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Prevalence, resistance phenotypes, and fluoroquinolone resistance genes of *Salmonella* isolates from raw milk of healthy dairy cows in Henan province, China

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Abstract. – OBJECTIVE: ***Salmonella*** **isolates have been discovered in many regions of the world. We investigated the prevalence and resistance of** ***Salmonella*** **isolates in raw milk of healthy dairy cows on farms in different regions of Henan Province, China.**

MATERIALS AND METHODS: From July 2020 to November 2021, 422 raw milk samples were collected. The minimum inhibitory concentrations (MICs) of 16 antimicrobial agents against 89 ***Salmonella*** **strains detected from the raw milk samples were determined using the broth microdilution method, and the resistance genes for fluoroquinolones were identified using polymerase chain reaction.**

RESULTS: Eighty-nine (21.09%) ***Salmonella*** **isolates were recovered from 422 raw milk samples. The** ***Salmonella*** **strains exhibited high resistance to amoxicillin (100.00%), tylosin (95.50%), and lincomycin (95.50%). Additionally, tigecycline showed good activity against** ***Salmonella*****, with an MIC_{50} of 0.25 μg/mL. All** ***Salmonella*** **isolates showed multidrug resistance (MDR), and >50% of the strains showed resistance to more than six antimicrobials. The strains from Jiaozuo exhibited 100% resistance to amoxicillin, terramycin, tylosin, and lincomycin. Two efflux pump genes, oqxA and oqxB, had the highest carrying rates of 66.29% and 64.04%, respectively. Additionally, the carrying rates of oqxA and oqxB were high in Shangqiu, Zhengzhou, and Jiaozuo. The carrying rates of aac(6')-Ib-cr in Shangqiu and Zhengzhou were 33.33% and 38.46%, respectively.**

CONCLUSIONS: This study revealed a high prevalence of ***Salmonella*** **isolates obtained from raw milk of healthy dairy cows in different regions of Henan Province, China. The** ***Salmonella*** **strains exhibited various degrees of MDR.** ***Salmonella*** **can be transmitted to humans via consumption of contaminated raw milk; thus, the presence of resistance genes poses a potential threat to public health, highlighting the need for vigilant monitoring of** ***Salmonella*** **isolates.**

Key Words:
Salmonella, Minimum inhibitory concentrations, Resistance phenotypes, Fluroquinolones, Resistance gene.

Introduction

Salmonella is a pathogenic microorganism that causes zoonotic diseases. It is also a type of major food-borne pathogen that is associated with severe infections that pose serious risks to human health[1,2]. *Salmonella* can infect people through the food chain, and contaminated animal food is an important source. This strain is prevalent throughout the world and often causes enteritis, which is especially harmful to young animals, such as young poultry. However, livestock and poultry and their dairy products are the main carriers of *Salmonella*[2,3], which can cause gastroenteritis, typhoid fever, septicemia, and intestinal diseases[4]. At present, there are a number of reports on the isolation and identification of *Salmonella*[5-9]. *Salmonella* has not only been isolated from chickens[5] and pigs[6,7], but also from humans[8] and the environment[9]. In addition,

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some *Salmonella* strains were found in children with diarrhea[10]. However, reports on *Salmonella* isolated from raw milk of healthy dairy cows are relatively scarce.

Antimicrobials, such as β-lactams, fluoroquinolones, aminoglycosides, and tetracyelines, are frequently used to treat systemic bacterial infections, including those caused by *Salmonella*[11].

Fluoroquinolones, in particular, are extensively used owing to their strong efficacy against a wide range of gram-negative bacteria[12]. Furthermore, fluoroquinolones are one of the last-line treatments for multidrug resistant (MDR) *Salmonella* infections in adults[13]. Currently, because of the long-term use of antimicrobial drugs, the increasing frequency of antibiotic resistance has become a universal problem[14]. The emergence of MDR strains poses several challenges to clinical facilities[15,16]. In recent years, an increasing number of MDR *Salmonella* isolates have been reported[11,17]. Moreover, most *Salmonella* isolates develop a large number of resistance genes, including *oqxA*, *oqxB*, *aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*[18,19]. However, there is limited information about the prevalence, antimicrobial susceptibility, and fluoroquinolone resistance genes of *Salmonella* isolates obtained from raw milk in Henan Province, China. Therefore, drugs that can be used to treat *Salmonella* infections should be urgently developed or improved through sensitivity and resistance gene testing.

Since these antimicrobials are an important part of drug therapy for humans and animals with bacterial infections, resistance to these drugs could ultimately pose a significant threat to human and animal health[20]. This study aimed at investigating the prevalence and resistance of *Salmonella* isolates obtained from healthy dairy cows on farms in different regions of Henan Province, China. The results will be of great significance to guide rational clinical drug use and monitor drug resistance.

