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Article

Article

Emergence of rapidly spreading antimicrobial-resistant *Salmonella* in traditional blood-based foods

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Abstract

Objectives: This study aims to investigate the survival ability and risk of *Salmonella* isolated from traditional blood-based food, using both phtnotypic and genotypic analysis.

Materials and Methods: We characterized and identified seventeen *Salmonella* isolates using 16s rRNA sequencing, real-time PCR and whole-genome sequencing (WGS). Cell counts were recorded to monitor growth ability and temperature tolerance. Virulence genes and whole genome phylogenies were confirmed by WGS. The minimum inhibitory concentration (MIC) of corresponding strains to different antibiotics and antimicrobial resistance (AMR) genes predicted by WGS were evaluated.

Results: In this study, we characterized 17 *Salmonella* isolates obtained from contaminated traditional blood-based food in China. Compared to laboratory strain *S.* Typhimurium ATCC 14028, these *Salmonella* isolates generally grew more rapidly and developed less biofilm, but their tolerance to food processing-associated cold and heat stresses was distinct. Whole-genome phylogenies pointed out the potential for cross-contamination during food practices. In addition, 21 antibiotic-resistance genes were found among blood-based food isolates, including high-prevalent resistance genes *gyrA* (including associated mutations D87G and S83F), bla_{TEM}, and *aadA1*. Antibiotic susceptibility test confirmed the *in-silico* prediction and revealed a 41.18% (7/17) multidrug resistance (MDR) rate. Resistance to ceftiofur, kanamycin, and nalidixic acid was observed without corresponding resistance genes, suggesting overlooked resistance mechanisms.

Conclusions: This study revisited microbial safety concerns associated with traditional blood-based food and underlined the emergence of high-risk MDR *Salmonella* strains. It also underscores the importance of implementing better hygiene practices in the production and handling of traditional food products.

Keywords: *Salmonella*; antimicrobial resistance; blood-based food; whole-genome sequencing; biofilm.

Introduction

Animal blood is rich in proteins, iron, and bioactive compounds, and has a long history of consumption as a traditional food all over the world (Kinley *et al.*, 2010; Davidson, 2014; Bah *et al.*, 2016; Toldrá *et al.*, 2016). Blood puddings hold significant cultural importance for British communities, as they are a distinctive feature of traditional breakfast. Blood sausage is a traditional blood-based dish in many countries, such as Italy, France, and Germany. Served as a by-product of the meat industry (Bah *et al.*, 2016; Toldrá *et al.*, 2016, 2021), blood-based food made via simple heating of fresh edible animal blood is prepared as 'blood tofu', a classic dish in China. Currently, blood-based food is mainly produced through traditional routes with limited food hygiene regulations. Animal blood is mainly collected from slaughterhouses and vulnerable contamination by animal-harbored foodborne pathogens, including *Salmonella*, pathogenic *Escherichia coli*, *Staphylococcus aureus*, and *Campylobacter* (Zhou *et al.*, 2020; Hong *et al.*, 2023; Wareth and Neubauer, 2021; Navacharoen *et al.*, 2009; Sorvillo *et al.*, 2011). The main process of blood-based food production that can reduce bacterial load is heating–coagulation. Although heating is conducted above 80 °C, to maintain the smooth texture, heating is intentionally maintained for a very short period which may result in incomplete sterilization. The Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) issued a public health alert for imported cooked duck blood curds due to pathogen contamination (FSIS, 2020). The USDA indicated that pathogen contamination was caused by improper food practices and inadequate hygiene conditions.

Salmonella is a leading foodborne pathogen that can cause salmonellosis, resulting in enteric fever (typhoid), enterocolitis/diarrhea, bacteremia, chronic asymptomatic carriage, or death (Ehuwa *et al.*, 2021). In 2020, *Salmonella* was among the most commonly reported infection causes in the European Union, affecting 52 702 people. The U.S. Centers

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for Disease Control and Prevention (CDC) recorded 46 623 cases of culture-confirmed *Salmonella* infections that were reported in 53 states in 2016 (CDC, 2016). Food animal poultry and swine can harbor *Salmonella* without any sign of disease (Thomson *et al.*, 2008; Ferrari *et al.*, 2019; CDC, 2022). *Salmonella* can be found in the blood of animals when systematic infection has developed (Uthe *et al.*, 2009; Huang *et al.*, 2011). Hence, animal blood-based food produced with high pathogen loads is potentially risky to consumers. Several reports have reported the identification of foodborne pathogens from blood-based food. In 2019, a *Campylobacter* outbreak linked to duck blood curd was found in Shunyi District, Beijing, China, and 16 of 130 workers were identified to be suspected cases (Chen *et al.*, 2021). In addition, due to the abuse of antibiotics on animal farms, contaminated blood-based food can potentially serve as a reservoir of antibiotic resistance, aggravating antimicrobial resistance (AMR) transfer. However, there is limited information regarding antibiotic-resistant *Salmonella* in traditional blood-based products.

Antibiotics are used as prophylaxis or therapeutics to reduce *Salmonella* load and control the development of infection. However, antibiotic abuse was found to be positively correlated with the evolution of resistance, leading to the emergence of multidrug-resistant (MDR) strains (Landers *et al.*, 2012; Verraes *et al.*, 2013; Jans *et al.*, 2018). The presence of AMR bacteria in blood-based food can be due to the use of antimicrobials on the farm, cross-contamination with antimicrobial-resistant bacteria during the slaughter process, and handling or improper methods of sterilization. Moreover, the horizontal transfer and acquisition of antibiotic resistance ability in *Salmonella* have been reported worldwide (Powell *et al.*, 2018; Judd *et al.*, 2019). Therefore, it is important to understand the antibiotic-resistant strains in traditional blood-based food.

The development of whole-genome sequencing (WGS) has enabled a more comprehensive characterization of food safety hazards (Taboada *et al.*, 2017; Oniciuc *et al.*, 2018; Rumore *et al.*, 2018). With the assistance of bioinformatics analysis, WGS provides a more powerful approach to molecular epidemiology in food safety (den Bakker *et al.*, 2011; Chen *et al.*, 2020; Liu *et al.*, 2020; Deng *et al.*, 2021). The CDC launched a WGS-based investigation (termed PulseNet) on foodborne pathogens including *Campylobacter*, Shiga toxin-producing *E. coli*, *Salmonella*, *Vibrio*, and *Listeria*. Campioni *et al.* (2022) linked the transmission of cattle-associated serovar *Salmonella* Dublin from Brazil to outbreaks in other countries using WGS-based phylogenetic analysis. They located virulence genes (i.e. *iroB* and *iroC*) specifically in *S.* Dublin isolated in Brazil.

