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ORIGINAL ARTICLE

Antimicrobial Resistance in Non-typhoidal *Salmonella* from Retail Foods Collected in 2020 in China

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Abstract

Objective: Non-typhoidal *Salmonella* (NTS) is a major cause of human salmonellosis globally. Food animals are major NTS reservoirs. An increase in antimicrobial resistance (AMR) in foodborne NTS has led to clinical treatment failures. Here, to examine the prevalence and perform characterization of foodborne NTS with AMR in China, we tested the antimicrobial susceptibility of 1,256 NTS isolates cultured from retail foods in 2020 in China.

Methods: The antimicrobial susceptibility of 26 antimicrobial agents representing 12 classes was evaluated with the broth-microdilution method; the presence of ten *mcr* genes was screened with multi-PCR. The complete closed genomes of *mcr*-gene-carrying isolates were generated by hybrid assembly through whole genome sequencing on both the PacBio and Illumina platforms. Genomic features and genetic environments of the *mcr-1* gene were analysed.

Results: The overall drug resistance rate was 92.28%, and the multi-drug resistance (MDR) rate was 76.53%. A total of 341 AMR profiles were determined, and resistance was highest to nalidixic acid (63.38%). Among 887 NTS isolates with MDR, 232 showed co-resistance to cefotaxime and ciprofloxacin, and 25 were resistant to ten classes of antimicrobial agents. The resistance of NTS isolated from different regions varied. Isolates from raw chicken sources most frequently showed resistance. Four NTS carried the *mcr-1* gene and represented four different serotypes. Four *mcr-1* gene-bearing plasmids from the four *Salmonella* isolates were classified into two replicon types (IncI2 and IncHI2A). Two *mcr-1* genes in IncI2 type plasmids were found to be located between a PAP2 family protein-encoding gene and a relaxase-encoding gene, whereas the other two *mcr-1* gene structures in IncHI2A type plasmids showed variations in the presence of insertion sequences.

Conclusion: Our data demonstrated severe AMR among foodborne NTS isolated from food in China, thus highlighting the importance of antimicrobial susceptibility surveillance to decrease the spread of AMR, particularly to critical drugs in human medicine.

Key words: Non-typhoidal *Salmonella* (NTS), antimicrobial resistance (AMR), multi-drug resistance (MDR), *mcr-1*

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INTRODUCTION

Foodborne diseases remain a global public health challenge posing a major burden. In 2010, 31 hazards in unsafe food caused 600 million cases of foodborne illnesses and 420,000 deaths worldwide; 40% of these deaths occurred among children younger than 5 years of age [1]. The most frequent causes of these foodborne illnesses were diarrhoeal agents, which were responsible for 230,000 deaths. Non-typhoidal *Salmonella* (NTS), a major cause of foodborne infections, gives rise to more than 93 million cases of gastroenteritis annually and 155,000 deaths globally, thus resulting in approximately 4 million disability-adjusted life years [2]. In the United States, 1.35 million illnesses, 26,500 hospitalizations and 420 deaths have been estimated to be attributable to NTS, thus leading to more than $400 million in medical costs each year [3]. In 2019, 27 European Union member states reported 5,175 foodborne outbreaks, among which NTS was the most commonly identified agent and accounted for 17.9% of the total outbreaks [4]. From 2002 to 2017, China reported 2,815 foodborne disease outbreaks associated with meat and meat products, thus resulting in 52,122 illnesses, 25,361 hospitalizations and 96 deaths, among which NTS was the most common cause of outbreaks (420/2815, 14.92%) and hospitalizations (7641/25,361, 30.13%). Hence, NTS is the most frequently reported bacterial species causing human gastrointestinal infections globally. Food animals, mainly poultry, serve as a major reservoir of NTS, and contaminated animal-based products are frequently associated with human salmonellosis.

The emergence and spread of NTS with antimicrobial resistance (AMR) have become major public health concerns over the past two decades. The presence of extended spectrum beta-lactamase genes in NTS plasmids and reports of carbapenemase-containing NTS isolates are particularly concerning [5,6], because both confer resistance to highly important antimicrobial agents. The acquisition of genes conferring AMR to both antimicrobial agents on foodborne NTS along the food chain is increasing. Treatment options for salmonellosis in animals and humans have been hindered by AMR in NTS. Data from China have indicated that the prevalence of NTS with multi-drug resistance (MDR) increased from 20–30% in the 1990s to 70% in the early 2000s; moreover, the overall incidence of foodborne NTS with AMR exceeded 70% between 2015 and 2016, and was notably observed in strains carrying plasmids with the *mcr-1* gene, which mediates resistance to colistin [7]. Food workers who are infected with NTS with AMR after consuming or handling contaminated food may serve as reservoirs, thus posing a high risk of further food contamination. To decrease the prevalence of NTS in foods and consequently the burden of human salmonellosis, China implemented a nationwide foodborne pathogen monitoring and control program. In this study, the antimicrobial susceptibility of 1256 NTS isolates cultured from retail foods in 2020 in China was tested. All isolates were subsequently screened for the presence of *mcr* genes through polymerase chain reaction (PCR), which was followed by whole genome sequencing of *mcr* gene-positive strains to provide further confirmation. Our aim was to gain genomic insight into antimicrobial mechanisms.