Materials and Methods

Collection of Samples

From July 2020 to November 2021, a total of 422 raw milk samples (Zhengzhou city, n = 102; Jiaozuo city, n = 76; Kaifeng city, n = 146; Xuchang city, n = 44; and Shangqiu city, n = 54) were obtained from healthy dairy cows on farms in different regions of Henan Province, China. All samples were transported to the laboratory under the required preservation conditions (in a cooler with ice) within 6 h of collection and were processed within 2 h to detect the presence of *Salmonella*.

Isolation and Identification of Salmonella

Isolation and identification of *Salmonella* were performed by enrichment and sequential plating onto selective plates, as previously described[21]. The samples were incubated in Luria-Bertani (LB) broth (Beijing Land Bridge Technology Co., Ltd, Beijing, China) at 37°C overnight. Colonies were purified on selective agar (XLD and SS) plates (Beijing Land Bridge Technology Co., Ltd, China). The broth was streaked onto CHROMagar *Salmonella* (CHROMagar, France) plates and incubated at 37°C for 24 h to obtain presumptive isolates of *Salmonella*. These presumptive *Salmonella* colonies were identified using the VITEK 2 compact automated identification system (BioMérieux, Marcy-I'Etoile, France). *E. coli* ATCC 25922 was used as a quality control strain.

Molecular Identification of the Host Cell Invasion (Inva) Gene

Bacterial genomic DNA was extracted from 2 mL of the bacterial cell suspension that was incubated overnight in SS agar. Single colonies were grown on LB agar and transferred to 2 mL of LB broth. The samples were heated in a thermocycler at 99.9°C for 15 min and then immediately incubated at -20°C for 15 min. This was followed by centrifugation at 12,000 ×g for 15 min. A total of 140 µL of the supernatant was removed without disturbing the pellet, and the pellet was then dissolved in ddH_2O. The extracted genomic DNA was stored at -20°C and subsequently used as a Polymerase Chain Reaction (PCR) template. The extraction of *Salmonella* genomic DNA was performed according to the previously described method[5]. Subsequently, a total of 89 strains were screened for *invA* through PCR using specific primers (F: 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and R: 5′-TCATCGCACCGTCAAAGGAACC-3′) as previously described by Malorny et al[22]. *E. coli* ATCC 25922 was used as a positive control.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of 16 antimicrobial agents (**Supplementary Table I**) against the 89 *Salmonella* strains were determined using the broth microdilution method according to the Clinical and Laborato-

ry Standards Institute (CLSI) guidelines[23]. Stock solutions of all antimicrobial compounds were prepared to a final concentration of 5,120 µg/mL. Each antimicrobial solution was sterilized by filtration using 0.2-µm-pore-size filters.

E. coli ATCC 25922 was used as a reference strain for quality control in the MIC determinations. The MIC_{50} and MIC_{90} values were determined, which represent concentrations of an antibiotic that inhibits bacterial growth by 50% or 90%, respectively. The MIC breakpoints for most antimicrobial agents were in accordance with the CLSI criteria[23], while those for olaquindox and mequindox were based on relevant references[24,25]. If CLSI criteria were unavailable for some antimicrobials, the results were interpreted according to criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[26].

Determination of Resistance Genes

A total of 89 *Salmonella* strains were screened for resistance genes through PCR using specific primers. Two efflux pump genes (*oqxA* and *oqxB*)[27] and resistance genes for fluoroquinolones (*aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*) were determined using PCR. The oligonucleotide sequences, primers, and targets for PCR amplification of resistance genes are shown in the **Supplementary Table II**. The PCR products were sequenced and compared with sequences in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Data Analysis

The 89 isolated strains were categorized as sensitive (S) or resistant (R) based on the MIC values and the CLSI interpretive criteria. In addition, the MIC range, MIC_{50}, and MIC_{90} were determined.

Results

Isolation and Identification of Salmonella

Between 2020 and 2021, a total of 89 (21.09%) *Salmonella* isolates were recovered from 422 raw milk samples obtained from healthy dairy cows on farms, which comprised 102 samples from Zhengzhou city (26 isolates, 25.49%), 76 from Jiaozuo city (26 isolates, 34.21%), 146 from Kaifeng city (23 isolates, 15.75%), 44 from Xuchang city (2 isolates, 4.55%), and 54 from Shangqiu city (12 isolates, 22.22%). Thus, the prevalence of *Salmonella* isolates was the highest in Jiaozuo city (34.21%).