In this study, we aimed to evaluate *Salmonella*-contaminated strains isolated from traditional blood-based food in China. The phenotypic and genotypic characteristics of these *Salmonella* isolates were comprehensively investigated using biochemical assays coupled with high-throughput WGS. This study raised concern about microbial food safety in traditional food products.

Materials and Methods

Sample collection

The samples collected from seven cities in China are summarized in Table S1. Strick sample collection and handling procedures were implemented to avoid false positive outcomes.

Isolation and identification of *Salmonella*

The *Salmonella* strain was isolated according to the China National Food Safety Standard Methods for Food Microbiological Examination—*Salmonella* (GB/T 4789.4–2016). The sample was weighed and placed into sterile customized bags with 225 mL buffered peptone water (BPW) (Hopebio, Co., Ltd., Qingdao, China) and homogenized according to standard requirements. The sample was incubated at 37 °C for 16 h. After pre-enrichment, the BPW was shaken, and 1 mL of the BPW cultures were subcultured in 10 mL of tetrathionate broth (Hopebio, Co., Ltd.) at 42 °C for 24 h, and 1 mL of BPW cultures were transferred to 10 mL of selenite cystine broth (Hopebio, Co., Ltd.). The medium was inoculated in bismuth sulfite agar (Hopebio, Co., Ltd.) plates and xylose lysine desoxycholate (XLD) agar (Hopebio, Co., Ltd.) plates. Next, they were incubated at 37 °C for 48 h and 24 h, respectively. Isolates with typical *Salmonella* phenotypes were confirmed by 16S rRNA sequencing using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 911R (5′-GCCCCCGTCAATTCMTTTGA-3′), and the result was analyzed using the Basic Local Alignment Search Tool (BLAST). The target *Salmonella* strains were stored at –80 °C in cryovials with 50% glycerol.

Identification of serovar

The isolates extracted from blood-based products were recovered on XLD and grown on Luria–Bertani (LB) agar (Hopebio, Co., Ltd.) at 37 °C for 24 h under aerobic conditions. After culturing, the DNA of the strains was extracted using the TIANamp bacteria DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions for sequencing. Then, the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration and purity of DNA. The quality requirements were concentration ≥50 μg/mL and OD_{260}/OD_{280} (purity measurement) between 1.6 and 1.8. *Salmonella* serovars were detected by real-time polymerase chain reaction (PCR) and WGS. Twelve serovar-specific primer pairs that can detect *Salmonella* serovars were selected according to Yang *et al.* (2021) (Table S2). The reaction volume of real-time PCR was 20 μL, which contained 10 μL of platinum SYBR green qPCR Super Mix-UDG with ROX (ThermoFisher Scientific), 1 μL of each primer pair, 2 μL of template DNA and 6 μL of DNase/RNase free water. Amplification was performed on a Real-Time PCR System (QuantStudio 3, Thermo Fisher Scientific) for 40 cycles of denaturation at 95 °C for 2 min, followed by 95 °C for 15 s and 60 °C for 30 s. The conditions of the melting curve were as follows: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 1 s. The remaining serovars that were not detected by real-time PCR were determined by WGS.

Cold stress and heat stress challenge

The effect of temperature on bacterial strains was evaluated by observing their growth at different temperatures. All *Salmonella* strains were determined overnight on LB agar at 37 °C for 24 h under aerobic conditions. The colonies were resuspended in phosphate-buffered saline (PBS) (Solarbio, Beijing, China) and diluted to 10^7 CFU/mL. Two hundred milliliters of bacterial solution were transferred to sterile 96-well plates, in which the outer wells were filled with PBS. The 96-well plates were subjected to a temperature of 4 °C for 14 d under aerobic conditions. The bacterial populations were counted at 0, 3, 7, and 14 d.

Bacteria were grown at 37 °C as a control in heat stress experiments. The resuspended bacterial suspension was heated in a water bath at 43, 48, 53, 58, and 63 °C for 20 min (Reissbrodt *et al.*, 2002), with *S.* Typhimurium ATCC 14028 as standard. The samples were placed to cool at the end of the heat treatment. After heating, the cell counts were recorded. Experiments were done with three biological replicates.

Growth curve

After overnight growth on the LB agar plate at 37 °C, all isolated strains were resuspended in LB broth (Hopebio, Co., Ltd.) and diluted to 10^7 CFU/mL. The standardized inoculum was transferred to a 96-well polystyrene microtiter plate to a final volume of 200 µL, which was then incubated at 37 °C with shaking at 300 r/min. The kinetic measurements of absorbance at 600 nm were taken at 0, 1, 2, 4, 6, 12, and 24 h by a microtiter plate reader (Untersbergster. 1A, A-5082 Groedig, Austria). The experiments were performed were done with three biological replicates. The growth curves were drawn by GraphPad Prism 8.0.2.

Bacterial biofilm formation assay

A standard crystal violet assay in a 96-well polystyrene microtiter plate was used to detect the ability of *Salmonella* strains to form bacterial biofilm. The overnight culture was diluted to 10^7 CFU/mL and inoculated in technical duplicates in 96-well plates. The wells at the border were filled with 200 µL of LB broth to prevent evaporation. To evaluate the biofilm formation capacity of *Salmonella* strains, we adopted the criteria described by Merino *et al.* (2019), which characterized the bacterial biofilm-forming ability into four categories: non-biofilm producer, weak biofilm producer, moderate biofilm producer and strong biofilm producer. The experiments were performed in triplicate.

