveness. Resistance to gentamicin (8.6%) and

Table 4
Distribution of *Salmonella* positive samples in different slaughterhouses in different visiting times.

Source	V 1			V 2			V3			Total %
	Environmental (%)[e]	Slaughtering pig (%)[d]	Total (%)	Environmental (%)[e]	Slaughtering pig (%)[d]	Total (%)	Environmental (%)[e]	Slaughtering pig (%)[d]	Total (%)	
A[c]	7[a] (38.9)[b]	22(41.5)	29(40.8)	7(38.9)	21(39.7)	28(39.4)	12(66.7)	39(73.6)	51(71.8)	51.6
B[c]	10(55.5)	20(37.7)	30(42.3)	7(38.9)	19(35.6)	26(36.6)	12(66.7)	34(64.2)	46(64.8)	47.9
C	7(38.9)	32(47.1)	39(45.3)	7(38.9)	27(39.7)	34(39.5)	10(55.5)	29(42.6)	39(45.3)	43.1
Total	24(44.4)	74(42.5)		21(38.9)	67(38.5)		34(63.0)	102(58.6)		

[a] The number of positive.
[b] Positive rate.
[c] Prevalence of contaminated carcasses was different amongst visits to the same slaughterhouse ($P < 0.05$).
[d] Prevalence of contaminated slaughtering pigs of three slaughterhouses was significantly between different visits ($P < 0.01$).
[e] Prevalence of contaminated environmental samples of three slaughterhouses was significantly between different visits ($P < 0.01$).

Table 5
The prevalence of *Salmonella* in different points of the slaughter line in three pig slaughterhouses.

Slaughterhouse									
Source		C1	C2	C3	B1	B2	B3	A1	A2	A3
Enviromental samples										
Lairage		0	2[a](40.0)[b]	3(60.0)	3(60.0)	1(20.0)	3(60.0)	1(20.0)	1(20.0)	3(60.0)
Scalding water		0	0	0	0	0	0	0	0	0
Cooling water		2(66.7)	1(33.3)	3(100.0)	2(66.7)	1(33.3)	3(100.0)	3(100.0)	3(100.0)	2(66.7)
Floor		3(60.0)	2(40.0)	2(40.0)	3(60.0)	4(80.0)	5(100.0)	3(60.0)	1(20.0)	5(100.0)
Waste water		1	1	1	1	0	0	0	1	1
Visceral processing countertop		1	1	1	1	1	1	0	1	1
Slaughtering pig										
Lymph node		0	2(25.0)	0	1(12.5)	0	0	1(12.5)	0	1(12.5)
Carcass swab	Submitting	4(20.0)	6(30.0)	4(20.0)	5(33.3)	1(6.7)	5(33.3)	4(26.7)	8(53.3)	12(80.0)
	Evisceration	15(75.0)	6(30.0)	11(55.0)	11(73.3)	9(60.0)	15(100.0)	12(80.0)	11(73.3)	12(80.0)
	After washing carcass	13(65.0)	13(65.0)	14(70.0)	3(20.0)	9(60.0)	14(93.3)	5(33.3)	2(13.3)	14(93.3)

[a] The number of positive.
[b] Positive rate.

Table 6
Serotype distribution of 322 *Salmonella* isolates in slaughterhouses.

Serovar	Slaughterhouse			Total (%)
	C	B	A	
Derby	48	35	87	170(52.8)
Meleagridis	24	19		43(13.4)
Typhimurium	11	3	16	30(9.3)
London	9	15		24(7.5)
Anatum	3	13	1	17(5.3)
Infantis		1	4	5(1.6)
St. Paul	4			4(1.2)
Riessen		2		2(0.6)
Give	1			1(0.3)
Worthington		1		1(0.3)
Corvallis	1			1(0.3)
Agona	1			1(0.3)
Senftenberg	1	2		3(0.9)
(3,10:_:1,5)[a]	9	11		20(6.2)
Total	112	102	108	322

[a] Undetermined.

kanamycin (8.0%) was sporadic. For chloramphenicol, 28 (11.3%) of the isolates were classified as resistant. Representing the sulfonamides, trimethoprim-sulfamethoxazole resistance was found in 69 of the *Salmonella* isolates. There were no differences in the resistance rates of tetracycline, trimethoprim-sulfamethoxazole, ampicillin, streptomycin and nalidixic acid between *Salmonella* isolated from the environment and the slaughtered pig samples, which ranged from 18.5% to 31.2% ($p > 0.05$).

