



Heavy Metal Resistance in *Salmonella* Typhimurium and Its Association With Disinfectant and Antibiotic Resistance

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Metals are widely used in animal feed for their growth-stimulating and antimicrobial effects, yet their use may potentially promote the proliferation of antibiotic resistance through co-selection. We studied the prevalence and associations of metal, antibiotic, and disinfectant resistances of 300 *Salmonella* Typhimurium isolates from pig meat, pig manure, chicken meat, poultry manure, and human stool from Sichuan, China. Seventy four percent of the 300 *Salmonella* Typhimurium isolates were considered resistant to Cu, almost 50% to Zn and Cr, over 25% to Mn and Cd, and almost 10% to Co. Most of the isolates carried at least one heavy metal resistance gene (HMRG). The Cr-Zn-Cd-resistance gene *czcD* was carried by 254 isolates and the Cu-resistance genes *pcoR* and *pcoC* by 196 and 179 isolates, respectively. Most of the isolates were resistant to at least one antibiotic and almost 80% were multidrug-resistant. The prevalence of resistance to six antibiotics was higher among the pig meat and manure isolates than among other isolates, and that of streptomycin and ampicillin were highest among the pig meat isolates and that of ciprofloxacin and ofloxacin among the pig manure isolates. From 55 to 79% of the isolates were considered resistant to disinfectants triclosan, trichloroisocyanuric acid, or benzalkonium chloride. The metal resistances and HMRGs were associated with resistance to antibiotics and disinfectants. Especially, Cu-resistance genes were associated with resistance to several antibiotics and disinfectants. The transfer of the Cr-Zn-Cd-resistance gene *czcD*, Cu-resistance gene *pcoC*, and Co-Ni-resistance gene *cnrA* into *Escherichia coli* and the increased Cu-resistance of the transconjugants implied that the resistance genes were located on conjugative plasmids. Thus, the excessive use of metals and disinfectants as feed additives and in animal care may have the potential to promote antibiotic resistance through co-selection and maintain and promote antibiotic resistance even in the absence of antibiotics.

Keywords: *Salmonella* Typhimurium, heavy metal resistance, disinfectant resistance, antibiotic resistance, heavy metal resistance gene, conjugation

INTRODUCTION

Salmonella infections are a major public health concern worldwide (Sodagari et al., 2020). *Salmonella* is the second most common cause of food-borne diseases worldwide and is associated with more deaths than any other food-borne disease in the developed countries (WHO, 2020). Food-borne Disease Active Surveillance Network (Foodnet) reported *Salmonella* as the leading cause of food-borne disease-related deaths in the United States (Barton Behravesh et al., 2011). Among the food-borne infections caused by a single etiologic agent in the United States in 2015, *Salmonella* was responsible for 34% of the outbreaks, 39% of the illnesses, 64% of the hospitalizations, and 60% of the deaths (Dewey-Mattia et al., 2018). The European Food Safety Authority (EFSA) confirmed salmonellosis as the second most common zoonosis with over 90,000 confirmed cases in 2017 (EFSA and ECDC, 2018). The food-borne surveillance system in China suggested that *Salmonella* was the second most common bacteria causing food-borne outbreaks during 2000–2014 (Luo et al., 2017). Thus, the *Salmonella* infection-related mortality and morbidity burden societies worldwide (Majowicz et al., 2010). For example, in England, the *Salmonella* infections, characterized by diarrhea, fever, and abdominal cramps, leading to over 11,000 annual GP consultations (Tam et al., 2012; Barr and Smith, 2014).

More than 2,600 serotypes of *Salmonella* have been identified (Takaya et al., 2020). *Salmonella* Typhimurium is one of the predominant serotypes in many countries, including China (Ceysens et al., 2015; Wang Y. et al., 2017; Simpson et al., 2018) and it has held first or second place in China for many years (Ran et al., 2011; Wang Y. et al., 2017). *Salmonella* Typhimurium was also reported as the most frequently isolated serotype of non-typhoidal *Salmonella* from food-borne illnesses in different provinces of China (Liang et al., 2015), which represented 25.5, 29.44, and 39.7%, of the isolates obtained from diarrheal disease surveillance between 2006–2010 in Shanghai, 2010–2014 and 2013–2018 in Shenzhen, China (Zhang et al., 2014; Li W. et al., 2017; Shen et al., 2020). *Salmonella* is associated with a wide variety of foods, yet animal products, especially pig and poultry, are the main source of *Salmonella* (Hugas and Beloeil, 2014; Antunes et al., 2016; Demirbilek, 2017; Heredia and García, 2018). The routes of transmission for *Salmonella* Typhimurium include contaminated meat, eggs, and manure (Antunes et al., 2016; Chousalkar et al., 2018; Kumar et al., 2019). With the development of China, the average intake of meat, especially pig increased from 37.1 g/day in 1992 to 64.3 g/day per person in 2012 and was expected to surpass 100 g/day people by 2020 which has indirectly increased the risk of food-borne zoonoses, including salmonellosis (He et al., 2016; CIIN, 2018a,b; Pan et al., 2018, 2019). In China, *Salmonella* Typhimurium strains are commonly isolated from retail meat, particularly pig, suggesting a link between human infections with this serotype and pig products.

Antibiotics have commonly been used to treat animal diseases and as growth promoters (Allen et al., 2013). However, a substantial amount of the antibiotics are excreted in feces and enter soil via manure (Kumar et al., 2005). Due to food safety