Whole-genome sequencing

The genomic DNA of the isolated strains was extracted using TIANamp bacteria DNA kit (Tiangen Biotech) following the manufacturer's instructions. The sequencing library was generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (Ipswich, MA, USA) following the manufacturer's instructions. Following PCR amplification, the genomic DNA fragmented by sonication to a size of 350 bp was ended polished, A-tailed, and ligated with a full-length adapter for Illumina sequencing. The PCR products were purified by the AMPure XP system (Beckman Coulter, Brea, CA, USA). DNA concentration was measured by Qubit®3.0 Fluorometer (Invitrogen, Waltham, MA, USA). After analysis of size distribution by NGS3K/Caliper and quantification by real-time PCR (3 nmol/L), the samples were loaded on a cBot Cluster Generation System using Nextera XT DNA library construction kit according to the manufacturer's instructions (FC-131-1024; Illumina, San Diego, CA, USA) and sequenced by the Illumina sequencing platform NovaSeq 6000. Then, the DNA libraries were sequenced on the Illumina platform and 2×150 bp paired-end reads were produced.

This Whole Genome Shotgun project has been deposited in GenBank under accession numbers JAVIUN000000000, JAVIUO000000000, JAVIUP000000000, JAVIUQ000000000, JAVIUR000000000, JAVIUS000000000, JAVIUT000000000, JAVIUU000000000, JAVIUV000000000, and JAVIUW 000000000.

Verification of serovar and construction of phylogenetic tree by WGS

The *in-silico* tool SeqSero2 v1.1.0 was used to determine the *Salmonella* serovars from WGS data. SeqSero used the raw sequencing reads as the input and assembly was used as an algorithm for analysis (Zhang *et al.*, 2019). Phylogenetic analysis was performed using the Codon Tree pipeline provided by the BV-BRC system based on the differences within 1000 genes. Forty-five genomes of different *Salmonella* serovar strains originated from humans and animals were obtained from the BV-BRC database, and selected for tree construction. The phylogenetic tree was visualized in the Interactive Tree of Life (iTOL) (Letunic *et al.*, 2021).

Antibiotic resistance genes and virulence factors

The antibiotic resistance genes of all the strains were detected by the tool Staramr in Galaxy Version 0.5.1 using the collective resource of ResFinder, PlasmidFinder, and PointFinder databases (Bharat *et al.*, 2022). The prediction of virulence genes was conducted based on the virulence factors database (VFDB; Liu *et al.*, 2022), and the *E*-value less than or equal to 1e-10 was used as the cutoff. GraphPad Prism 8.0.2 was used to construct a heatmap of the antibiotic resistance genes and virulence factors.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the 17 strains was tested by ampicillin (1280 µg/mL), ceftiofur HCl (1280 µg/mL), chloramphenicol (1280 µg/mL), gentamicin (640 µg/mL), kanamycin (1280 µg/mL), nalidixic acid (1280 µg/mL), streptomycin (1280 µg/mL), and tetracycline (1280 µg/mL). Antibiotic concentrations started from the original concentration, with 10×2-fold dilution by Mueller–Hinton broth (MHB) (Hopebio, Co., Ltd.). A volume (100 µL) of the diluted antibiotics was added to the first 10 columns of the 96-well polystyrene microtiter plate. The MHB and the lowest concentration of antibiotics were added to the last two columns. *Salmonella* strains were detected using optical density (OD_{600}) values by the microplate spectrophotometer (Tecan, Männedorf, Switzerland) and were diluted by the MHB to a final OD_{600} of 0.1. Then, the strains were added to the microplate. *S.* Typhimurium ATCC 25922 was used as the quality control. The final volume in each well was 200 µL and the 96-well plate was incubated at 37 °C for 24 h. The MIC, which was the lowest concentration of antimicrobial agent that completely inhibited the growth of the bacteria in the 96-well plate, was detected by the microplate spectrophotometer according to the 2019 Clinical and Laboratory Standards Institute (CLSI) recommendations. There is no CLSI-defined clinical breakpoint for streptomycin because of the inability of streptomycin to treat enteric infections. The National Antimicrobial Resistance Monitoring System (NARMS) program has long used a narrow dilution range (32–64 mg/L) as the breakpoint. However, further testing revealed that ≥32 mg/L was better to define resistance in *Salmonella* (Tyson *et al.*, 2016). All measurements were performed in duplicate and each experiment was repeated at least three times.

Results

Identification of *Salmonella* isolates from traditional blood-based food

We collected blood-based food samples in 2017 from seven cities in China (across Jiangsu, Chongqing, Heilongjiang, Guangdong, Guizhou, Henan, and Liaoning provinces). Selective cultivation recovered 17 putative *Salmonella* isolates. Then, 16S rRNA sequencing confirmed the identity of these isolates as *Salmonella* (data not shown). Of the 17 strains, 70.6% (12/17) were recovered from pig blood-based food, 17.6% (3/17) were recovered from chicken blood-based food, and the remaining 11.8% (2/17) were recovered from duck blood-based food (Table S1). Real-time PCR was used in this study to detect the serovar of the isolated strains. Seven serotypes were identified within the 17 *Salmonella* strains, including seven Derby, three I 4,[5], 12: i: –, three Kentucky, one Anatum, one Carno, one Mbandaka, and one Senftenberg. The serovar recovered from pig blood-based food were I 4,[5], 12: i: –, Mbandaka and Senftenberg (Figure 1). Most of the *Salmonella* Derby strains (85.71%; 6/7) were recovered from pig blood-based food. Anatum and Carno were identified from chicken blood-based food and duck blood-based food, respectively.

Growth parameters of blood-based food *Salmonella* isolates

Temperature fluctuations can cause significant stress for *Salmonella*, some of which were considered to be thermotolerant foodborne pathogens. We assessed the heat tolerance of the isolated strains in the study compared with that of the standard strain *S.* Typhimurium ATCC 14028. Our results showed that seven strains (Sal-021, Sal-043, Sal-046, Sal-047, Sal-080, Sal-081, and Sal-122) were more sensitive to heat stress compared with *S.* Typhimurium ATCC 14028 (Figure S1). Except for strains Sal-020, Sal-042, and Sal-080, most strains kept at 63 °C for 20 min were reduced to nondetectable levels.

In general, foodborne pathogens can grow rapidly at temperatures ranging from 5 to 60 °C (Ricke *et al.*, 2018). Thus, we hypothesized that the isolated strains have the ability to tolerate heat stress and cold stress. We measured the survival ability of the isolated *Salmonella* strains at refrigeration temperature (4 °C) for 14 d. The results showed that 52.9% (9/17) of the strains were tolerant to 4 °C in PBS (Figure S2).