There were 51 and 181 isolates resistant to at least one antimicrobial, which presented 20 and 68 kinds of drug-resistant patterns respectively, and the most common type were TET with the percentages of 25.5% and 12.2% (Table 9). For MDR phenotypes, 32 and 131 different patterns were found among the strains from the environment and slaughtered pig samples. One *Salmonella* Typhimurium isolate collected from slaughtered pig samples was resistant to 11 antimicrobials (AMP, CFZ, CHL, GEN, KAN, MEZ, NAL, NOR, STR, SXT and TET). The most common MDR patterns were SXT TET (25%, 8/32) and STR TET (9.3%, 3/32) in environmental isolates, while TET (13%, 17/131) and STR TET (5.3%, 7/131) were found in isolates from slaughtered pig samples.

3.3. Multilocus sequence typing (MLST) analysis

We characterized 322 *Salmonella* isolates using MLST analysis. Sixteen different ST patterns were identified among the 322 *Salmonella* isolates (Fig. 1). ST40 was the most common ST in this study, represented by 164 *Salmonella* isolates, followed by ST463 (43) and ST34 (28). Most STs obtained in this study were correlated with certain serovars, such as ST40 with *Salmonella* Derby, ST13 with *Salmonella* Agona, and ST46 with *Salmonella* Newport. Through cluster analysis, two clone complexes CC34 and CC40 were found in the minimum spanning tree. In addition, ST34 and ST818 were the single-locus variants of ST19 and ST40, respectively. Through a minimum spanning tree, we also found that slaughterhouse C had the most ST patterns while the least ST patterns were found in slaughterhouse A.

3.4. PFGE analysis

As shown in Fig. 2, 56 ST40 *S.* Derby isolated from slaughterhouse and pork were identified as thirty-five PFGE patterns, which were grouped into seven clusters. PFGE fingerprinting profiles grouped the isolates by origin, such as the strains typed as PF1 and PF2 with the closer genetic distances all isolated from slaughterhouse A, and the strains with PF21 and PF22 types in Cluster 1 were from slaughterhouse C. However some isolates from the same slaughterhouse belonged to different clusters. For example, two isolates grouped into cluster 7, while the remaining isolates grouped mainly into cluster 1 and 3. Of particular interest was the finding that strains with the same PFGE types also shared the same drug-resistance pattern, such as the isolates typed as PF1, PF3, PF12 and PF14. Nearly half of the *S.* Derby isolates obtained from pork showed the same PFGE types as the isolates in slaughterhouse C. Moreover, the same PFGE type can also be found in isolates from Yangzhou pork and the carcass samples in two slaughterhouses. In addition, four *S.* Derby isolates typed as PF26 were detected from the cooling water and slaughtered pig samples in slaughterhouse A.

Sixteen *Salmonella* Typhimurium isolates showed ten patterns and three clusters using a cutoff value of 85% similarity (Fig. 3). Isolates in each cluster also showed similar resistance phenotypes and ST types. The first cluster contained five *S.* Typhimurium isolates of slaughtered pig samples from submitting, evisceration and the carcasses. Four PFGE types (PF5, PF6, PF7 and PF10) were identified from the six isolates in Yangzhou pork, four of which showed the closest genetic relatedness with isolates from slaughterhouse C and were clustered in group 2. However, the remaining two strains have a further genetic distance than the others.

4. Discussion

In the present study, the results demonstrated that the prevalence of *Salmonella* contamination in slaughtered pigs in Yangzhou

Table 7
The serotype of Salmonella in different points of the slaughter line in three pig slaughterhouses.