MATERIALS AND METHODS

Bacterial strains

A total of 1256 foodborne NTS isolates were cultured from various retail foods, primarily meat and meat-based products, collected from 30 provinces (municipalities or autonomous regions) in China in 2020. The presumptive colonies were confirmed to be *Salmonella* according to both their morphology and *invA* gene amplification by PCR, as described previously; those with negative amplification were further validated with GN card and Vitek2 compact (BioMérieux, France) analysis [8]. All isolates confirmed to be *Salmonella* were preserved in brain heart infusion broth with 40% (v/v) glycerol (HopeBio, Qingdao, China) at -80°C before analysis. *Escherichia coli* ATCC®25922 was used as the control in antimicrobial susceptibility testing (AST).

Antimicrobial susceptibility testing

All *Salmonella* isolates were subjected to AST with Biofosun® Gram-negative panels (Fosun Diagnostics, Shanghai, China) through the broth microdilution method. The following panel of 26 antimicrobial compounds representing 12 classes was selected: ampicillin (AMP), ampicillin/sulbactam (SAM), cefepime (FEP), ceftazidime (CAZ), ceftriaxone (CRO), cefoxitin (FOX), cefotaxime (CTX), aztreonam (ATM), ertapenem (ETP), imipenem (IMP), meropenem (MEM), colistin (CT), polymyxin B (PB), gentamicin (GEN), amikacin (AK), tetracycline (TET), doxycycline (DC), tigecycine (TGC), ciprofloxacin (CIP), nalidixic acid (NAL), sulfamethoxazole-trimethoprim(SXT), sulfonamides (SMX), trimethoprim (TMP), chloramphenicol (CHL), florfenicol (FFC) and nitrofurantoin (NIT). The data were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute guidelines (CLSI, M100-S32, version 2022) [9]. Additionally, CLSI (M31-A3, version) and European Committee on Antimicrobial Susceptibility Testing documents were consulted for FFC and TGC, respectively [10,11].

mcr gene screening

All 1256 foodborne NTS isolates were screened for the presence of *mcr* genes (*mcr-1* to *mcr-10*) with multi-target PCR methods, as previously reported [12]. Isolates carrying any *mcr* genes were selected for further whole genome sequencing.

Whole genome sequencing

DNA extraction and whole genome sequencing were conducted for *mcr*-gene-carrying isolates to obtain complete genomes. Briefly, single colonies of NTS isolates were cultured in brain heart infusion broth and incubated

at 37°C overnight. A TIANamp bacterial DNA extraction kit (DP302, TIANGEN BIOTECH, Beijing, China) was used to extract the bacterial genomic DNA according to the manufacturer's instructions, and library preparation was then performed with an NEBNext® Ultra DNA Library Prep Kit for Illumina (NEB#E7370) and sonication fragmentation (350 bp insert). Sequencing was performed commercially on the Illumina HiSeq platform with a PE 150 sequencing strategy (Novogene, Beijing, China) and a HiSeq X Ten Reagent Kit v2.5 (Illumina, San Diego, CA). The *mcr* gene carrying isolates were also sequenced on the SMRT® Pacific Biosciences (PacBio) Sequel platform (Tianjin Biochip Corporation, Tianjin, China), with a 10-kbp template library preparation step with a PacBio® Template Prep Kit. SMRT Analysis v2.3.0 was used for de novo assembly according to the RS Hierarchical Genome Assembly Process (HGAP) workflow v3.0. Subsequently, Consed version 28.0 was used to manually inspect and trim duplicate ends to generate single, complete and closed sequences for each chromosome and plasmid. For data error correction, Pilon v1.23 was used with Illumina MiSeq sequencing read data. The closed genomes were then annotated with prokka (version 1.14.6).

Bioinformatic analysis

The predicted serotypes and multi-locus sequence typing (MLST) types were identified with the *Salmonella* In Silico Typing Resource (SISTR). Plasmid replicon types (Incompatibility groups or Inc groups) were identified through the Center for Genomic Epidemiology (CGE) website with PlasmidFinder (v2.0). All gene, plasmid and chromosome sequences used in this study were managed, aligned and analysed in Geneious prime (v2023.1.2) software. The genetic environments of the *mcr-1* gene were analysed and displayed with Easyfig (v2.2.2).

RESULTS

Antimicrobial resistance of 1256 NTS isolates

The key AMR trends in 1256 NTS isolates recovered from various foods is shown in Table 1. A total of 1159 (1159/1256, 92.28%) isolates exhibited resistance to at least one antimicrobial compound, whereas 97 (97/1256, 7.72%) isolates showed no resistance to any antimicrobial compounds tested. The studied strains most frequently showed resistance to nalidixic acid (796/1256, 63.38%), sulfonamides (782/1256, 62.26%), tetracycline (714/1256, 56.85%), doxycycline (710/1256, 56.53%), ampicillin (705/1256, 56.13%), ampicillin/sulbactam (530/1256, 42.20%), florfenicol (463/1256, 36.86%), chloramphenicol (455/1256, 36.23%) and trimethoprim (427/1256, 34.00%); resistance to the other tested drugs was observed at a prevalence below 30% (Table 1). Relatively lower resistance to amikacin (93/1256, 7.40%) and cefoxitin (33/1256, 2.63%) was observed. Notably, among the foodborne NTS, we observed a high prevalence of resistance to drugs categorized as critically important antimicrobial agents for human medicine by the World Health Organization [13], including cephalosporin, quinolones, aminoglycosides, lipopeptides, monobactams and penicillins (Table 1). In particular, we observed high resistance to the first line antimicrobial compounds for salmonellosis treatment: cephalosporins—including the 3rd generation agents cefotaxime (322/1256, 25.64%), ceftriaxone (316/1256, 25.16%) and ceftazidime (223/1256, 17.75%), and the 4th generation agent cefepime (282/1256, 22.45%)—and quinolones, such as ciprofloxacin (333/1256, 26.51%). Our findings indicated that the resistance of NTS to both cephalosporin and quinolones was markedly higher than previously reported [7]. In addition, we observed a high percentage of intermediate resistance to polymyxin B (944/1256, 75.16%), colistin (933/1256, 74.28%) and ciprofloxacin (655/1256, 52.15%), and a slightly lower incidence of intermediate resistance to nitrofurantoin (353/1256, 28.11%), chloramphenicol (240/1256, 19.11%), ampicillin/sulbactam (150/1256, 11.94%) and cefoxitin (117/1256, 9.32%), thus suggesting that resistance to the above antimicrobial agents is likely to increase in the near future. No isolate was resistant to tigecycine or any carbapenem compounds tested (ertapenem, imipenem and meropenem).