Antimicrobial Susceptibility Testing

The results of the antimicrobial susceptibility testing for 89 *Salmonella* strains are presented in Tables I and II. *Salmonella* strains showed high resistance to amoxicillin (100.00%), tylosin (95.50%), lincomycin (95.50%), and oxytetracycline (80.90%). Moderate resistance was observed for doxycyclin (55.00%), erythromycin (46.10%),

Table I. Distribution of MICs of *Salmonella* isolates (n = 89).

Antimicrobials	Distribution of MIC (µg/ml)											
	512[a]	256[a]	128[a]	64	32	16	8	4	2	1	0.5	0.25[b]
Terramycin	4	13	42	11	1	1	6	5	2		1	3
Ceftiofur		5	3	5	4	1			8	21	24	18
Cefquinome		1		1	3	4	5	2	5	1	10	57
Doxycyclin				8	18	23	18	3	8	6	1	4
Gentamicin	1			1	2	3	10	8	16	7	8	33
Amikacin						1	1	10	14	29	19	15
Erythromycin			1	9	31	32	8	4	2	1		1
Tylosin	38	23	18	6				1	2			1
Enrofloxacin								1	6	11	8	63
Florfenicol			12	10	5	2	4	13	29	10	3	1
Ciprofloxacin	1		1	9	19	1			9	1	5	43
Amoxicillin	47	2	10	20	7	1	2					
Lincomycin	34	37	9	1			1	3	3	1		
Tigecycline							1	1	3	3	24	57
Mequindox				2	15	34	11	8	13	6		
Olaquindox			1	6	12	13	18	21	11	4	3	

[a]Including higher than this tested MIC value; [b]Including lower than this tested MIC value.

Table II. Susceptibility of *Salmonella* isolates (n = 89).

Antimicrobials	Resistance breakpoints (μg/mL)	Senstivity breakpointsa (μg/mL)	MIC Range (μg/mL)	R (%)	S (%)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Oxytetracycline	≥ 16	≤ 4	0.25-> 512	80.9%	12.4%	128	256
Ceftiofur	≥ 8	≤ 2	0.25-> 256	20.2%	79.8%	1	64
Cefquinome	≥ 8	≤ 2	0.25-> 256	15.7%	82%	0.25	16
Doxycyclin	≥ 16	≤ 4	0.25-64	55%	24.7%	16	32
Gentamicin	≥ 16	≤ 4	0.25-> 512	7.9%	80.9%	1	8
Amikacin	≥ 64	≤ 16	< 0.25-16	0%	100%	1	4
Erythromycin	≥ 32	≤ 8	0.25->128	46.1%	18%	16	64
Tylosin	≥ 32	≤ 8	0.25-> 512	95.5%	4.5%	256	512
Enrofloxacin	≥ 4	≤ 0.5	< 0.25-4	1.1%	79.8%	0.25	1
Florfenicol	≥ 32	≤ 2	0.25-> 128	30.3%	48.3%	4	128
Ciprofloxacin	≥ 4	≤ 1	0.125-> 512	34.8%	55.1%	0.5	64
Amoxicillin	≥ 1	≤ 0.12	8-> 512	100%	0%	512	512
Lincomycin	≥ 4	≤ 0.5	1-> 512	95.5%	0%	256	512
Tigecyclineb	> 0.5	≤ 0.5	< 0.25-8	8.99%	91.01%	0.25	0.5
Mequindoxc	≥ 64	≤ 16	1-64	2.2%	80.9%	16	32
Olaquindoxc	≥ 64	≤ 16	0.5->128	7.86%	78.7%	8	32

Note: [a]MIC (μg/mL) results were calculated according to CLSI (2020) breakpoint criteria; [b]The breakpoints for tigecycline was interpreted according to criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022); [c]The breakpoints for olaquindox and mequindox were based on reference values (Hansen et al, 2004; Hansen et al, 2005).

ciprofloxacin (34.80%), and florfenicol (30.30%). The rate of resistance to gentamicin, enrofloxacin, mequindox, olaquindox, and tigecycline was below 10%. All *Salmonella* isolates were susceptible to amikacin. In addition, tigecycline, a new class of glycylcyclines, showed good activity against *Salmonella*, with an MIC_{50} of 0.25 μg/mL. Tigecycline is known to exhibit broad-spectrum activity against most gram-negative bacteria.

As shown in Table III, all *Salmonella* isolates in this study had an MDR phenotype, and more than half of the strains were resistant to more than six antimicrobials. Among them, the strains from Jiaozuo city exhibited the most severe resistance, with the largest number of resistant drugs, and 100% resistance to amoxicillin, terramycin, tylosin, and lincomycin was observed.