		C1	C2	C3	B1	B2	B3	A1	A2	A3
Lairage			Derby (2)	Senftenberg (1) Derby (1) St. Paul (1)	Anatum (2) Infantis (1)	Derby (1)	Meleagridis (1) Derby (2)	Derby (1)	Derby (1)	Derby (3)
Cooling water		Derby (2)	Derby (1)	Typhimurium (3)	Meleagridis (1) U (1)	Derby (1)	Meleagridis (1) Derby (2)	Typhimurium (1) Derby (2)	Derby (3)	Derby (2)
Floor		Derby (2) U (1)	Meleagridis (2)	London (1)	Anatum (1) Derby (1) U (1)	Derby (3) London (1)	Meleagridis (2) Senftenberg (1) London (1) U (1)	Typhimurium (2) Derby (1)	Derby (1)	Typhimurium (1) Derby (4)
Waste water		Derby (1)	Meleagridis (1)	Typhimurium (1)	Derby (1)				Derby (1)	Derby (1)
Visceral processing countertop		Derby (1)	Meleagridis (1)	Derby (1) Anatum (1)	Derby (1)	Derby (1)	Riessen (1)		Derby (1)	Derby (1)
Lymph node			Meleagridis (2)		Derby (1)			Typhimurium (1)		Derby (1)
Carcass swab	Submiting	Derby (3) London (1)	Meleagridis (3) Agona (1) U (2)	Typhimurium (3) Meleagridis (1)	Anatum (4) U (1)	Derby (1)	Typhimurium (2) Derby (1) U (2)	Infantis (2) Anatum (1) Derby (1)	Derby (8)	Typhimurium (1) Derby (11)
	Evisceration	London (4) Derby (9) U (2)	Corvallis (1) Derby (5)	Derby (6) St. Paul (2) Typhimurium (2) Anatum (1)	Anatum (4) Derby (4) London (2) U (1)	London (6) Derby (3)	Typhimurium (1) Meleagridis (9) Anatum (1) Riessen (1) Derby (3)	Derby (7) Typhimurium (4) Infantis (1)	Derby (10) Infantis (1)	Typhimurium (1) Derby (11)
	After washing carcass	London (2) Derby (7) U (4)	Derby (2) Meleagridis (11)	Meleagridis (3) Typhimurium (2) London (1) St.Paul (1) Anatum (1) Derby (5) Give (1)	Derby (2) Anatum (1)	London (5) Derby (4)	Meleagridis (5) Senftenberg (1) Worthington (1) Derby (3) U (4)	Typhimurium (3) Derby (2)	Derby (2)	Typhimurium (3) Derby (11))

U:Undetermined.

Table 8
Antimicrobial resistance phenotypes of 322 *Salmonella spp.* isolates.

Antimicrobial agents	Number of resistant isolates (%)	
	Sample from	
	Environment (n = 79)	Slaughtering pig (n = 243)
Beta-lactamase		
Ampicillin	16(20.3)	83(34.1)
Mezlocillin	3(3.8)	16(6.6)
Cefazolin	1(1.3)	5(2.0)
Ceftriaxone	0	4(1.6)
Cefotaxime	0	0
Quinolone		
Nalidixic acid	10(12.7)	44(18.1)
Ofloxacin	1 (1.3)	1(0.4)
Enrofloxacin	1 (1.3)	3(1.2)
Norfloxacin	0	2(0.8)
Ciprofloxacin	0	5(2.0)
Tetracycline	44 (55.7)	144(59.3)
Chloramphenicol	9(11.3)	38(15.6)
Aminoglycosides		
Kanamycin	6(7.6)	29(11.9)
Gentamicin	3(3.7)	18(7.4)
Streptomycin	13(16.5)	76(31.2)
Sulfonamides		
Trimethoprim-sulfamethoxazole	18(22.8)	69(28.4)

Table 9
Multidrug resistance observed among *Salmonella* isolates from various sources.

Source	Number of resistant to indicated number of antimicrobials (%)					
	0	1–3	4–6	7–9	>9	Number of resistant isolates (%)
Slaughtering pig (243)	62(25.6)	138(56.8)	31(12.8)	10(4.2)	2(8.2)	181 (74.5)
Environment (79)	28(35.4)	42(53.2)	6(7.6)	2(2.5)	1(1.3)	51 (64.6)
Total (322)	90 (27.9)	180 (55.9)	37 (11.5)	12 (37.2)	3 (0.9)	232 (72.0)