and health issues, many countries, e.g., in the European Union, have banned the use of antibiotics in the feed of animals as growth promoters (Burch, 2006). Both WHO and individual countries have introduced guidelines to withdraw medicated feed additives to combat antimicrobial resistance from animal resources (Hu and Cowling, 2020). Metal-containing compounds are also widely used animal feed additives (Cavaco et al., 2011; Argudín et al., 2016). Metals, e.g., copper, zinc, cobalt, chromium, and manganese, are widely used in animal feed for their growth-stimulating and antimicrobial effects; copper and silver are commonly used as disinfectants and preservatives, and mercury, lead, arsenic, and cadmium can be found as contaminants in animal feed (Seiler and Berendonk, 2012; Korish and Attia, 2020). However, the use of heavy metals at high concentrations causes problems due to their toxicity, bioaccumulation, and biomagnification in the food chain (Figure 1; Eisler, 1993). Heavy metal contamination in meat and the prevalence of these pollutants in the environment is a risk for both food safety and human health (Khan et al., 2008; Bamuwamyte et al., 2015; Lu et al., 2015). Notably, the use of metals may potentially promote the proliferation of antibiotic resistance through co-selection; the metal and antibiotic resistance are often linked either due to co-location of the resistance genes, a shared resistance mechanism, or co-regulation of resistance pathways (Baker-Austin et al., 2006; Deng et al., 2017; Pal et al., 2017; Yang et al., 2020). The disinfectant resistance genes and heavy metal resistance genes (HMRGs) are commonly located in mobile genetic elements (MGEs) (Frost et al., 2005). The role of metals, antibiotics, and disinfectants in the development and spread of antimicrobial resistance has raised concerns (Bragg et al., 2014; Roosa et al., 2014; Zou et al., 2014; Di Cesare et al., 2016). However, the co-occurrence of heavy metal, antibiotic and disinfectant resistance in *Salmonella* Typhimurium isolates from retail foods, animal manure, and human stool has not been widely investigated. Therefore, we studied the prevalence and associations of resistances of *Salmonella* Typhimurium isolates from pig meat, pig manure, chicken meat, poultry manure, and human stool from Sichuan, China.

MATERIALS AND METHODS

Bacterial Strains

The 300 *Salmonella* Typhimurium strains analyzed in this study were isolated from January 2016 to December 2018 in Sichuan, China, from pig meat ($n = 182$), pig manure ($n = 23$), chicken meat ($n = 30$), poultry manure ($n = 27$), and stool of hospitalized diarrhea patients ($n = 38$). The details of sampling and location of the strains are available in **Supplementary Tables 4A,B**.

Isolates and Serotyping

Collected specimens were tested for *Salmonella* using the following protocol. Stool samples from diarrhea patients were enriched in Selenite Brilliant Green broth (SBG, CHROMagar, Paris, France) for 16–22 h at 37°C. For the isolation of *Salmonella* from pig and chicken meat and manure samples, the method described by the United States Department of Agriculture Food

TABLE 1 | Incidence of metal resistance among 300 *Salmonella* Typhimurium strains isolated from pig meat, pig manure, poultry manure, chicken meat, and human stool samples.

| | MIC | | | | | | | | | | Resistance % |
|----|------|-----|-----------|-----|-----|-----------|------------|-----------|------|--|--------------|
| | 12.5 | 25 | 50 | 100 | 200 | 400 | 800 | 1600 | 3200 | | |
| Cu | | | | 1 | 1 | 76 | 222 | | | | 74 |
| Zn | | | 4 | | | 152 | 143 | | | | 47.7 |
| Mn | | | 1 | | | | 216 | 83 | | | 27.7 |
| Cd | 5 | 207 | 88 | | | | | | | | 29.3 |
| Co | | 1 | | 2 | 270 | 27 | | | | | 9 |
| Cr | | | | | | 163 | 137 | | | | 45.7 |

The strains with MIC higher than that of control strains *E. coli* ATCC 10536 and *Salmonella* H9812 were considered resistant. The numbers of resistant strains are in bold.

out in a 25 μ L reaction volume, comprising 10 μ L of 2 \times T5 Super PCR Mix, 2 μ L of 5 \times Enhancer Buffer, 1 μ L of each forward and reverse primer, 2U of Taq-polymerase (Promega, Madison, WI, United States) and 2 μ L of template DNA or 2 μ L of sterile deionized water as a negative control. The thermal program included initial denaturation at 98°C for 3 min, followed by 34 cycles of denaturation at 98°C for 10 s, annealing at the T_m of primer pair (**Supplementary Table 1**) for 10 s and extension at 72°C for 20 s, and a final extension at 72°C for 2 min. The success of amplification was verified using electrophoresis in 0.8% agarose gel (GENEI, Bengaluru, India) with a 2,000 bp DNA molecular weight marker as reference (Fermentas, Waltham, MA, United States) and visualized using a gel documentation system (BIO-RAD, Hercules, CA, United States). Appropriate positive controls for amplification were selected from retail meat *Salmonella* Typhimurium isolates. The positive controls were confirmed by sequencing the amplicons (GENEWIZ, Inc., Germantown, MD, United States). All results were confirmed by at least two independent assays.

Determination of Minimal Inhibitory Concentrations (MICs)

The minimal inhibitory concentrations (MICs) of metal ions, disinfectants, and antibiotics for *Salmonella* Typhimurium were

determined using the agar microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) (Weinstein and Lewis, 2020). Mueller-Hinton agar plates were inoculated with bacteria suspended in 0.85% NaCl to a turbidity equivalent to a 0.5 McFarland using a multipoint inoculator (Oxoid, Lenexa, KS, United States) with approximately 10^4 CFU per spot. The plates were incubated at 37°C for 18–24 h. The MICs were determined as the lowest concentration of the metal that inhibited the growth of strains completely after 18–24 h of culture at 37°C. All experiments were run in triplicate.

The MICs of copper ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), chromium ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$), cobalt (CoCl_2), cadmium (CdCl_2), zinc (ZnSO_4), and manganese Mn (MnCl_2) (Alfa Aesar, Shanghai, China) were determined. Doubling dilutions of the heavy metal stock solutions were incorporated into Mueller-Hinton agar plates with final concentrations ranging from 0.25 to 3200 mg L^{-1} . *Escherichia coli* ATCC 10536 and *Salmonella* H9812 were used as the quality control strain in the tests (Zou et al., 2014; Deng et al., 2017; Yang et al., 2020). The MICs of benzalkonium chloride (BC), Trichloroisocyanuric acid (TCCA), (Chengdu Best-Reagent Company, Chengdu, China; > 98% purity), Triclosan (TCS) (J&K Chemical; > 98% purity), were determined at concentration ranges of 0.125–1024 mg L^{-1} for BC and TCCA and 0.03125–1.0 mg L^{-1} for TCS. *Escherichia coli* ATCC 10536 and *Salmonella* H9812 were used as the quality control strain in the tests (Zou et al., 2014; Deng et al., 2017; Yang et al., 2020). In the MIC assays of streptomycin (S), sulfonamides (S3), tetracycline (TET), ampicillin (AMP), nalidixic acid (NA), chloramphenicol (C), sulfamethoxazole (SXT), trimethoprim (TMP), gentamicin (CN), amoxicillin/clavulanic acid (AMC), ciprofloxacin (CIP), ofloxacin (OFX), ceftazidime (CAZ) and cefotaxime (CTX) (Hangzhou Microbial Reagent Co., Ltd., China), the breakpoints for antibiotic resistance and/or susceptibility were determined as recommended by the Clinical and Laboratory Standards Institute (CLSI). *Escherichia coli* ATCC 25922 and 35218 strain were used for quality control (Wang J. et al., 2017).