Co-resistance and AMR profiles

Among 1159 resistant NTS isolates, MDR (resistance to three or more antimicrobial classes) was present in approximately 76.53% (887/1159) of isolates on average. Among these, 146 (146/887, 16.46%), 138 (138/887, 15.56%), 140 (140/887, 15.78%), 127 (127/887, 14.32%), 88 (88/887, 9.92%), 95 (95/887, 10.71%), 128 (128/887, 14.43%) and 25 (25/887, 2.82%) isolates were resistant to 3, 4, 5, 6, 7, 8, 9 or 10 classes of antimicrobial agents tested, respectively. Notably, 248 isolates (27.96%, 248/887) were resistant to eight or more classes of antimicrobial agents, and 232 (26.16%, 232/887) MDR isolates were co-resistant to cefotaxime and ciprofloxacin, the first-line antimicrobial agents used in the clinical treatment of human salmonellosis. In total, 341 AMR profiles were recorded. The top five AMR profiles were AMP-SAM-FEP-CAZ-CRO-CTX-ATM-GEN-AK-TET-DC-CIP-NAL-SXT-SMX-TMP-CHL-FFC (4.23%, 49/1159), TET-DC (3.71%, 43/1159), AMP-SAM-CT-PB-NAL-SMX-NIT (3.62%, 42/1159), CT-PB-NAL (3.62%, 42/1159) and SMX (3.62%, 42/1159) (S1 Table).

Geographical distribution of NTS with AMR isolates

A total of 30 provinces were selected as sampling sites. Among sampling locations, the frequency of AMR ranged from 78.57% to 100%, and the average was 92.28%. The geographical distribution of AMR frequency (Fig 1) indicated that more than half of the NTS isolates had MDR, and the range was 56.45% to 100% (average: 76.53%, 887/1159). The frequencies of NTS isolates with MDR exceeded 80% (range: 80.95%–92.42%) in 11 provinces (Hebei, Anhui, Ningxia, Yunnan, Liaoning, Henan,

TABLE 1 | Antimicrobial susceptibility of 1256 *Salmonella* isolates to 26 antimicrobial agents representing 12 classes.

Antimicrobial class	Antimicrobial agent	AST results, number of strains (%)			WHO Category*
		Resistant	Intermediate	Susceptible	
Penicillins	AMP	705 (56.13)	1 (0.08)	550 (43.79)	CIA
β-Lactam combination agents	SAM	530 (42.20)	150 (11.94)	576 (45.86)	HIA
Cephems	FEP	282 (22.45)	28 (2.23)	946 (75.32)	CIA
	CAZ	223 (17.75)	14 (1.11)	1019 (81.13)	CIA
	CRO	316 (25.16)	1 (0.08)	939 (74.76)	CIA
	FOX	33 (2.63)	117 (9.32)	1106 (88.06)	HIA
	CTX	322 (25.64)	2 (0.16)	932 (74.20)	CIA
Monobactams	ATM	302 (24.04)	19 (1.51)	935 (74.44)	CIA
Carbapenems	ETP	0 (0.00)	0 (0.00)	1256 (100.00)	CIA
	IMP	0 (0.00)	1 (0.08)	1255 (99.92)	CIA
	MEM	0 (0.00)	0 (0.00)	1256 (100.00)	CIA
Lipopeptides	CT	323 (25.72)	933 (74.28)	-	CIA
	PB	312 (24.84)	944 (75.16)	-	CIA
Aminoglycosides	GEN	309 (24.60)	7 (0.56)	940 (74.84)	CIA
	AK	93 (7.40)	4 (0.32)	1159 (92.28)	CIA
Tetracyclines	TET	714 (56.85)	7 (0.56)	535 (42.60)	HIA
	DC	710 (56.53)	22 (1.75)	524 (41.72)	HIA
	TGC	0 (0.00)	1 (0.08)	1255 (99.92)	HIA
Quinolones and fluoroquinolones	CIP	333 (26.51)	655 (52.15)	268 (21.34)	CIA
	NAL	796 (63.38)	-	460 (36.62)	CIA
Folate pathway antagonists	SXT	351 (27.95)	-	905 (72.05)	HIA
	SMX	782 (62.26)	-	474 (37.74)	HIA
	TMP	427 (34.00)	-	829 (66.00)	HIA
Phenicols	CHL	455 (36.23)	240 (19.11)	561 (44.67)	HIA
	FFC	463 (36.86)	106 (8.44)	687 (54.70)	HIA
Nitrofurans	NIT	229 (18.23)	353 (28.11)	674 (53.66)	IA

*CIA: critically important antimicrobial agents; HIA: highly important antimicrobial agents; IA: important antimicrobial agents.