The kinetics of the growth curves of the isolated *Salmonella* strains were determined initially. The growth curves of all the isolates were similarly shaped, displaying lag, exponential and stationary phases, suggesting that all the strains grew normally during the experiment (Figure S3). Moreover, all the strains grew faster than *S.* Typhimurium ATCC 14028.

The evaluation of biofilm forming ability using the standard crystal violet assay in a 96-well plate revealed that six isolated *Salmonella* strains (Sal-20, Sal-47, Sal-81, Sal-83, Sal-131, and Sal-132) produced biofilm, which were all isolated from pig blood-based food (Figure 2). Among the six isolated strains, Sal-081 and Sal-132 could produce high quantities of biofilm. The other strains were weak biofilm producers, as well as ATCC 14028.

Virulence gene screening and phylogenetic analysis

The genes associated with virulence were screened using WGS and the VFDB. Among the *Salmonella* strains, 137 virulence-related genes were detected and the pathogenicity mechanisms of the virulence genes were also evaluated, which is summarized in Table S3. Sal-081 and Sal-123 exhibited the highest prevalence (78.10%; 107/137) of virulence genes, and Sal-023 contained 95 (69.34%) virulence genes. It is known that *Salmonella* Pathogenicity Islands (SPIs) encoding type III secretion system (T3SSs) play a crucial role in *Salmonella* pathogenicity (Mohakud *et al.*, 2022). The isolated strains that were positive for the genes encoding for the T3SS effector is shown in Figure 3 and Table S3. The results showed that genes *sopA*, *slrP*, *sopB*/*sigD*, *sipA*/*sspA*, *sptP*, and *sseL* were detected in all isolates. In addition to the six genes mentioned above, the two *Salmonella* Derby strains also harbored genes *sseK1*, *nleB1*, *steC*, *sseJ*, *sspH2*, *sopE2*, *sspH1*, *ssel*/*srfH*, *pipB*, and *sopD2*. Moreover, genes *sseK1*, *nleB1*, *steC*, *sseJ*, *sseK2*, *sspH1*, *sspH2*, *ssel*/*srfH*, and *gogB* were all detected in *Salmonella* I 4,[5], 12: i: – strains.

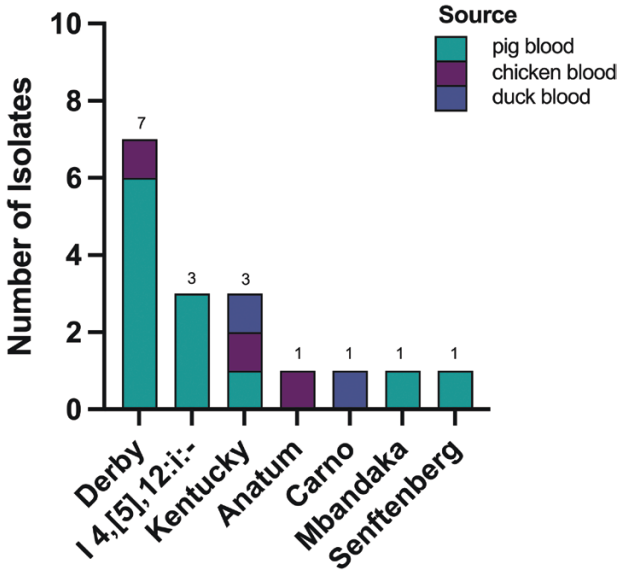

Figure 1. The sources of different serotypes of *Salmonella* isolates.

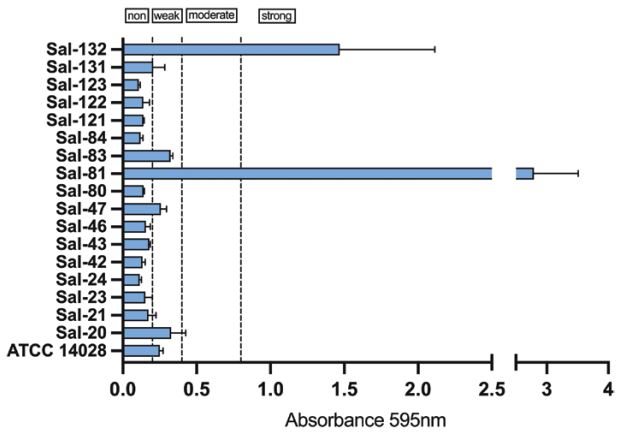

Figure 2. Biofilm formation capacity of *Salmonella* isolates. Biofilm formation capacity was classified as strong, moderate, weak, and no biofilm formers, as indicated by the dashed line.