Conjugation Experiment

The transfer of HMRGs was determined in a conjugation experiment using mixed broth cultures as previously described

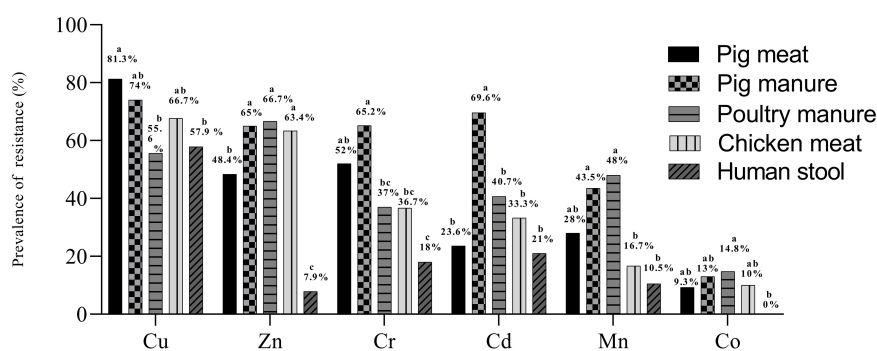


FIGURE 2 | Prevalence of resistance to Cu, Zn, Mn, Cd, Co, and Cr among *Salmonella* Typhimurium isolates from pig meat, pig manure, poultry manure, chicken meat, and human stool. Different lowercase letters indicate statistically significant differences between groups ($P < 0.05$).

TABLE 2 | Prevalence of heavy metal resistance genes among 300 *Salmonella* Typhimurium isolates.

| Heavy metal resistance gene | Number of isolates (n) | Prevalence (%) |
|-----------------------------|------------------------|----------------|
| <i>czcD</i> | 254 | 84.7 |
| <i>pcoR</i> | 196 | 65.3 |
| <i>pcoC</i> | 179 | 59.7 |
| <i>cnrA</i> | 155 | 51.7 |
| <i>nccA</i> | 153 | 51 |
| <i>cadD</i> | 131 | 43.7 |
| <i>merA</i> | 104 | 34.7 |
| <i>pbrA</i> | 63 | 21 |
| <i>pcoA</i> | 61 | 20.3 |
| <i>chrB</i> | 18 | 6 |
| <i>czcB</i> | 17 | 5.7 |
| <i>arsB</i> | 15 | 5 |

(Cai et al., 2008). Isolates with the highest resistance against copper and other metals were chosen as donors. Plasmid-free *Escherichia coli* J53 strain resistant to sodium azide and sensitive to the metals used in this study was selected as a recipient. Donor and recipient strains were grown on trypticase soy agar (TSA) plates overnight, single colonies of donor and recipient were inoculated into 30 mL of Mueller Hinton Broth (MHB) and grown at 37°C for 18 h, after which the recipient and donor strains were mixed at 10:1 (v = v) proportion. Subsequently, 1 mL of the mixture was inoculated onto a sterilized membrane on Mueller Hinton Agar (MHA) and incubated at 37°C for 18 h. The trans-conjugant bacteria were suspended into 3 mL of 0.9% NaCl, and serial dilutions were spread on MHA plates containing 200–400 µg/mL copper and 100 µg/mL sodium azide (Wang et al., 2003; Xu et al., 2007). Plates were incubated at 37°C and inspected at 24 and 48 h. The transfer of heavy metal resistance determinants was determined by amplifying the Cr-Zn-Cd-resistance gene *czcD*, Cu-resistance gene *pcoC*, and Co-Ni-resistance gene *cnrA* using DNA from trans-conjugant bacteria as a template. The MIC of copper (CuCl₂·2H₂O) for the trans-conjugants were determined with final concentrations ranging from 100 to 800 µg L⁻¹. The conjugation experiment was repeated at least twice.

Statistical Analysis

Association of the metal resistances and HMRGs with antibiotic and disinfectant resistance in *Salmonella* Typhimurium were determined using the χ^2 -test of independence or Fisher's exact test was performed to analyze data using SPSS v. 21. *P*-value less than 0.05 was considered statistically significant.

The data about prevalence were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range tests in SAS statistical software (SAS Institute; Cary, NC, United States). Differences were considered statistically significant at $P \leq 0.05$. The results were visualized using GraphPad prism 8.0.1. (GraphPad Software, San Diego, CA, United States).

RESULTS

Metal Resistance

Over 98% of the 300 *Salmonella* Typhimurium isolates had MICs of 400–800 mg L⁻¹ for Cu, 400–800 mg L⁻¹ for Zn, 800–1600 mg L⁻¹ for Mn, 25–50 mg L⁻¹ for Cd, 200–400 mg L⁻¹ for Co and 400–800 mg L⁻¹ for Cr (Table 1). Compared to *E. coli* ATCC 10536 and *Salmonella* H9812, 74% ($n = 222$), 47.7% ($n = 143$), 45.7% ($n = 137$), 27.7% ($n = 83$), 29.3% ($n = 88$), and 9% ($n = 27$) of the isolates had higher MIC for Cu, Zn, Cr, Mn, Cd and Co, respectively, and were considered resistant. The prevalence of Cu resistance was higher among the isolates from pig meat than among the human stool and poultry manure isolates ($P < 0.05$) (Figure 2). The prevalence of Zn resistance was highest among the isolates from pig manure, poultry manure, and chicken meat, second highest among the pig meat isolates, and lowest among the human stool isolates ($P < 0.05$).