Jiangsu, Inner Mongolia, Shanxi, Jiangxi and Chongqing). Frequencies of 70.37%–78.48% were found in 11 other provinces (Shandong, Gansu, Beijing, Heilongjiang, Guizhou, Zhejiang, Sichuan, Shaanxi, Guangxi, Fujian and Tianjin), and frequencies of 56.45%–69.01% were found in Hunan, Guangdong, Shanghai and Hubei. Although the MDR rates of isolates from four regions (Qinghai, Hainan, Xinjiang and Jilin) were high, but it might due to the deviation caused by the low number of NTS isolates, rather than real high MDR level existing in isolates from these regions. In the 18 provinces with an isolate number above 35, the highest total resistance frequency was found in Gansu, at 100.00% (38/38), together with an MDR frequency of 76.32% (29/38); the next highest frequencies were found in Hebei (total: 98.51%, 66/67; MDR: 92.42%, 61/66) and Jiangsu (total: 96.77%, 60/62; MDR: 83.33%, 50/60).

Antimicrobial resistance of NTS from food samples

NTS isolates were recovered from six categories of foods in this study. Among the food categories, resistance to any of the 26 tested compounds was most frequently observed in raw chicken sources (approximately 93.85% resistant to one or more agent class, 565/602), followed by other raw poultry sources (92.04%, 104/113) and raw duck (88.19%, 254/288). NTS cultured from prepared meat exhibited the same trend of resistance to one class of drug as that observed in raw poultry meat (93.55%, 58/62 for red meat sources; 93.85%, 122/130 for poultry meat sources). MDR was most frequently found in isolates from prepared poultry meat (76.15%, 99/130) and raw chicken (74.42%, 448/602), followed by prepared red meat (72.58%, 45/62), other raw poultry

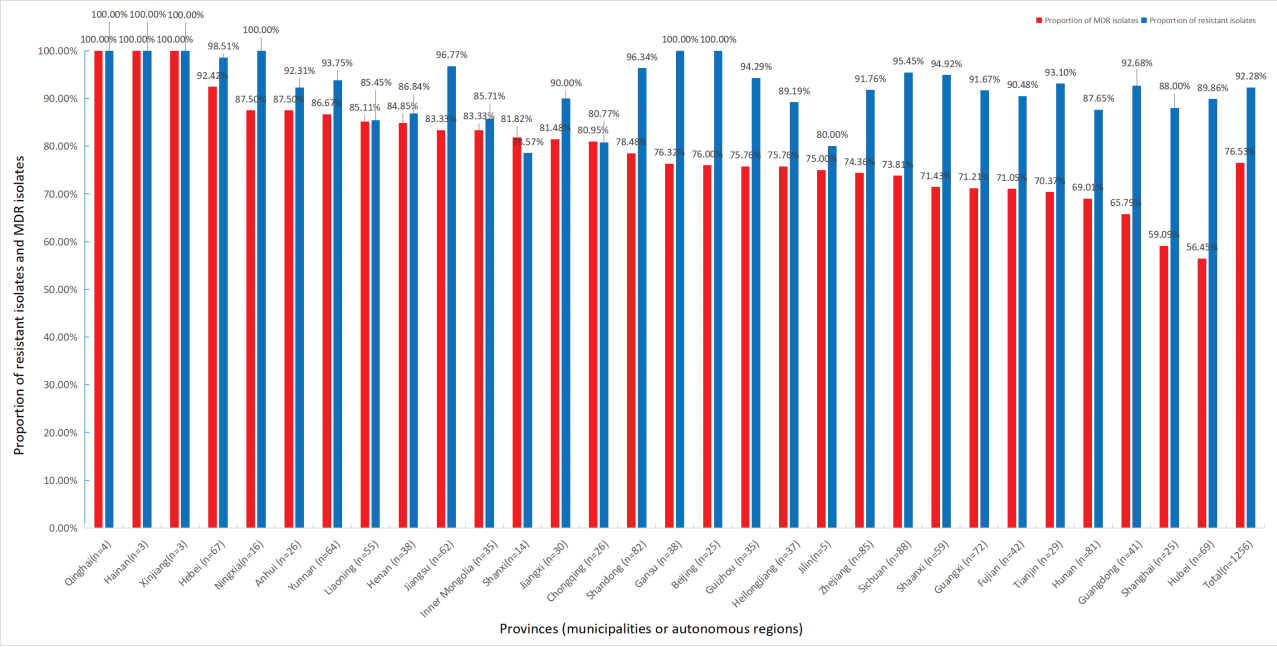

FIGURE 1 | Antimicrobial resistance rates and MDR rates of *Salmonella* recovered from different regions.

meat (66.37%, 75/113) and raw duck (61.46%, 177/288). Although high prevalence of MDR (78.38%, 29/37) in isolates from other foods (including sushi, cake and bread, milk, beverages and processed algae) was observed, a small number of NTS isolates might have contributed to this large variation. Among 25 isolates resistant to ten classes of antimicrobial agents, 24 were recovered from raw poultry samples, and only one was recovered from prepared beef (Table 2). These findings indicated that NTS from poultry sources tended to be more resistant than that from other sources.

mcr gene screening

PCR results together with whole-genome sequencing data indicated that 4 of 1256 (0.32%) NTS isolates carried the *mcr-1* gene. No other *mcr* genes (*mcr-2* to *mcr-10*) were detected. All four *mcr-1* positive NTS isolates were recovered from prepared meat samples. Sample information

TABLE 2 | Distribution of antimicrobial resistance among 1256 NTS isolates from various sample sources.