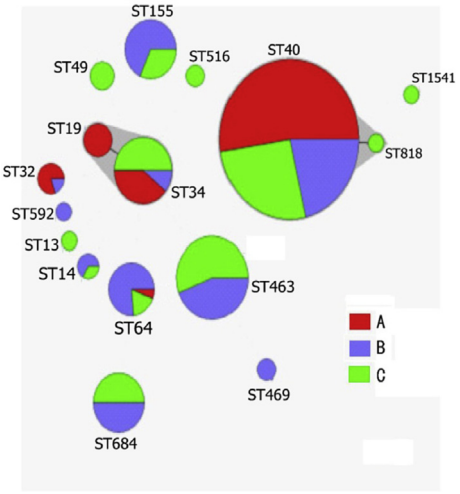

Fig. 1. Minimum spanning tree analysis of *Salmonella* isolated from different sources. Circles correspond to sequence types (STs), while the size of each circle is proportional to the number of isolates in each ST. Clonal complexes (STs with single allele differences) are shaded gray.

was high. *Salmonella* was recovered from slaughtered pigs and in their slaughterhouse environmental samples with an overall prevalence of 46.6% and 48.4%, which was similar to that of previous studies conducted in slaughterhouses in Vietnam (48.8%) and Belgium (48.2%) (De Busser et al., 2011; Thai, 2007), but higher than those reported in the other areas of China and some developing countries such as Italy, Denmark, Spain and Southwest China (Baptista, Dahl, & Nielsen, 2010; Bonardi et al., 2013; De Busser et al., 2011; Piras, Brown, Meloni, Mureddu, & Mazzette, 2011; Hou, Liu, Han, & Wu, 2013). This could be because the poor *Salmonella* control measures taken during the slaughtering processing caused serious *Salmonella* cross-contamination in the three slaughterhouses. Contrary to what was expected, albeit with better hygienic conditions and newer devices, we were unable to find a significant difference in *Salmonella* prevalence between the three slaughterhouses. This illustrates that the key points of the slaughtering process in different slaughterhouses should be ascertained and well controlled.

Slaughtered pigs and environmental samples were higher at risk of being contaminated during visit 3, which was from April 2012 to July 2012, compared with the rest of the survey period. The effect that these same sampling months increased the risk of surface contamination of carcasses was previously reported (EFSA, 2008a). It illustrates that the prevalence of *Salmonella* in slaughterhouses may be greatly affected by seasonal factors, but this hypothesis should be studied further and confirmed by additional studies. In our research, the prevalence of *Salmonella* in lymph node samples ranged from 12.5% to 25%, in accordance with 13.9% reported from EFSA (EFSA, 2008a,b). In literature, several authors emphasize the role of *Salmonella*-infected pigs in causing contamination and cross contamination during the slaughtering process, so it is quite necessary to perform pre-slaughter inspections and quarantines (Arguello, Carvajal, Collazos, García-Feliz, & Rubio, 2012). There were large differences in *Salmonella* between slaughtered pig samples at different sampling sites, such as in B3 the prevalence of *Salmonella* in submitting samples was only 6.7%, but in carcass samples the positive rate raised was as high as 60% ($p < 0.01$), suggesting that an incorrect slaughter process and cross-contamination within the slaughterhouse can lead to *Salmonella* contamination in pigs that were not carriers (Arguello et al., 2012).

As far as the environment was concerned, no *Salmonella* was detected in all scalding water samples, while an 80% positive rate was observed in a slaughterhouse in Vietnam (Thai, 2007). Based on the temperature measurement, we found the temperature of the water in the scalding pool was maintained at above 60 °C, which effectively kills the *Salmonella* in the water. However, *Salmonella* was identified from the cooling water samples in all visits, especially C3, B3, A1 and A2 in which all samples were detected as *Salmonella*-positive. The slow circulation speed of the pooling water and *Salmonella* leakage from the cavity of slaughtered pigs may account for this result. In the summer, because of high temperatures and poor water circulation, cooling water could become a broth enriched with bacteria and contaminate all of the slaughtered pigs that pass through the water continuously.

The prevalence of *Salmonella* in the lairages was similar to a previous report from EFSA. Studies show that *Salmonella* can be detected in the intestines of a slaughtered pig after just a 30 min stay in the lairage, which can be an important cause of *Salmonella* contamination at the surface of a slaughtered pig's body (EFSA, 2011).