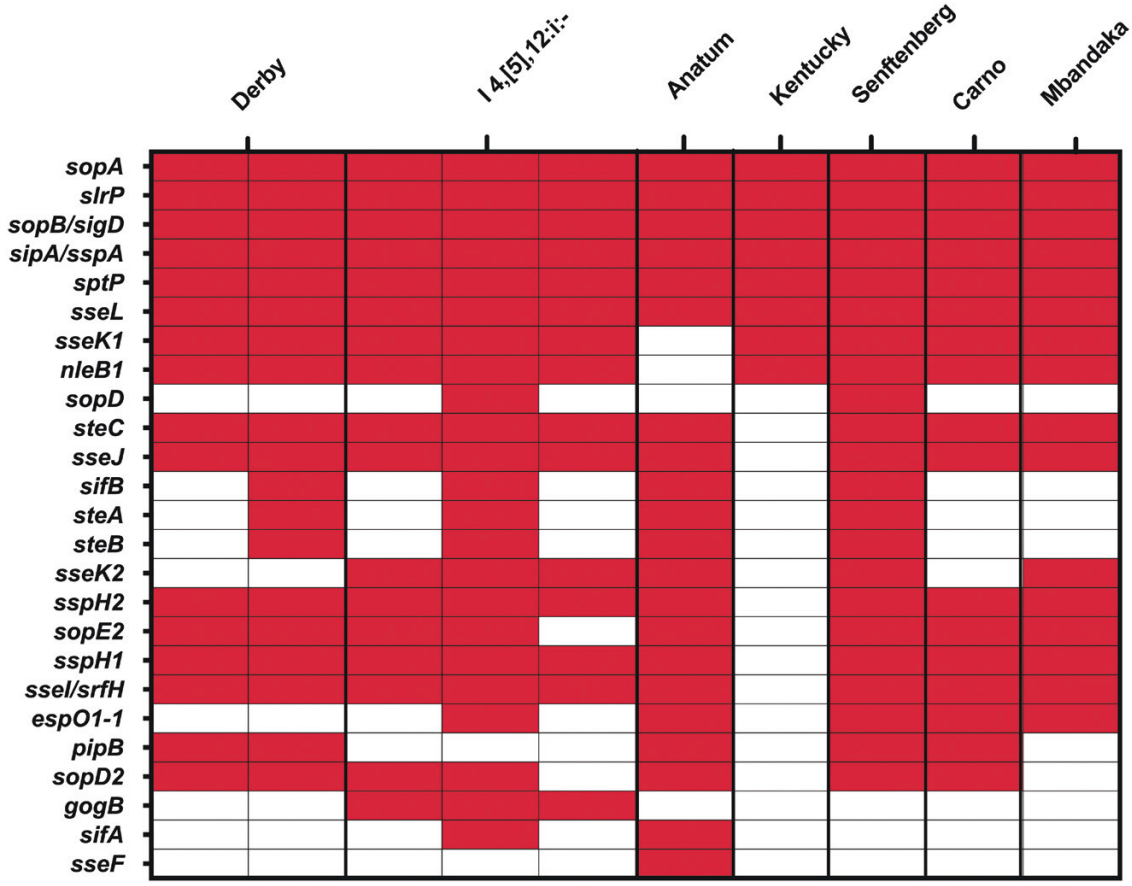

Figure 3. The presence of virulence genes is shown in the heatmap among the isolated *Salmonella* strains (n=10). The detected virulence genes in the corresponding strains are highlighted in red.

Additionally, the *Salmonella* Kentucky strain harbored the fewest genes.

Phylogenetic analysis was used to assign the possible origin of blood-based food isolates. The genome sequences of blood-based food isolates were collectively analyzed with 45 randomly selected *Salmonella* genome sequences from the National Center for Biotechnology Information database. The codon tree method was applied with 100 genes for tree building. According to Figure 4, the origins of contamination were dispersed. Three *Salmonella* I 4,[5], 12: i: – strains (Sal-043, Sal-081, and Sal-132) showed close relatedness with strains from clinical origins, as well as strains Sal-020 and Sal-023. At the same time, *S*. Derby isolates were all potentially of animal origins.

Antimicrobial resistance profiles of *Salmonella* isolates

The antimicrobial resistance profiles of selected *Salmonella* strains were searched and identified using a collective resource from ResFinder, PlasmidFinder, and PointFinder databases. The results revealed a high prevalence of MDR, in which 7 of the 10 isolates contained at least four resistance genes (Figure 5). Among the detected resistance genes, $bla_{\text{TEM-1B}}$ mediating ampicillin resistance, *aph(6)*-Id mediating kanamycin resistance, *aph(3′′)*-Id mediating streptomycin resistance and *tet(A)* mediating tetracycline resistance were among the most detected (10%; 4/10) AMR genes. Three of 10 MDR strains included *cmlA1*, *aac(3)-IV*, *aadA1*, and *tet(B)* genes, conferring resistance to chloramphenicol, gentamicin, streptomycin, and tetracycline, respectively. The genes *gyrA(D87G)* and *gyrA(S83F)* associated with nalidixic acid resistance were also found. *S*. Senftenberg, Carno, and Mbandaka (Sal-023, Sal-042, and Sal-131) strains were free of resistance genes in the collection of AMR databases.

In order to validate the *in-silico* prediction of antimicrobial resistance profiles, we performed an antimicrobial susceptibility test with corresponding antibiotics. The resistance level was classified as susceptible, intermediate, and resistant according to the criteria of the CLSI guidelines. The minimum inhibitory concentration (MIC) of the corresponding strains to different antibiotics is summarized in Table 1. The results revealed a high resistance rate, including tetracycline (70.6%; 12/17), ampicillin (41.2%; 7/17), streptomycin (41.2%; 7/17), and gentamicin (23.5%; 4/17). There were seven isolated *Salmonella* strains that were resistant to at least three antimicrobial agents (Table S4). The origin of the seven MDR strains was mainly pig blood-based food (85.7%; 6/7).

The results from antimicrobial susceptibility testing and *in-silico* prediction were consistent on MDR strains (Table S5). However, inconsistency was also observed. Strains Sal-020 and Sal-081 were tested as resistant to ceftiofur and strains Sal-080 and Sal-081 were tested as resistant to kanamycin and nalidixic acid, but the corresponding resistance genes were not detected. On the other hand, strains Sal-043, Sal-081, Sal-132, Sal-080, and Sal-123 harbored genes conferring resistance to