The prevalence of Cr resistance was highest among the isolates from pig manure and lowest among the human stool isolates ($P < 0.05$). The prevalence of Cd resistance was higher among the isolates from pig manure than among the other isolates ($P < 0.05$). The prevalence of Mn resistance was highest among the isolates from pig and poultry manure and lowest among the chicken meat and human stool isolates ($P < 0.05$). None of the human stool isolates showed Co resistance and among the other isolates, the prevalence of resistance was on the same level.

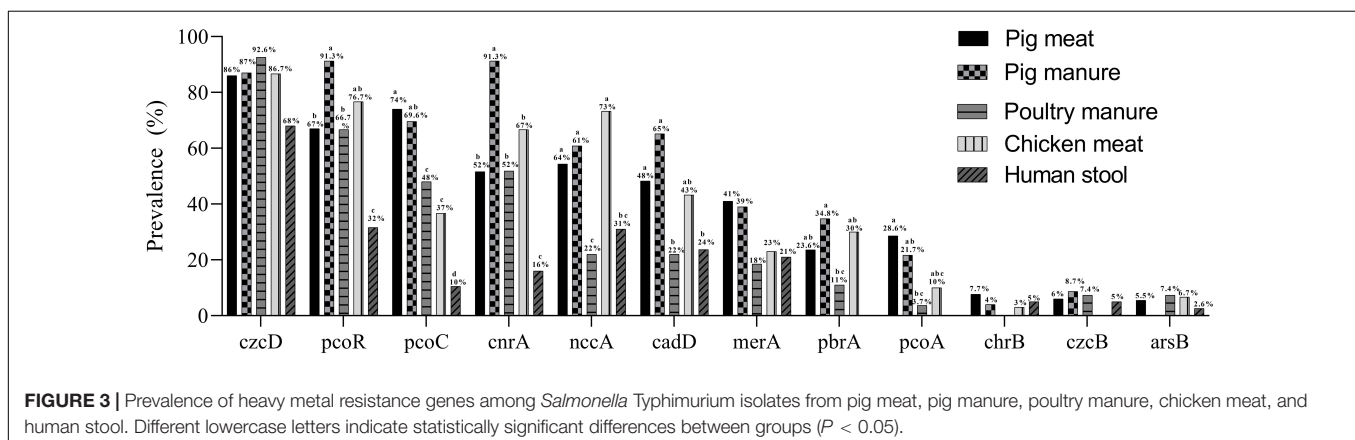


FIGURE 3 | Prevalence of heavy metal resistance genes among *Salmonella* Typhimurium isolates from pig meat, pig manure, poultry manure, chicken meat, and human stool. Different lowercase letters indicate statistically significant differences between groups ($P < 0.05$).

TABLE 3 | Prevalence of antibiotic resistance among 300 *Salmonella* Typhimurium isolates.

| Antibiotic resistance | Number of isolates | Prevalence (%) |
|-----------------------|--------------------|----------------|
| S | 237 | 79 |
| S3 | 234 | 78 |
| TET | 228 | 76 |
| AMP | 216 | 72 |
| NA | 194 | 64 |
| C | 180 | 60 |
| SXT | 171 | 57 |
| TMP | 165 | 55 |
| CN | 123 | 41 |
| AMC | 54 | 18 |
| CIP | 27 | 9 |
| OFX | 18 | 6 |
| CAZ | 3 | 1 |
| CTX | 3 | 1 |

In total, 97% ($n = 291$) of the isolates carried at least one heavy metal resistance gene (HMRG). A total of 132 gene combinations were found (Supplementary Table 2). The Cr-Zn-Cd-resistance gene *czcD* was carried by 254 isolates. The Cu-resistance genes *pcoR* and *pcoC* were found in 196 and 179 isolates, respectively. The Co-Ni-resistance gene *cnrA*, Ni-Cr-Cd-resistance gene *nccA*, and Cd-resistance gene *cadD* were carried by 155, 153, and 131 the isolates, respectively. The Hg-resistance gene *merA*, Pb-resistance gene *pbrA*, Cu-resistance gene *pcoA*, and Cr-resistance gene *chrB* were carried by 104, 63, 61, and 18 isolates, respectively. The Cr-Zn-Cd-resistance gene *czcB* and As-resistance gene *arsB* were carried by 17 and 15 isolates (Table 2).

The prevalence of *pcoR* and *cnrA* were highest among the pig manure isolates and lowest among the human stool isolates ($P < 0.05$) (Figure 3). The prevalence of *pcoC* was highest among the pig meat and manure isolates and lowest among the human stool isolates ($P < 0.05$). The prevalence of *nccA* was highest among the chicken meat isolates and lowest among the poultry manure isolates ($P < 0.05$). The prevalence of

cadD was higher among the pig meat, pig manure, and chicken meat isolates than among the poultry manure and human stool isolates ($P < 0.05$). The prevalence of *pbrA* was higher among the pig manure isolates than among the poultry manure isolates ($P < 0.05$). The prevalence of *pcoA* was higher among the pig meat isolates than among the poultry manure isolates ($P < 0.05$).

Antibiotic Resistance

A total of 287 *Salmonella* Typhimurium isolates were resistant to at least one antibiotic; all the 23 pig manure isolates, 98.4% of the pig meat isolates ($n = 179$), 97.4% of the human stool isolates ($n = 37$), 86.7% of the chicken meat isolates ($n = 26$) and 81.5% of the poultry manure isolates ($n = 22$). Over 200 isolates were resistant to streptomycin (S), sulfonamides (S3), tetracycline (TET), or ampicillin (AMP), over 120 isolates to nalidixic acid (NA), chloramphenicol (C), sulfamethoxazole (SXT), trimethoprim (TMP) or gentamicin (CN), and less than 55 to amoxicillin/clavulanic acid (AMC), ciprofloxacin (CIP), ofloxacin (OFX), ceftazidime (CAZ) or cefotaxime (CTX) (Table 3).