The most prevalent serotypes were *S.* Derby and *S.* Typhimurium followed by *S.* Anatum and *S.* Meleagridis. These results are in accordance with other similar studies carried out in slaughterhouses (Arguello et al., 2012; Bonardi et al., 2013; De Busser et al., 2011; Gomes-Neves et al., 2012; Piras et al., 2011). In recent years, *S.* Derby has become more frequent in worldwide pork production. Based on a baseline EU survey, *S.* Derby was the most frequently isolated serovar in both breeding and production holdings and can become the potential source of the bacteria disseminated to slaughtered pigs (EFSA, 2011). Moreover, *S.* Derby is also isolated from cases of human salmonellosis and have been reported in China and other countries (Hauser et al., 2011; Kerouanton, Rose, Weill, Granier, & Denis, 2013; Ling et al., 2001). In our study, *S.* Derby was also widely spread in carcass samples, which would then be sold in public markets after splitting and weighing. Therefore, we conclude that *S.* Derby can survive in certain niches of the three slaughterhouses, and may become part of the resident flora (house strains) and can result in subsequent carcass contamination of slaughtered pigs passing along the slaughter line. In this way, all the serotypes associated with swine species may lead to *Salmonella* contamination of pork and cases of non-typhoidal salmonellosis have been described in humans. There were various kinds of serotype identified in this study, many of which were reported in this city for the first time. This could be related to the diverse source of pigs in different slaughterhouses (Pan et al., 2010; Wang, Jiao, Liu, Chen, & Huan, 2007; Zhou et al., 2013). We hypothesize that the slaughterhouse can serve as a transport hub, where different *Salmonella* serotypes carried by pigs from different regions can be released and subsequently through the pork on sale in the public market, which causes a serious threat to public health.

The presence of antimicrobial resistant pathogens in food and food products could enable the bacteria to spread via the food chain to humans, causing infections (Angulo, Baker, Olsen, Anderson, & Barrett, 2004). Observations in this study on the prevalence of antimicrobial resistance in *Salmonella* isolates are in accordance with other studies carried out at slaughterhouses (Bonardi et al., 2013; Botteldoorn, Herman, Rijpens, & Heyndrickx, 2004), where resistance to tetracycline was the most common phenotype observed in 75.6% of the 716 isolates of *S. enterica*, followed by ampicillin, trimethoprim-sulfamethoxazole and streptomycin. Similar results were found in *Salmonella* isolates from food-producing animals in our country (Han, Liu, Hou, Chen, & Peng, 2014; Yang et al., 2010). These drugs are commonly used in veterinary clinical medicine, which means the using of antibiotics in pig production process is both complex and frequently in China.