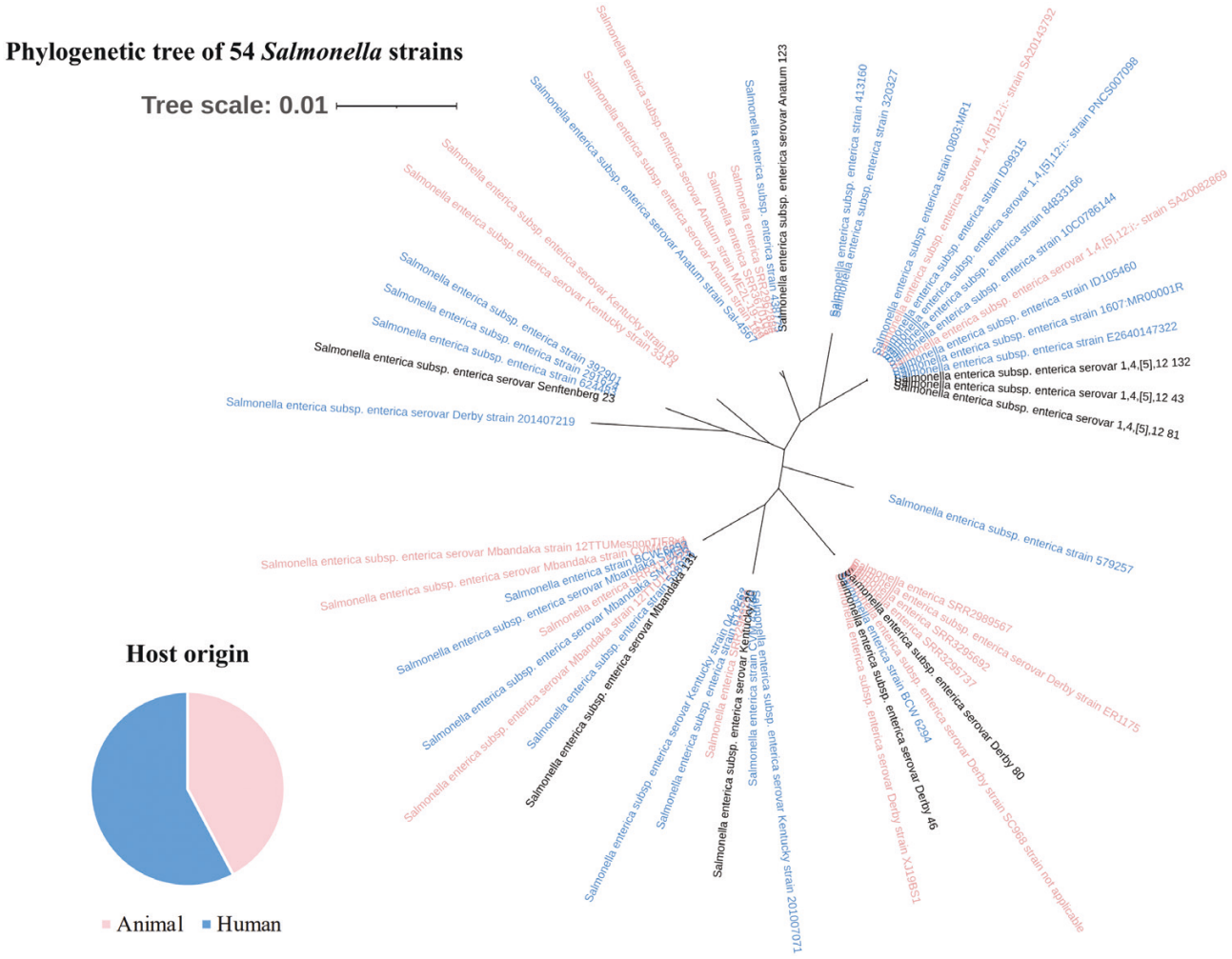

Figure 4. Phylogenetic relationships of 54 *Salmonella* strains (including 45 whole-genome sequenced blood-based food isolates). The origins of publicly available genome sequences are labeled as indicated colors, and strains isolated in this study are partly shown in black.

kanamycin and streptomycin but were tested as sensitive. In general, the presence of the *cmlA1* gene showed good consistency of tested chloramphenicol resistance (Figure 6).

Discussion

In China, traditional blood-based foods are popular due to their good flavor and rich nutrients (Bah *et al.*, 2013; Lynch *et al.*, 2017). Blood used for the production of blood-based food is mainly from poultry and pig which may carry zoonotic pathogens, such as *Salmonella*. The blood-based products are kept in a hot pool at 80–90 °C for 20–30 min and immediately kept in ice water during the traditional manufacturing process. Limited hygiene standards and improper handling may lead to incomplete sterilization and aggravate the risk of pathogen contamination. Foodborne pathogens are often exposed to various environmental stresses during food processing, and temperature fluctuations can cause significant stress to these bacteria. *Salmonella* is generally considered to be thermotolerant foodborne pathogens (Bunning *et al.*, 1990; O'Connor *et al.*, 2009; Nguyen *et al.*, 2017). Generally, they can grow rapidly at temperatures ranging from 5 to 60 °C (Ricke *et al.*, 2018). *Salmonella* isolates studied in the project could survive a wide range of temperatures ranging from 4 to 53 °C, which covered the cold and heat stress range used in food processing. Bunning *et al.* (1990) examined the tolerance of *Salmonella* typhimurium to heat shock at 42, 48, and 52 °C, and found that *Salmonella* started to lose viability at 52 °C. *Salmonella*-contaminated blood-based food can survive heating for solidification and cooling at 4 °C, which may be a potential threat to food safety.

Salmonella can develop biofilm to colonize and resist harsh environments. A biofilm is defined as a community of cells sticking to each other, embedding in a slimy extracellular matrix composed of extracellular polymeric substances (Funari and Shen, 2022). Allison *et al.* (2000) showed that biofilms were 100–1000 times more tolerant than planktonic cells to antimicrobial agents, such as isothiazolones, quaternary ammonium compounds, halogens, and halogen-release agents. We noticed that several *Salmonella* isolates from contaminated blood-based food could form biofilm (Figure 2). In addition, MDR isolates Sal-081 and Sal-132 could form strong biofilm, indicating the potential food safety and resistance transfer risk that can persist in food. Blood may stimulate the biofilm formation ability of the strains. Wiktorczyk *et al.* (2018) found that strains isolated from blood formed biofilm more efficiently when sheep blood was added. Medium with sheep blood at a concentration of up to 20.0% increased