No. of antimicrobial classes with observed resistance	Raw poultry meat (n=890)			Prepared meat (n=192)		Others* (n=37)	Information unavailable (n=24)	Total (n=1256)
	Raw chicken (n=602)	Raw duck (n=288)	Others (n=113)	Red meat (n=62)	Poultry meat (n=130)			
0	37 (6.15)	34 (11.81)	9 (7.96)	4 (6.45)	8 (6.15)	2 (5.41)	3 (12.5)	97 (7.72)
1	56 (9.30)	45 (15.63)	16 (14.16)	6 (9.68)	7 (5.38)	5 (13.51)	2 (8.33)	137 (10.91)
2	61 (10.13)	32 (11.11)	13 (11.50)	7 (11.29)	16 (12.31)	1 (2.70)	5 (20.83)	135 (10.75)
3	72 (11.96)	41 (14.24)	13 (11.50)	1 (1.61)	13 (10.00)	3 (8.11)	3 (12.5)	146 (11.62)
4	64 (10.63)	29 (10.07)	11 (9.73)	13 (20.97)	17 (13.08)	2 (5.41)	2 (8.33)	138 (10.99)
5	72 (11.96)	19 (6.60)	10 (8.85)	11 (17.74)	19 (14.62)	7 (18.92)	2 (8.33)	140 (11.15)
6	58 (9.63)	25 (8.68)	11 (9.73)	3 (4.84)	22 (16.92)	5 (13.51)	3 (12.5)	127 (10.11)
7	38 (6.31)	16 (5.56)	9 (7.96)	8 (12.90)	12 (9.23)	5 (13.51)	0 (0)	88 (7.01)
8	50 (8.31)	23 (7.99)	4 (3.54)	3 (4.84)	11 (8.46)	3 (8.11)	1 (4.17)	95 (7.56)
9	76 (12.62)	21 (7.29)	14 (12.39)	5 (8.06)	5 (3.85)	4 (10.81)	3 (12.5)	128 (10.19)
10	18 (2.99)	3 (1.04)	3 (2.65)	1 (1.61)	0 (0)	0 (0)	0 (0)	25 (1.99)
Total	602 (100)	288 (100)	113 (100)	62 (100)	130 (100)	37 (100)	24 (100)	1256 (100)

*Others include sushi (n=6), cakes and breads (n=11), milk (n=2), beverages (n=4) and processed algae (n=14).

and resistance phenotypes against a panel of 26 antimicrobial compounds are shown in Table 3. Of note, three strains, 2020s302, 2020s327 and 2020s329, cultured from two prepared chicken samples and one prepared beef sample, respectively, were from the same region (Quzhou, Zhejiang province), whereas strain 2020s542 was isolated from prepared chicken meat in Suizhou, Hubei province. All four *mcr-1* positive isolates showed resistance to ampicillin, ceftriaxone, cefotaxime and colistin, and susceptibility to cefoxitin, ertapenem, imipenem, meropenem, amikacin, tigecycine and nitrofurantoin. The four *mcr-1* positive strains showed an MDR phenotype for at least three classes of antimicrobial compounds. The strains 2020s302, 2020s327, 2020s329 and 2020s542 were resistant to three, nine, ten and eight classes of antimicrobial agents, respectively, with AMR profiles

TABLE 3 | AST in four *mcr-1* positive *Salmonella* isolates.

Antimicrobial class	Antimicrobial agent	AST results (R/I/S)*			
		2020s302	2020s327	2020s329	2020s542
Penicillins	AMP	R	R	R	R
β-Lactam combination agents	SAM	I	R	R	R
Cephems	FEP	R	R	R	I
	CAZ	S	S	R	S
	CRO	R	R	R	R
	FOX	S	S	S	S
	CTX	R	R	R	R
Monobactams	ATM	S	I	R	R
Carbapenems	ETP	S	S	S	S
	IMP	S	S	S	S
	MEM	S	S	S	S
Lipopeptides	CT	R	R	R	R
	PB	R	R	I	R
Aminoglycosides	GEN	S	R	R	I
	AK	S	S	S	S
Tetracyclines	TET	S	R	R	R
	DC	S	R	R	R
	TGC	S	S	S	S
Quinolones and fluoroquinolones	CIP	S	R	R	I
	NAL	S	R	R	S
Folate pathway antagonists	SXT	S	R	S	R
	SMX	S	R	R	R
	TMP	S	S	S	R
Phenicols	CHL	S	R	R	R
	FFC	S	R	R	R
Nitrofurans	NIT	S	S	S	S
AMR profiles (number of antimicrobial class)		AMP-FEP-CRO-CTX-CT-PB (3)	AMP-SAM-FEP-CRO-CTX-CT-PB-GEN-TET-DC-CIP-NAL-SXT-SMX-CHL-FFC (9)	AMP-SAM-FEP-CAZ-CRO-CTX-ATM-CT-GEN-TET-DC-CIP-NAL-SMX-CHL-FFC (10)	AMP-SAM-CRO-CTX-ATM-CT-PB-TET-DC-SXT-SMX-TMP-CHL-FFC (8)

*Note: R, resistant; I, intermediate; S, susceptible.

of AMP-FEP-CRO-CTX-CT-PB, AMP-SAM-FEP-CRO-CTX-CT-PB-GEN-TET-DC-CIP-NAL-SXT-SMX-CHL-FFC, AMP-SAM-FEP-CAZ-CRO-CTX-ATM-CT-GEN-TET-DC-CIP-NAL-SMX-CHL-FFC and AMP-SAM-CRO-CTX-ATM-CT-PB-TET-DC-SXT-SMX-TMP-CHL-FFC, respectively.