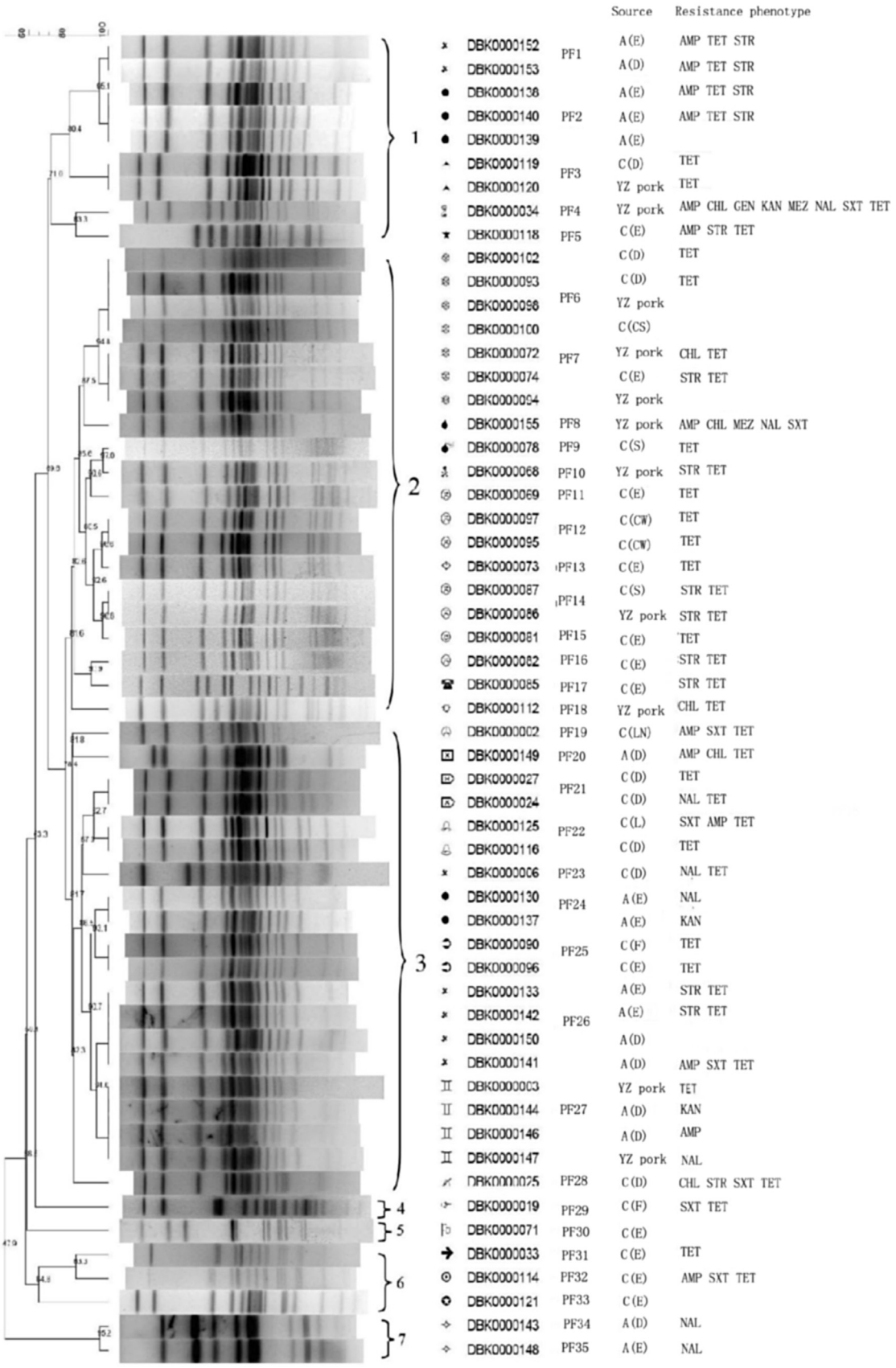

Fig. 2. Dendrogram of PFGE patterns of 65 *S*. Derby isolates recovered from slaughterhouses and pork, and their association with source and drug-resistance types. Fifty-six ST40 *S*. Derby isolated from slaughterhouse and pork was identified as thirty-five PFGE patterns, which were grouped into seven clusters. In the source column, the letters in parentheses represent the different links of slaughterhouses A and C (E for evisceration, D for after washing carcass, S for submit, CS for waste water, CW for cooling water, F for floor sample, LN for lymph nodes).