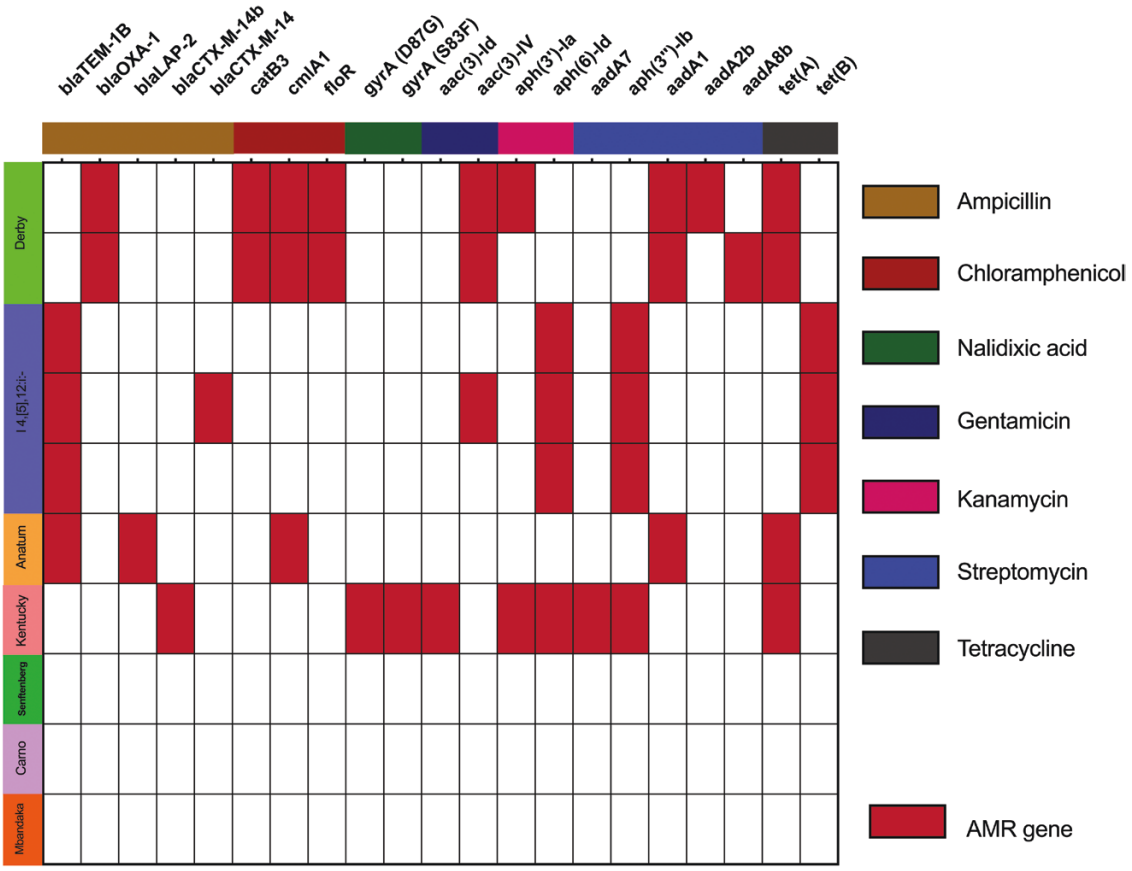

Figure 5. The presence of antimicrobial resistance genes in the corresponding isolates is shown as a heatmap and highlighted in red.

Table 1. Antimicrobial susceptibility results of the isolated *Salmonella* strains (*n*=17)

Antimicrobial agents	MIC Breakpoints (μg/mL)			Results in percentage		
	S	I	R	S	I	R
Ampicillin	≤8	16	≥32	58.8% (10/17)	0% (0/17)	41.2% (7/17)
Ceftiofur	≤2	4	≥8	88.2% (15/17)	0% (0/17)	11.8% (2/17)
Gentamicin	≤4	8	≥16	76.5% (13/17)	0% (0/17)	23.5% (4/17)
Chloramphenicol	≤8	16	≥32	0% (0/17)	82.4% (14/17)	17.6% (3/17)
Kanamycin	≤16	32	≥64	82.4% (14/17)	0% (0/17)	17.6% (3/17)
Nalidixic acid	≤16	—	≥32	64.7% (11/17)	23.5% (4/17)	11.8% (2/17)
Streptomycin	≤8	16	≥32	5.9% (1/17)	52.9% (9/17)	41.2% (7/17)
Tetracycline	≤4	8	≥16	29.4% (5/17)	0% (0/17)	70.6% (12/17)

S: susceptible; I: intermediate; R: Resistant.

the number of *Listeria monocytogenes* strains recovered from biofilm, whereas only the strains isolated from blood formed more biofilm when 50% blood was added to the medium. Low biofilm-forming strains can possibly originate from productive processes other than chronic stress exposure, resulting in the formation of less biofilm.

Salmonella as a pathogen contains various virulence genes mediating functions, such as host adhesion and invasion, survival within host cells, and biofilm formation (Main-Hester *et al*., 2008; Huehn *et al*, 2010; Thung *et al*, 2018; Nikiema *et al*, 2021; Sobota *et al*, 2022). *Salmonella* pathogenesis is mainly conferred through the expression of T3SSs, which facilitate bacterial invasion into host cells (Mohakud *et al*., 2022). In this study, WGS coupled with bioinformatics was used to detect virulence genes in the isolated strains. Strains collected in this study all contained the typical T3SS virulence factors. This indicated the potential for invasion and survival inside a variety of mammalian cells as suggested by LaRock *et al*. (2015). Strains Sal-081 and Sal-123 contained the most virulence genes, as much as 107 virulence genes. Among these virulence genes, more than 20 were SPI genes, which indicated a higher pathogenicity potential. In order to understand the contamination origin of the isolated *Salmonella* strains, we constructed a WGS-based phylogenetic tree and found