Genomic features of *mcr-1*-bearing *Salmonella* isolates

Combined whole genome sequencing data from the Illumina and PacBio platforms showed that the serovars (MLST type) of these four *mcr-1* positive isolates were *S.* Bredeney (ST241) for 2020s302, *S.* Schwarzengrund (ST 241) for 2020s327, *S.* Kentucky (ST198) for 2020s329 and *S.* Newport (ST45) for 2020s542, on the basis of predictions by the SISTR platform. Each isolate consisted of a single circular chromosome (4.59–4.82 Mbp) and at least one plasmid (30–263 kbp). Notably, *S.* Newport 2020s542 contained four plasmids (one large plasmid of 258 kbp and three small plasmids of 30–85 kbp). Four *mcr-1* gene-bearing plasmids were classified into two replicon types (IncI2 and IncHI2A). The genomic information, including serotype, MLST type, genome size, GC content and incompatibility (Inc) group of each sequence of these four *Salmonella* isolates is listed in Table 4.

To better understand the genetic environment of the *mcr-1* loci of the plasmids bearing the *mcr-1* gene, we compared and analysed sequences extracted from various plasmids from this study and previous studies, belonging to two replicon types (Fig 2). The analysis revealed that the *mcr-1* genes in five IncI2 type plasmids (pCFSA244-2, pCFSA664-3, pHNSHP45, p2020s542-3 and p2020s302-1) were located between a PAP2 family protein-encoding gene (yellow arrow) and a relaxase-encoding gene (dark green arrow). In plasmid pHNSHP45 (KP347127), an IS*30* family element IS*Apl1* was followed by a relaxase-encoding gene downstream. In plasmids p2020s542-3 and p2020s302-1 in this study, together with pCFSA244-2 and pCFSA664-3, the *mcr-1* genes were found to have a PAP2 family protein-encoding gene distal to the right site of the *mcr-1* gene, without any insertion sequences (ISs).

In comparison with these IncI2 type plasmids, seven IncHI2A plasmids (two plasmids in this study and five other plasmids from previous studies) did not have a relaxase-encoding gene upstream of the *mcr-1* gene, but encoded some hypothetical proteins and contained some open reading frames (ORFs). Beyond the ORF differences, the main difference in the gene structures near *mcr-1* among the plasmids was the varying presence of insertion sequences. pCFSA1096 had no IS; pCFSA122-1, pCFSA629, pHNSHP45-2 (KU341381) and p2020S329-2 contained only one IS*Apl1*; and a tellurium resistance gene cluster was located downstream of the PAP2 encoding gene in pCFSA629, pHNSHP45-2 and p2020S329-2. Moreover, pWW012 (CP022169), the *mcr-1*-carrying plasmid from our previous study, and p2020s329-2, from the present study, contained an IS-*mcr-1*-PAP2-IS module, which is an IS*Apl1*-flanked composite transposon (Tn6330). Notably, the PAP2 encoding gene of p2020s329-2 had exactly the same sequence as the same gene in pWW012 but in an opposite orientation.

TABLE 4 | Chromosome and plasmid sequence information for four *Salmonella* isolates bearing the *mcr-1* gene.

Strain/plasmid	Description	Serotype	MLST type	Region	Food source	Size (bp)	G+C content	Plasmid replicon type (Inc group)[b]
2020s302	Chromosome	Bredeney	241	Zhejiang	Chicken	4,746,813	52.2%	N/A
p2020s302[a]	Plasmid					64,501	42.5%	IncI2
2020s327	Chromosome	Schwarzengrund	241	Zhejiang	Chicken	4,590,316	52.0%	N/A
p2020s327-1[a]	Plasmid					263,461	46.0%	IncHI2A
p2020s327-2	Plasmid					36,368	52.6%	IncR
2020s329	Chromosome	Kentucky	198	Zhejiang	Beef	4,821,018	52.2%	N/A
p2020s329-1	Plasmid					84,611	50.0%	IncI1-I (Alpha)
p2020s329-2[a]	Plasmid					209,722	46.5%	IncHI2A
2020s542	Chromosome	Newport	45	Hubei	Chicken	4,625,084	52.2%	N/A
p2020s542-1	Plasmid					258,726	46.9%	IncHI2A
p2020s542-2	Plasmid					85,305	50.1%	IncI1-I (Alpha)
p2020s542-3[a]	Plasmid					60,961	42.4%	IncI2
p2020s542-4	Plasmid					30,166	52.9%	N/A

[a]These plasmids contain the *mcr-1* gene.
[b]NA indicates that the plasmid replicon-typing was not applicable to the chromosome. p2020s542-4 was not predicted to have an Inc group by PlasmidFinder.