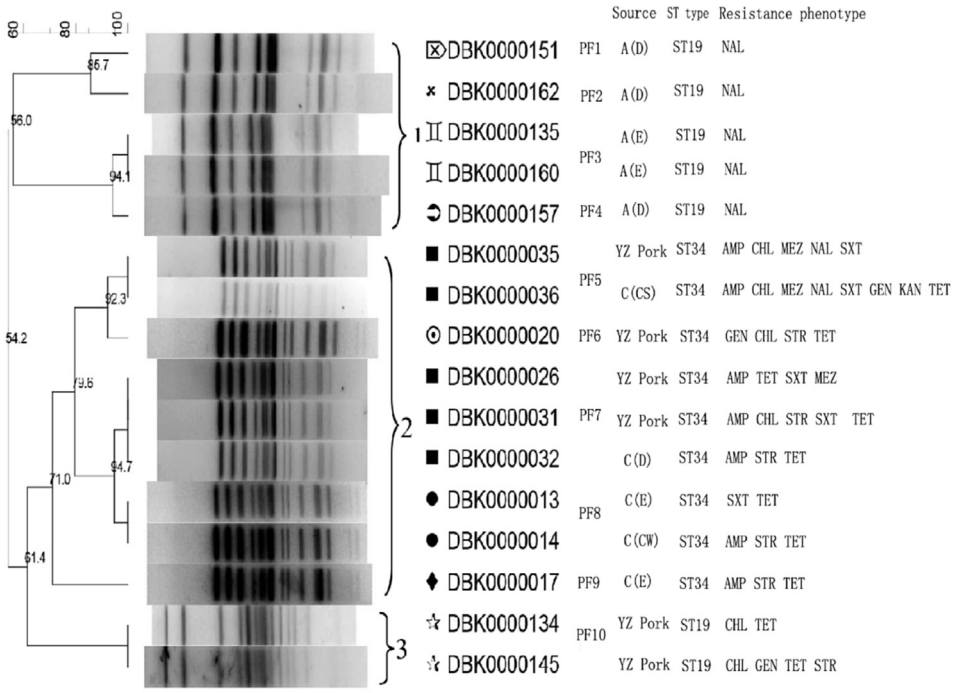

Fig. 3. Dendrogram of PFGE patterns of 16 *S.* Typhimurium isolates recovered from slaughterhouses and pork, and their association with source, sequence type, and drug-resistance types. Sixteen *Salmonella* Typhimurium isolates showed ten patterns and three clusters using a cutoff value of 85% similarity. In the source column, the letters in parentheses represent the different links of slaughterhouses A and C (E for evisceration, D for after washing carcass, S for submit, CW for cooling water).

MLST could be used to overcome the limitations of the traditional serotyping method and provide reliable identification of the most common *Salmonella* serotypes and their associated evolutionary information. In our study, we used MLST as a regular tool for *Salmonella* preliminary classification and for rapid and accurate identification of serotyping (Achtman et al., 2012). ST40 was the most common genotype in isolates recovered from the three slaughterhouses in this study, which was in accordance with our study on retail pork in Yangzhou (Li et al., 2014). According to the previous report, ST40 *Salmonella* isolates may spread widely throughout the pork production chain. The remaining common STs in *Salmonella* from the three slaughterhouses such as ST34, ST155 and ST64 were also frequently in *Salmonella* from the retail pork (Li et al., 2014). However, *S.* Typhimurium ST34 and ST19, which were associated with the ACSSuT type (resistance to amoxicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines) showing higher mortality rates were frequently detected in our study (Molbak, 2005; Wong et al., 2013), which indicated serious contaminations of such *S.* Typhimurium in the pork production chain and may pose a great threat to public health.

As an important method of the molecular typing of *Salmonella*, PFGE plays an important role in tracking the sources of infection and epidemic control, which is known as the “gold standard” in molecular typing of bacteria (Villalón et al., 2011). In the present study, PFGE had a higher discriminatory power than MLST, and the *Salmonella* with the same PFGE type mostly derived from the same slaughterhouse and was placed in the same cluster, showing evident regional characteristics. In addition, the isolates with the same PFGE type pattern shared similar resistant phenotype and ST types, which support previous reports (Harbottle, White, McDermott, Walker, & Zhao, 2006).

The same PFGE types could be found in carcass samples of both slaughterhouses and pork, proving the spread of *Salmonella* between slaughterhouses and pork in the public market. Four *S.* Derby isolates with the same PFGE type (PF26) were detected in the samples of the environment and the slaughtered pigs in slaughterhouse A, indicating cross contamination between the two sources. Six *S.* typhimurium in cluster 1 with the same ST type (ST19), which can be considered as the same *Salmonella* clone, came from different points of the slaughtered pigs, and took place during horizontal transmission along the slaughtering processing. This illustrates that carcass contamination originates from both the environment and the slaughtered pig. In addition, by comparing the PFGE type between the slaughterhouses and the pork, it was revealed that *Salmonella* in Yangzhou pork were mostly correlated with the strains in slaughterhouse C.

5. Conclusion

In summary, we examined the epidemiology of *Salmonella* from three slaughterhouses in Yangzhou located in eastern China. The results of this study illustrated the distribution, antimicrobial susceptibility profiles, serotype and genotype in *Salmonella* isolates from three slaughterhouses in different time periods, and revealed the clonal relationships of recovered strains and their involvement in three slaughterhouses and pork from related public markets. Contamination and cross contamination frequently occurred inside the slaughterhouse and can also be important sources of *Salmonella* contamination in pork sold in the public market. Further research in more complete pork production chains in Jiangsu are required, which can help us to identify the source and character of the most prevalent *Salmonella* isolates in production chains and confirm critical control points in the whole production chain so that contamination risks in hog and pork products can be reduced.

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