Prevalence of Resistance Genes

As shown in Table IV, a total of 89 *Salmonella* isolates were evaluated for 9 resistance genes, and the presence of resistance genes was found to be varied among strains from different areas. Two efflux pump genes, *oqxA* and *oqxB*, had the highest carrying rates of 66.29% and 64.04%, respectively. In addition, the carrying rates of the

Table III. The data of resistant *Salmonella* isolates (n = 89) from different regions.

Regions	Resistance rate 100%	Resistance rate 0%	The ratio of 6 kinds of tolerance drugs
Zhengzhou (n = 26)	Amoxicillin	Ceftiofur, Cefquinome, Amikacin, Mequindox, Tigecycline	34.62%
Jiaozuo (n = 26)	Amoxicillin, Terramycin, Tylosin, Lincomycin	Amikacin, Enrofloxacin, Mequindox	84.62%
Kaifeng (n = 23)	Amoxicillin	Ceftiofur, Cefquinome, Amikacin, Enrofloxacin, Florfenicol, Ciprofloxacin	26.1%
Xuchang (n = 2)	Amoxicillin, Tylosin	Ceftiofur, Cefquinome, Doxycyclin, Amikacin, Enrofloxacin, Florfenicol, Ciprofloxacin, Tigecycline, Mequindox, Olaquindox	0%
Shangqiu (n = 12)	Amoxicillin, Terramycin	Ceftiofur, Cefquinome, Enrofloxacin, Mequindox, Olaquindox	58.34%

Table IV. The carrying rate of resistant genes in *Salmonella* isolates (n = 89) obtained from different regions.

Resistance genes	Carrying rate in different regions					Carrying rate
	Zhengzhou (n = 26)	Jiaozuo (n = 26)	Kaifeng (n = 23)	Xuchang (n = 2)	Shangqiu (n = 12)	
oqxA	88.46%	69.23%	30.43%	0%	91.67%	66.29%
oqxB	84.61%	69.23%	26.08%	0%	91.67%	64.04%
qepA	0%	0%	0%	0%	0%	0%
aac(6')-Ib-cr	38.46%	3.84%	13.04%	0%	33.33%	20.22%
qnrA	0%	0%	0%	0%	0%	0%
qnrB	7.7%	0%	0%	0%	8.3%	3.37%
qnrC	0%	0%	0%	0%	0%	0%
qnrD	0%	0%	0%	0%	0%	0%
qnrS	26.9%	3.8%	26.1%	0%	16.7%	17.97%

fluoroquinolone resistance genes *aac(6')-Ib-cr*, *qnrS*, and *qnrB* were 20.22%, 17.97%, and 3.37%, respectively. However, the carrying rates of other fluoroquinolone resistance genes, namely, *qepA*, *qnrA*, *qnrC*, and *qnrD*, were all 0%.

In addition, the carrying rates of resistant genes differed considerably among strains from different areas; for example, the carrying rates of *oqxA* and *oqxB* in strains from Shangqiu, Zhengzhou, and Jiaozuo cities were higher than those in strains from the other cities. The carrying rates of *aac(6')-Ib-cr* in strains from Shangqiu and Zhengzhou cities were 33.33% and 38.46%, respectively, whereas that in strains from Xuchang city was 0%.

Discussion

This study revealed a high prevalence of *Salmonella* isolates in healthy dairy cows on farms from different regions of Henan Province, China. The prevalence of the isolates (21.90%) in this study was higher than that previously reported studies[5,6]. However, the prevalence of *Salmonella* isolates in this study was lower than that previously reported in slaughtered pigs (29.21%)[7]. The classical method for the isolation of *Salmonella* involves pre-enrichment, selective enrichment, and biochemical identification and is difficult to use for a large number of samples. *invA* has a highly conserved sequence associated with *Salmonella* invasion, and PCR amplification of *invA* can greatly reduce the working time and improve work efficiency[28].

In this study, we observed high resistance rates. The resistance rates to doxycyclin (55.00%) and ciprofloxacin (34.80%) were similar to those observed in previous studies[2,14]. The primary reason for the high resistance rates may be the use of antimicrobials on dairy farms. The rate of resistance to amoxicillin was up to 100%, which may be associated with the frequent use of amoxicillin as an antibacterial as well as the sources and number of samples. However, the rate of resistance to tylosin was higher than 95%, indicating that long-term clinical application of amoxicillin and tylosin resulted in low sensitivity of *Salmonella* to tylosin or even complete drug resistance. In this study, the rates of resistance to fluoroquinolones were not high, but the rate of intermediation was high, which suggested that long-term use of these antimicrobials would lead to resistance, resulting in an increased resistance rate. These results were similar to the drug resistance of strains isolated from medical, food, and animal sources in various countries[29-31]. In this study, strains from Jiaozuo city showed the most severe drug resistance, with the largest number of resistant antimicrobials, and showed 100% resistance to amoxicillin, terramycin, tylosin, and lincomycin, which may be related to the frequency or habits of drug use.