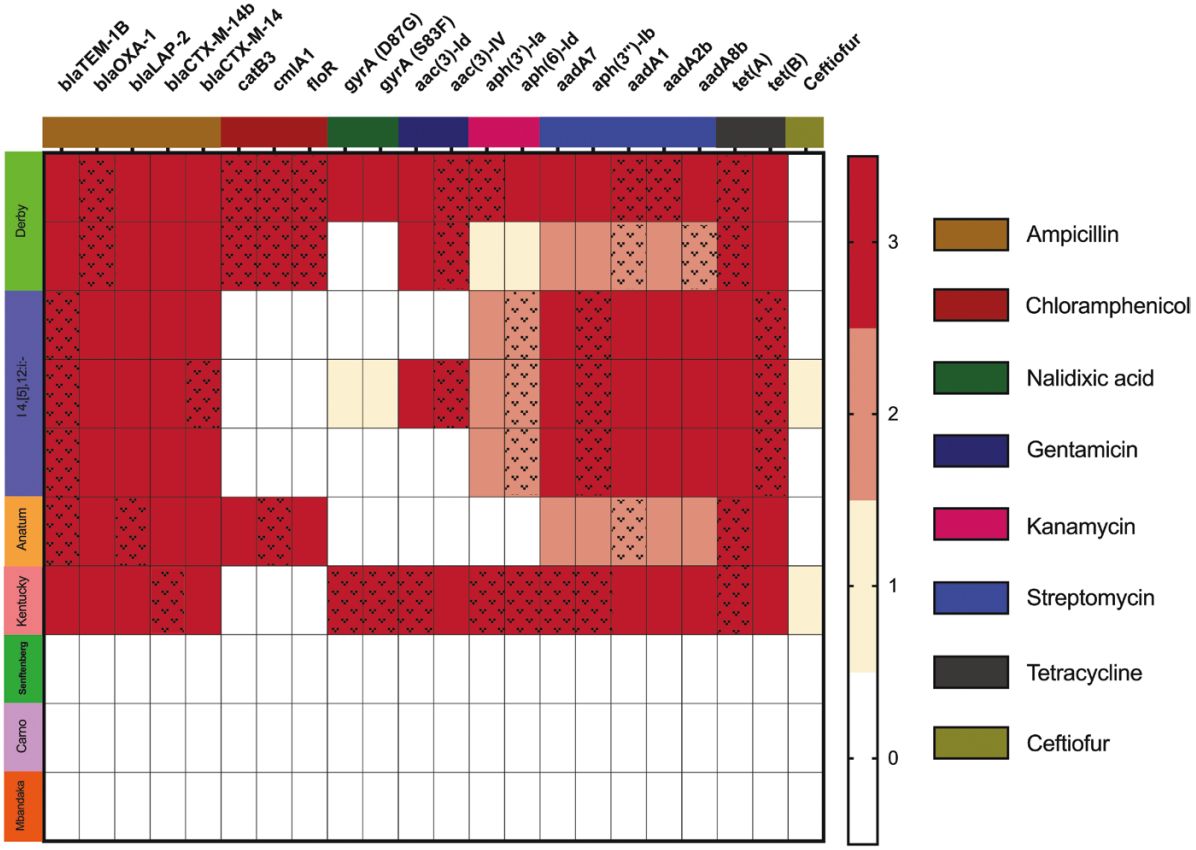

Figure 6. The correlation of phenotypical and genotypical antimicrobial resistance. The dashed area indicates the relevant antimicrobial resistance genes detected in the strains. '0' means that neither phenotypical nor genotypical antimicrobial resistance genes were detected. '1' means that only the phenotypical antimicrobial resistance genes were detected. '2' means that only the genotypical antimicrobial resistance genes were detected. '3' means that both the phenotypical and genotypical genes were detected.

that 55.56% (5/9) of *Salmonella* strains had the human origin of contaminations, which hints at the potential hazard of cross-contamination in the food industry.

Antimicrobials have been widely used to prevent or treat infections on animal farms to enhance feed proficiency and promote growth, which is linked to economic gains (Van *et al.*, 2020). However, the abuse of antimicrobials induces the emergence of antimicrobial resistance, and resistance can be spread and transmitted through the food chain. Bacteria can acquire exogenous genes, including resistance genes through horizontal gene transfer (HGT) (Read *et al.*, 2014). The increasing prevalence of MDR *Salmonella* has recently become an emerging problem worldwide (Abd-Elghany *et al.*, 2022; Antibiotic Resistance Surveillance Reference Laboratory, 2022; Salvador *et al.*, 2022). Therefore, it is necessary to detect the MDR *Salmonella* strains to monitor the transmission of AMR genes. The MIC results showed that 41.18% (7/17) of the isolates were MDR *Salmonella*. Two MDR *Salmonella* strains were resistant to nalidixic acid, which is the most common antibiotic used in humans (Pham *et al.*, 2019; Millanao *et al.*, 2021). This result highlighted the existence of cross-contamination during slaughtering or transportation and storage. According to the phenotypical results, all the MDR strains were resistant to ampicillin, streptomycin, and tetracycline, which made them less-effective antimicrobial agents. Various studies have reported a consistent trend of increasing resistance to ampicillin, streptomycin, and tetracycline in *Salmonella* (Harb *et al.*, 2018; Neuert *et al.*, 2018). WGS-based methods were used to detect antimicrobial resistance genes among blood-based food isolates and 21 antibiotic-resistance genes were found. Among these resistance genes, we found point mutation genes in *gyrA* genes *gyrA(D87G)* and *gyrA(S83F)*, which encode nalidixic acid resistance. *gyrA* is an essential gene, encoding quinolone resistance and determining the region of the A subunit of DNA gyrase (Eaves *et al.*, 2022). Many kinds of mutations in *gyrA* have been found (Gopal *et al.*, 2016) and the mutations can serve as substitutions to confer quinolone resistance in *Salmonella* (Piddock, 2002).

In this study, we also investigated the consistency between genotype and phenotype of antimicrobial resistance. Neuert *et al.* (2018) compared the phenotypic susceptibility testing and WGS prediction of MDR profiles, and their results completely matched both approaches. Zankari *et al.* (2013) observed similar high consistency between genotypic and phenotypic resistance in a study of 50 *Salmonella* isolates from swine. In this study, we observed three isolates that carried kanamycin resistance genes but which were susceptible phenotypically, and two isolates were phenotypically susceptible but carried streptomycin resistance genes. The existence of silent resistance genes may explain the inconsistency between *in-silico* prediction and phenotypic antimicrobial susceptibility test, as silence of *aadA* and *catA1* variants has been reported (Ma *et al.*, 2007; Deekshit *et al.*, 2012; Adesiji *et al.*, 2014). In addition, many antibiotic resistance genes were found in gene cassettes, segments of

DNA with one or two opening reading frames that lack promoters (Hall, 2013). Gene cassettes are components of an integron, a two-component gene capture and dissemination system (Warren *et al.*, 2013). The complex structure of gene cassettes and the absence of promoter attest to the difficulty of antimicrobial resistance genes expressing in the region of the integron, leading to the phenotypical susceptibility. Moreover, resistance to ceftiofur, kanamycin, and nalidixic acid was observed, although corresponding resistance genes were not detected, which suggested overlooked resistance mechanisms.

Conclusions

In this research, we successfully isolated and identified 17 *Salmonella* strains from blood-based food in seven cities in China. All the isolated *Salmonella* strains could survive under cold (4 °C) and heat (53 °C) stresses. By analyzing the contamination origin of the strains, we underlined the possibility of cross-contamination during slaughtering or transportation and storage. Ongoing efforts and further well-designed epidemiological studies to prevent MDR transmission of foodborne pathogens between the environment and animals are essential for traditional blood-based products.

Supplementary Material

Supplementary material is available at *Food Quality and Safety* online.

Author Contributions

Tian Yang, Ricardo A. Wu-Chen, and Runrun Zhang performed the assays, collected and analyzed the data, and drafted the manuscript. Xinyu Liao, Yiyang Lou, and Greta Gölz analyzed the data and contributed to editing the manuscript. Tian Ding and Jinsong Feng designed the research, supervised the work, and carried out revisions.

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Conflict of Interest

The authors declare that there are no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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