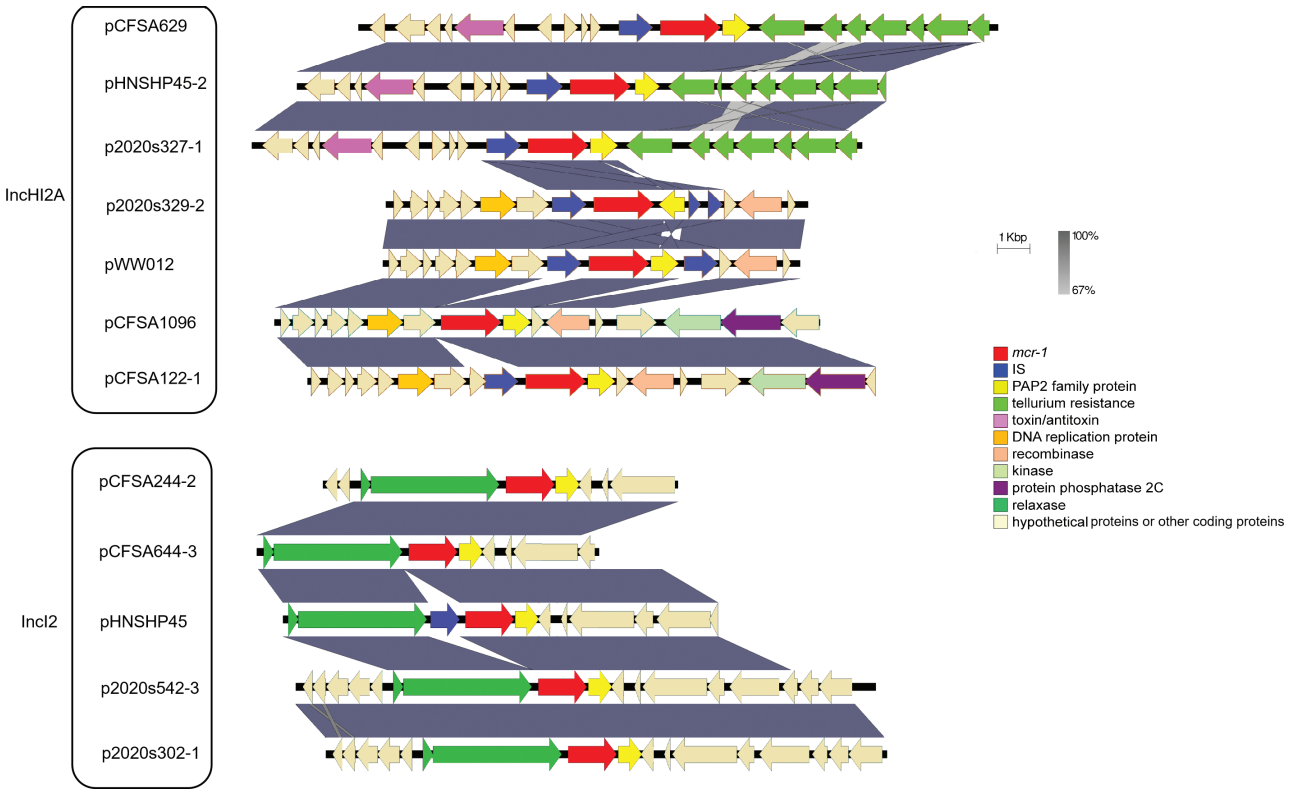

FIGURE 2 | Genetic environments associated with the *mcr-1* gene in different bacterial plasmids. The figure was generated in Easyfig (v2.2.5). Plasmids marked with "pCFSA" were carried by the *mcr-1* positive *Salmonella* isolates, according to our previous research [1,2], and the plasmid pWW012 belonged to a *Salmonella* isolate, according to previous research from our laboratory (accession number: CP022169) [3], whereas plasmids pHNSHP45 and pHNSHP45-2 (accession number: KP347127 and KU341381) belonged to *Escherichia coli* strain SHP45, the first reported isolate bearing the *mcr-1* gene [4]. Replicon types are shown in two groups for all plasmids. Confirmed and putative open reading frames (ORFs) are indicated by block arrows, their orientations are indicated by different colours, and arrow size is proportional to the predicted ORF length. The *mcr-1* gene is indicated by a red arrow, whereas genes encoding mobile elements (insertion sequence, IS) are indicated by blue arrows. Regions of homology among plasmids, ranging from 67% to 100% sequence identity are indicated by the graded shaded regions between sequences.

DISCUSSION

AMR poses an important, complex, and high-priority global public health challenge. China has one of the largest food animal production economies worldwide. To decrease the potential consequences of foodborne AMR risk to humans, animal and plant health, China has implemented a national AMR monitoring system. The status of resistance in *Salmonella* is assessed annually in many samples, primarily retail meat products. In this study, we characterized NTS isolates cultured in 2020, which were tested for resistance to a panel of 26 antimicrobial agents. The drug resistance rate of food-borne NTS in 2020 was 92.28%, and the MDR rate was 76.53%, in agreement with those reported in 2015 [7]. However, a higher resistance frequency was found for certain antimicrobial agents, such as cephems, quinolones, fluoroquinolones, lipopeptides, penicillins and aminoglycosides, which have a long history of use in food production chains in China. Quinolones are the preferred first-line drugs for clinical treatment or prevention/prophylaxis of *Salmonella* disease. The frequency of drug resistance to nalididic acid and ciprofloxacin was 63.38% and 26.51%, respectively—values slightly higher than those obtained in 2016 (52.5% and 21.3%) [14]. Therefore, much greater attention should be paid to the continuing increase in quinolone resistance, which could lead to a risk of clinical treatment failure.