The prevalence of resistance to S and AMP was higher among the pig meat isolates than among the poultry manure, chicken meat, and human stool isolates ($P < 0.05$) (Figure 4). The prevalence of resistance to S3, TET, C, SXT, TMP, and CN was highest among the pig meat and manure isolates ($P < 0.05$). The prevalence of resistance to CIP and OFX was highest among the pig manure isolates ($P < 0.05$). The prevalence of resistance to AMC was higher among the pig meat and manure isolates than among the human stool isolates ($P < 0.05$). The prevalence of resistance to NA was higher among the pig and chicken meat isolates than among the poultry manure isolates ($P < 0.05$). None of the pig and chicken meat and poultry manure isolates were resistant to CAZ, and none of the pig meat and pig and poultry manure isolates were resistant to CTX.

A total of 77 resistance profiles were observed among the isolates. A multiple resistance profile TET-AMP-SXT-C-NA-TMP-CN-S3-S was carried by 36 isolates, TET-AMP-SXT-C-TMP-S3-S by 25 isolates,

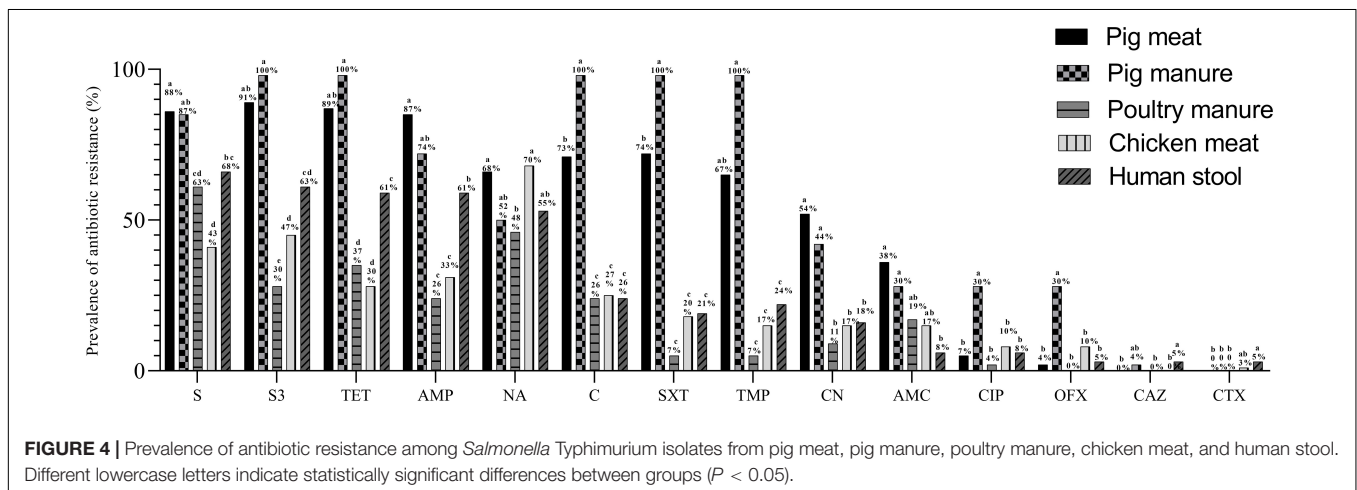


TABLE 4 | Incidence of disinfectants among 300 *Salmonella* Typhimurium strains isolated from pig meat, pig manure, poultry manure, chicken meat, and human stool samples.

| | MIC | | | | | | | | | | Resistance | |
|------|-----|---|----|------------|-----------|-----------|-----|------------|-----------|------|------------|------|
| | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | | % |
| BC | 17 | 3 | 42 | 181 | 18 | 39 | | | | | | 79.3 |
| TCCA | | | | | 33 | 4 | 45 | 149 | 69 | | | 72.7 |

| | MIC | | | | | | Resistance | |
|-----------------|--------|--------|-----------|-----------|-----------|----------|------------|----|
| | 0.0313 | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | | % |
| Triclosan (TCS) | 112 | 24 | 46 | 77 | 39 | 2 | | 55 |

The strains with MIC higher than that of control strain *E. coli* ATCC 10536 and *Salmonella* H9812 were considered resistant. The numbers of resistant strains are in bold.

TET-AMC-AMP-SXT-C-NA-TMP-CN-S3-S by 23 isolates, and TET-AMP-S3-S by 16 isolates (Supplementary Table 3).

A total of 238 isolates were multidrug-resistant (MDR) with resistance to at least three classes of antibiotics. The prevalence of MDR was highest among pig manure isolates (100%, $n = 23$) followed by pig meat isolates (92.3%, $n = 168$), human stool isolates (68.4%, $n = 26$), poultry manure isolates (40.7%, $n = 11$) and chicken meat isolates (36.6%, $n = 11$).

Disinfectant Resistance

The MICs of *E. coli* ATCC 10536 and *Salmonella* H9812 for benzalkonium chloride (BC), trichloroisocyanuric acid (TCCA), and triclosan (TCS) were 8, 256, and 0.0625, mg L⁻¹, respectively. A total of 238, 218, and 164 of the *Salmonella* Typhimurium isolates had higher MICs for BC, TCCA, and TCS, respectively, than *E. coli* ATCC 10536 and *Salmonella* H9812 (Table 4) and were considered resistant. The prevalence of BC and TCS resistance was lowest among the human stool isolates ($P < 0.05$) (Figure 5). The prevalence of BC resistance was higher among the pig manure isolates than among the chicken meat isolates ($P < 0.05$), whereas the prevalence of TCS resistance was lower among the pig manure isolates than among the poultry manure isolates ($P < 0.05$).

Association of Metal Resistance and Heavy Metal Resistance Genes With Antibiotic and Disinfectant Resistance

All the six metal resistances were associated with resistance to at least one antibiotic (Table 5 and Supplementary Table 5A). Cr-resistance was associated with resistance to eight antibiotics and to two disinfectants, Cu-resistance was associated with resistance to eight antibiotics, and Zn-resistance was associated with resistance to six antibiotics and to two disinfectants.