Salmonella isolates that were resistant to three or more classes of antimicrobials were defined as MDR. In our study, the emergence of MDR strains were consistent with previous studies[2,11,12,32,33].

A total of 89 *Salmonella* isolates were MDR, and more than half of the strains were MDR to six or more antimicrobials, among which amoxicillin resistance was the most severe, which is consistent with previous reports[11,34]. In addition, tigecycline showed good activity against *Salmonella* in this study. However, tigecycline resistance was recently identified for different pathogens, especially in MDR strains[15,35]. At pres-

ent, the emergence and spread of *Salmonella* resistance, especially those of MDR *Salmonella*, has been inevitable, which has posed a great threat to human and animal health. Antimicrobial agents should be used with caution during food animal breeding. Therefore, rational drug use should be practiced according to actual needs to avoid abuse. Regional differences and other factors, such as drug crossing or drug combinations, should be considered to avoid severe resistance caused by a single drug.

Plasmid-mediated fluoroquinolone resistance genes *aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*) are responsible for the low-level resistance to fluoroquinolones. *qnrB* and *qnrC* are the most widely distributed genes, whereas *qnrA* and *aac(6')-Ib-cr* are rarely reported in Europe, America, and Japan[36]. The distribution of *qnrC*, *qnrD*, *qepA*, *oqxA*, and *oqxB* is relatively limited[37,38]. In this study, the rate of detection of the resistance genes *qepA*, *qnrA*, *qnrC*, and *qnrD* was 0%, that of *aac(6')-Ib-cr* and *qnrS* was approximately 20%, and that of *qnrB* was only 3.37%, which is consistent with previous findings[39]. In addition, the rate of detection of *oqxA* and *oqxB* in this study was higher than 60%, which is consistent with the findings of a previous study[20]. The fluoroquinolone resistance genes identified in this study included *oqxA*, *oqxB*, *aac(6')-Ib-cr*, and *qnrS*. We found that the resistance genes carried by the strains differed among the five, and strains from Zhengzhou and Shangqiu cities had a higher carrying rate, which may be attributed to differences in the number and frequency of fluoroquinolone application in different regions.

In this study, two efflux pump genes, *oqxA* and *oqxB*, had a high rate of detection, but the resistance phenotype was associated with very low resistance rates. According to a previous study[40], even if bacteria carry resistance genes, the resistance phenotype is not highly resistant, which may be attributed to gene expression, the antibacterial activities of different antimicrobials, differences in enzyme stability and genotype popularity, or the presence of chromosomal mutations. There may also be other resistance mechanisms, warranting further investigation.

Conclusions

This study provides insight into the resistance characterization of clinical *Salmonella* isolates from raw milk of healthy dairy cows. *Salmonella* isolates exhibited varying degrees of MDR. Cephalosporins, aminoglycosides, and other antibiotics can be used as antimicrobials for clinical consideration or cross-use with fluoroquinolones to avoid the development of severe drug resistance. It is crucial to supervise the prevalence of *Salmonella* strains in raw milk. Furthermore, *Salmonella* can be transmitted to humans through the consumption of contaminated raw milk, which raises food safety concerns. The presence of resistance genes poses a potential threat to public health, highlighting the need for vigilant monitoring of *Salmonella* isolates. Future epidemiological studies should include a larger number of strains and samples, and supervision for resistance detection should be strengthened.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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Availability of Data and Materials
The data supporting the findings of this study are included within the manuscript and its supporting information.

Authors' Contribution
BGL, GZH, and EPX participated in study conception and design and prepared the manuscript. BGL, MX, YTG, YD, and HW participated in sample collection and performed the experiments. GMZ, HW, MB, and GZH analyzed the results and reviewed the manuscript. BGL, GZH, and EPX revised the manuscript and coordinated the whole project. All authors read and reviewed the final manuscript.

Consent for Publication
Not applicable.

Ethics Approval and Consent to Participate

With regard to our study's use of animals, this study protocol was reviewed and approved by the Henan University of Chinese Medicine animal ethics committee, and all experiments were performed in accordance with the regulations and guidelines established by this committee. The owners of the farm animals from which samples were collected consented to their animals being used in this study.

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