AMR varied among regions and food categories. Foodborne NTS showed regional differences in drug resistance, ranging between 100% and 78.57% in this study. A total of 341 AMR profiles were found in the tested NTS isolates, thus indicating high polymorphism. Additionally, more than 90% of the *Salmonella* isolates were resistant to at least one antimicrobial agent, and resistance to commonly used compounds including ampicillin, ampicillin-sulbactam, nalidixic acid and tetracycline was observed among substantial numbers of study isolates. For example, the frequency of resistance to ciprofloxacin (26.51%) and extended-spectrum cephalosporins, including ceftazidime (17.75%) and cefotaxime (25.64%), was much higher than values reported for NTS cultured from raw chicken carcasses between 2011 and 2012 (16.47% for ciprofloxacin, 4.71% for ceftazidime and 11.18% for cefotaxime) [8]. Given NTS from poultry sources in this study tended to be more resistant than that from other sources

and also than that from ten years ago, *Salmonella* isolated from raw chicken samples collected after 2020 might have higher level of drug resistance. Hence, administration and management of the use of antimicrobial agents in the food production chain is essential. Carbapenems are not used in Chinese agriculture, nor are they approved for use in food-producing animals in any country. No carbapenemase-producing *Salmonella* was found in the present study, thus suggesting that carbapenems may still be effective when tested in vitro. However, resistance to carbapenem compounds must be monitored, because these compounds might have suboptimal efficacy in the clinical treatment of *Salmonella* infection in vivo in some cases.

Polymyxins are important lipopeptide antibiotics that serve as the last line of defence against multidrug-resistant Gram-negative bacterial infections. The clinical utility of polymyxins is currently facing a highly concerning threat with the global spread of mobile colistin resistance (MCR) and the relevant *mcr* genes, which are the main determinant of polymyxin resistance in *Escherichia coli*. High prevalence of these genes in agriculture persists globally, and particularly in China, owing to high polymyxin usage. The transferability of *mcr* is of considerable concern, because of the potential of multidrug-resistant Gram-negative bacteria to acquire *mcr*-bearing plasmids and thus evade antimicrobial treatment with the last-line polymyxins. The *mcr-1* gene was first reported in November 2015 in China [15]. Although the use of colistin as a feed additive for animals has been banned for agriculture purposes in China since 30 April 2017 [16], NTS carrying the *mcr-1* gene was isolated from lettuce, beef and pork products in various foods at a frequency of 1.07% (3/280) in 2017, and from goose eggs and field snails at a frequency of 0.69% (4/579) in 2018 (data not published). No *mcr-1* positive foodborne NTS was detected in 2019. In the present study, *mcr-1* in foodborne NTS collected in 2020 was detected at a low level of 0.32% (4/1256), similarly to the 0.23% (6/2555) in isolates reported by Hu et al. [17]. Compared with our previous data on food sources of *mcr-1*-bearing *Salmonella* isolates (pork, chicken, egg, and dumpling sources) [17], the four strains of *Salmonella* carrying the *mcr-1* gene in this study were cultured from either poultry or beef, thereby indicating that *Salmonella*, as a reservoir of the *mcr*-1 gene, may have complex diversity in food sources, and the *mcr-1* positive clone may be largely limited to meat. Additionally, the widespread presence of *mcr-1* positive *Salmonella* in chicken, beef, pork, egg and vegetables also suggested potential transmission via the food chain, particularly by chickens.

The most common *mcr-1* gene locus structure is a 2609 bp DNA sequence consisting of an *mcr-1* gene and a putative PAP2 super family protein gene, along with two copies of IS*Apl1*, which is a member of the IS*30* family; this structure forms the composite transposon Tn6330, which is IS*Apl1*-flanked and is also believed to mediate the initial *mcr-1* gene mobilization event [18,19]. Although Tn6330 was commonly found among many *mcr-1*-bearing isolates, the *mcr-1* gene can also be disseminated through just a single end of IS*Apl1* or other means not involving this IS element. Dissemination can occur with different plasmid replicon types, including IncI2-, P-, X4- and HI-type plasmids, thus contributing to four general *mcr-1* structures identified to date [18,20]. In this study, the *mcr-1* region located on p2020s327-1 had a highly similar single-ended Tn6330 variant structure and tellurium resistance gene coding region to those in two other *mcr-1* locus structures on pCFSA629 and pHNSHP45-2, from a *S.* Typhimurium and an *E. coli* isolate, respectively. Moreover, both the *mcr-1* regions belonging to p2020s329-2 and pWW012 (*S.* Typhimurium) contained more than one IS. However, the IS from pWW012 had the standard Tn6330 structure, except for one difference in p2020s329-2, in which the PAP2 encoding gene had an opposite orientation, possibly because of genetic rearrangement caused by the loss and gain of IS*Apl1* from the transposon during multiplication. Plasmid-to-chromosomal transfer of *mcr-1* has also been suggested to have occurred recently, and Tn6330 on the chromosome might provide a relatively stable *mcr-1* state, and the loss of IS*Apl1* from the transposon may occur during the mobilization event, thus posing an additional challenge in preventing the spread of colistin resistant bacteria [21].

This report showed that all four *mcr-1* positive *Salmonella* isolates had MDR phenotypes, three of which were resistant to more than seven (or as many as ten) of the 12 examined antibacterial classes. Because polymyxins are the last therapeutic option for life-threatening infections caused by Gram-negative 'superbugs', all possible effort must be made to minimize the emergence of resistance, particularly that due to *mcr*. Hence China should consider integrated monitoring and surveillance of foodborne antimicrobial use as well as AMR in humans, animals and plants/crops, on the basis of a "One Health" approach—a strong multi-sectoral collaborative and institutional system [22]. Furthermore, more studies should focus on mechanisms and transmission of resistance of food-borne *Salmonella* to important antimicrobial drugs, to provide a theoretical basis for the rational use of antimicrobial drugs and governmental supervision to ensure food safety.

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CONFLICTS OF INTEREST

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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