All the 12 HMRGs were associated with resistance to at least two antibiotics (Table 6 and Supplementary Table 5B). The Hg-resistance gene *merA* was associated with resistance to 12 antibiotics and to three disinfectants, the Cu-resistance gene *pcoA* with resistance to ten antibiotics and to two disinfectants, the Pb-resistance gene *pbrA* with resistance to seven antibiotics and to two disinfectants, and the Ni-Cr-Cd-resistance gene *nccA* and the Cu-resistance gene *pcoC* with resistance to seven antibiotics and to one disinfectant ($P < 0.05$).

Transfer of Heavy Metal Resistance Genes

A conjugation experiment was carried out to determine the transferability of HMRGs. The genes *cnrA*, *pcoC*, and *czcD* were successfully transferred from *Salmonella* Typhimurium isolates S15 and S24 to *Escherichia coli* J53 (Figure 6). The transfer rate was 1×10^{-3} per donor. The MIC of Cu was 100 $\mu\text{g L}^{-1}$ for the *Escherichia coli* J53, 800 $\mu\text{g L}^{-1}$ for the isolates S15 and S24, 200 $\mu\text{g L}^{-1}$ for the J53 with genes transferred from S15 and 300 $\mu\text{g L}^{-1}$ for J53 with genes transferred from S24 (Table 7).

DISCUSSION

The increasing resistance of *Salmonella* strains to antimicrobial agents has become a major public health concern worldwide. However, little information is available on the resistance and co-resistance to heavy metals, disinfectants, and antibiotics among *Salmonella* Typhimurium from retail meat and manure. Thus, we studied the prevalence and associations of resistances of *Salmonella* Typhimurium isolate from pig meat,

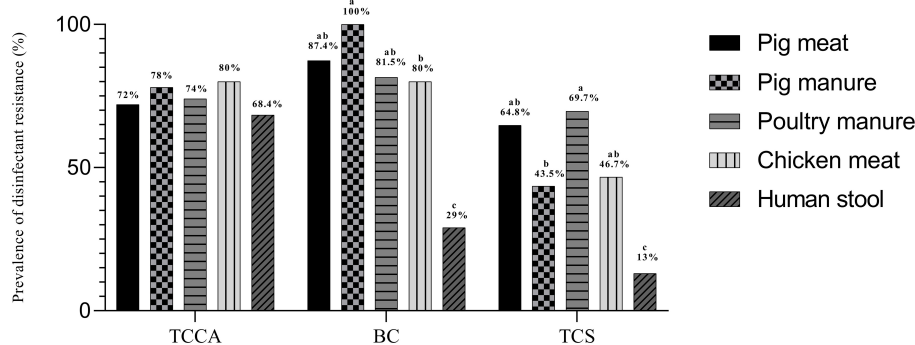


FIGURE 5 | Prevalence of disinfectant resistance among *Salmonella* Typhimurium isolates from pig meat, pig manure, poultry manure, chicken meat, and human stool. Different lowercase letters indicate statistically significant differences between groups ($P < 0.05$).

TABLE 5 | The association of metal resistances with antibiotic and disinfectant resistance in *Salmonella* Typhimurium.

| Metal | Antibiotic/Disinfectant | P-value |
|-------|-------------------------|-----------|
| | Antibiotic | |
| Cd | CIP | < 0.00001 |
| | OFX | < 0.00001 |
| Co | CAZ | 0.0003 |
| | AMP | 0.0229 |
| Cr | AMP | 0.0019 |
| | SXT | < 0.00001 |
| | CIP | < 0.05 |
| | C | 0.0329 |
| | OFX | 0.0002 |
| | TMP | < 0.05 |
| | CN | 0.0028 |
| | S3 | 0.0167 |
| | TET | < 0.00001 |
| Cu | AMP | 0.0001 |
| | SXT | < 0.00001 |
| | C | 0.0007 |
| | TMP | < 0.00001 |
| | CN | 0.0002 |
| | S3 | 0.0022 |
| | S | < 0.00001 |
| | AMP | 0.044 |
| | SXT | 0.004 |
| Mn | TMP | 0.0279 |
| | CN | 0.0013 |
| | SXT | 0.0036 |
| | CIP | 0.0019 |
| Zn | C | 0.0249 |
| | OFX | 0.0007 |
| | TMP | 0.0037 |
| | CN | 0.0062 |
| | BC | 0.0397 |
| | TCCA | < 0.00001 |
| Mn | BC | 0.0012 |
| | TCCA | < 0.00001 |
| Zn | BC | < 0.00001 |
| | TCCA | < 0.05 |

pig manure, chicken meat, poultry manure, and human stool from Sichuan, China.

In our study, 74% of the 300 *Salmonella* Typhimurium isolates were considered resistant to Cu, almost 50% to Zn and Cr, over 25% to Mn and Cd, and almost 10% to Co. Approximately similar prevalence of Cu resistant isolates has been detected among *Salmonella enterica* isolates from meat and meat-based products (Figueiredo et al., 2019). The high prevalences of Cu and Zn resistant isolates may have been due to selection by heavy metal micronutrients in the feed; the use of heavy metal micronutrients resulted in high concentrations of Cu and Zn in pig feces in the U.S. (Medardus et al., 2014). Alarming, the resistant strains in the feces may contaminate the meat, as suggested by the high Cu resistance prevalence among the pig and chicken meat isolates. The high prevalence of resistant manure isolates suggested that the resistance may spread further by the use of manure as a soil amendment in agriculture. Further studies are needed to

TABLE 6 | The association of HMRGs with antibiotic and disinfectant resistance in *Salmonella* Typhimurium.

| HMRG | Antibiotic/Disinfectant | P-value |
|------|-------------------------|-----------|
| | Antibiotic | |
| cadD | AMP | 0.0064 |
| | SXT | < 0.00001 |
| | S3 | 0.0106 |
| chrB | TMP | 0.0002 |
| | AMP | 0.0293 |
| | TMP | 0.0135 |
| cnrA | SXT | 0.0003 |
| | CIP | 0.0075 |
| | OFX | 0.0101 |
| merA | TMP | 0.0001 |
| | TET | < 0.00001 |
| | AMC | 0.0044 |
| nccA | AMP | < 0.00001 |
| | SXT | 0.0033 |
| | CIP | 0.0003 |
| | C | 0.0063 |
| | OFX | 0.0001 |
| | NA | 0.0079 |
| | TMP | 0.0286 |
| | CN | < 0.00001 |
| | S3 | < 0.00001 |
| | S | 0.011 |
| | TET | 0.0313 |
| | AMP | 0.0144 |
| pbrA | SXT | 0.0072 |
| | OFX | 0.0356 |
| | NA | 0.0119 |
| | TMP | 0.0106 |
| | CN | 0.001 |
| | SXT | 0.0009 |
| pcoA | CIP | 0.0005 |
| | C | 0.044 |
| | OFX | 0.0001 |
| | NA | 0.0012 |
| | TMP | 0.0069 |
| | CN | 0.0005 |
| | TET | 0.0002 |
| | AMC | 0.0083 |
| | AMP | 0.000114 |
| | SXT | < 0.00001 |
| | CIP | 0.0375 |
| | C | 0.0081 |
| NA | 0.0369 | |
| TMP | 0.0003 | |
| CN | 0.0002 | |
| S3 | 0.0002 | |
| pcoC | TET | 0.000012 |
| | AMP | < 0.00001 |
| | SXT | < 0.00001 |
| pcoR | C | < 0.00001 |
| | TMP | < 0.00001 |
| | CN | 0.000025 |
| | S3 | 0.000024 |
| | SXT | 0.0098 |
| | CIP | 0.0002 |
| | OFX | 0.0027 |
| | TMP | 0.0104 |

(Continued)

TABLE 6 | Continued

| HMRG | Antibiotic/Disinfectant | | P-value |
|------|-------------------------|--|----------|
| | Disinfectant | | |
| cnrA | BC | | 0.0066 |
| merA | BC | | 0.0349 |
| | TCS | | 0.000027 |
| nccA | TCCA | | 0.0007 |
| | BC | | 0.0016 |
| pbrA | BC | | 0.0013 |
| | TCCA | | <0.00001 |
| pcoA | BC | | 0.0072 |
| | TCCA | | 0.000045 |
| pcoC | BC | | 0.0004 |
| pcoR | BC | | 0.0005 |
| | TCCA | | 0.0214 |

TABLE 7 | Transconjugation of metal resistance genes from *Salmonella* Typhimurium to *Escherichia coli* J53.

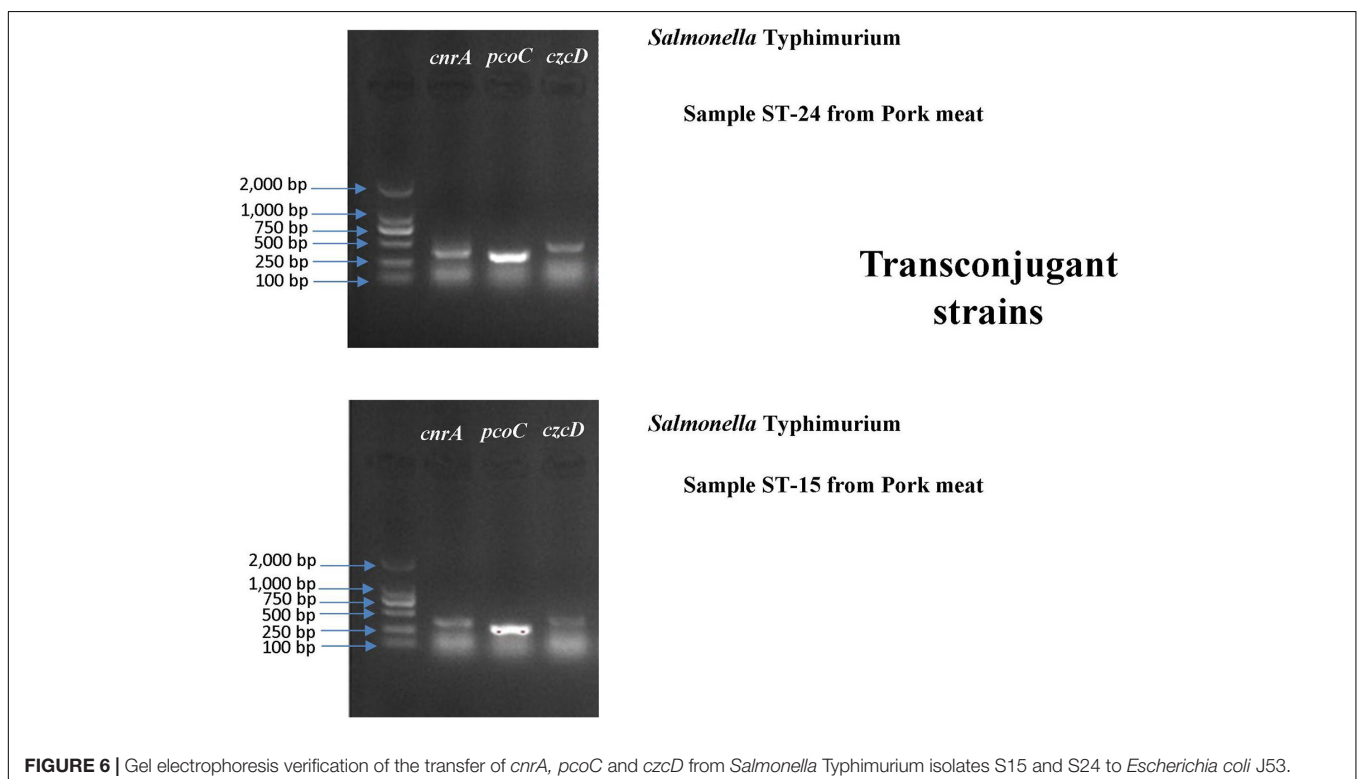
| | Strain number | Genes | MIC of Cu ($\mu\text{g L}^{-1}$) | | | | |
|----------------|--------------------------------------|-------------------------|------------------------------------|-----|-----|-----|-----|
| | | | 100 | 200 | 300 | 400 | 800 |
| Recipient | <i>Escherichia coli</i> J53 | | 100 | | | | |
| Donor | <i>Salmonella</i> Typhimurium | | | | | | |
| | ST-24 | <i>cnrA, pcoC, czcD</i> | | | | | 800 |
| | ST-15 | <i>CnrA, pcoC, czcD</i> | | | | | 800 |
| Transconjugant | <i>Escherichia coli</i> J53-ST-24 | <i>cnrA, pcoC, czcD</i> | | | | | 300 |
| | J53-ST-15 | <i>cnrA, pcoC, czcD</i> | | | | | 200 |

ascertain whether the high prevalence of Cu resistance among human stool isolates had resulted from meat products.

In line with the prevalence of metal resistances, most of the isolates carried at least one heavy metal resistance gene (HMRG), and the prevalences of the Cu-resistance genes *pcoR* and *pcoC* and Cu resistance were at the same level. HMRGs are found in a wide variety of bacteria from various environments (Li L.-G. et al., 2017; Pal et al., 2017). The *czcD* gene is involved in the regulation of an efflux system that mediates the resistance to metal ions (Anton et al., 1999). Almost 70% of the *Salmonella* Typhimurium isolates from pig feed and feces carried *czcD* (Medardus et al., 2014). In our study, the prevalence of the Cr-Zn-Cd-resistance gene *czcD* was 85%, higher than that of resistances

to those metals. Possibly the gene was not effectively expressed under the test conditions. However, ascertaining this necessitates further analyses.

The metal resistant isolates are often also antibiotic and disinfectant resistant; the resistance genes may be co-located, e.g., on a plasmid, or the resistance mechanism, e.g., an efflux pump, may provide resistance against both metals, and antibiotics (Deng et al., 2017; Pal et al., 2017). In our study, most of the *Salmonella* Typhimurium isolates were resistant to at least one antibiotic and almost 80% were multidrug-resistant. Similarly, among *Salmonella* isolates from retail food of animal origin in Romania and China, over 90% were resistant to at least one antibiotic, and from 43 to over 80% were multidrug-resistant (Mihaiu et al., 2014; Deng et al., 2017). The prevalence of resistance to



streptomycin, sulfonamides, tetracycline, and ampicillin were all over 70% among our isolates. In agreement with Deng et al. (2017), pigs were a major source of antibiotic-resistant isolates. The prevalence of resistance to six antibiotics was higher among the pig meat and manure isolates than among other isolates, and that of streptomycin and ampicillin were highest among the pig meat isolates and that of ciprofloxacin and ofloxacin among the pig manure isolates. In our study, from 55 to 79% of the isolates were considered resistant to disinfectants triclosan (TCS), trichloroisocyanuric acid (TCCA), or benzalkonium chloride (BC). The MICs for BC resistance were lower than those of the meat *Salmonella* isolates, among which almost 60% had a MIC of 128 mg L⁻¹ for BC (Deng et al., 2017). For three of the disinfectants, the prevalence of resistance was lowest among the human stool isolates, suggesting that the resistance had not been passed on in the food chain.

Due to the genes and mechanisms shared between metal, antibiotic, and disinfectant resistance, the development of resistance against metals may be associated with the development of antibiotic and disinfectant resistance (Deng et al., 2017; Pal et al., 2017). Similar to previous studies (Deng et al., 2017; Di Cesare et al., 2016; Yang et al., 2018), in our study, the metal resistances and HMRGs were associated with resistance to antibiotics and disinfectants. Especially, Cu-resistance genes were associated with resistance to several antibiotics and disinfectants. Thus, the excessive use of metals and disinfectants as feed additives and in animal care may have the potential to promote antibiotic resistance through co-selection. Alarming, this co-selection can maintain and promote antibiotic resistance even in the absence of antibiotics, and e.g., Cu may co-select for resistance to last-resort antibiotics such as colistin (Pal et al., 2017).

Knowing the genetic linkage of resistance genes and their association with MGEs is critical to fully understand the risks of horizontal transfer of resistance genes between bacteria (Martínez et al., 2015; Pal et al., 2015). The operons encoding resistance against different metals have been confirmed to be located on the same plasmid (Fang et al., 2016). In our study, the transfer of the Cr-Zn-Cd-resistance gene *czcD*, Cu-resistance gene *pcoC*, and Co-Ni-resistance gene *cnrA* into *Escherichia coli* and the increased Cu-resistance of the transconjugants implied that the resistance genes were located on conjugative plasmids and may be expressed in a receiving strain. Similarly, Hasman and Aarestrup (2002) and Amachawadi et al. (2013) reported that the copper resistance gene *tcrB* is horizontally transferable and linked to macrolide and glycopeptide resistance. Thus, the metal resistance genes may spread between bacteria and, due to the connection between metal and antibiotic resistance, further increase the prevalence of antibiotic resistance in the environment.

The study had the few limitations. Firstly, *Salmonella* Typhimurium, as a control strain, was not available and therefore we used standard control strains like *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 10536 and *Salmonella* H9812 as some previous studies who did the same in a scenario where the control strain was not available due to the reason such as faced by us.

Secondly, understanding differences in metal tolerances among pig feces isolates is certainly worthwhile and could have been done. However, group-wise comparison has previously been performed by a few researchers before with some limitations. In the current study, we made group-wise comparison and tried answering some of the limitations that were found in those previous studies. Since our group is constantly working on this particular aspect, future study will certainly be conducted in the subject manner as proposed.

CONCLUSION

We found a co-occurrence of heavy metal, antibiotic and disinfectant resistance in *Salmonella* Typhimurium isolates originating from retail foods, animal manure, and human stool. Such an increased prevalence of metal resistance and its corresponding genes among *Salmonella* Typhimurium isolates has not been reported previously and therefore provides a baseline study to further investigate the subject matter. Further, a prevalence of resistance and its genes indicates that meat and manure could be potential sources of human exposure to multiple strains of resistant *Salmonella* and other food-borne diseases. An excessive as well as an irrational use of metals and disinfectants either as feed additives or in an animal care setting may promote antibiotic resistance through co-selection and the transfer of the resistance genes through MGEs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

GM and KZ wrote the manuscript under the supervision of LZ. GM, KZ, XH, SC, SL, AM, LH, YY, XY, XA, AL, and XX performed sample collection and research work. GM and KZ performed data analysis. GM, KZ, LZ, MS, PP, and AM revised the manuscript. LZ was correspond to the author furthest up on the author list. All authors approved the manuscript's final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.702725/full#supplementary-material>

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