Prevalence and characteristics of multidrug-resistant *mcr-1*-positive *Escherichia coli* isolates from broiler chickens in Tai'an, China

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ABSTRACT Colibacillosis, caused by *Escherichia coli*, is one of the most common bacterial diseases of chickens. The high incidence and considerable economic losses associated with colibacillosis make it a significant concern worldwide. In recent years, the efficacy of colistin has been severely impacted by the emergence of plasmid-mediated colistin resistance genes, especially *mcr-1*. Therefore, monitoring of antibiotic resistance, particularly colistin resistance, amongst *E. coli* strains is vitally important to the future growth and sustainability of the poultry industry. In this study, a total of 130 *E. coli* strains were isolated from the livers of chickens displaying symptoms of colibacillosis in Tai'an, China. Isolates were screened for their susceptibility to various antibiotics and for the presence of mobile colistin resistance genes and other antibiotic resistance genes. Overall, 75 (57.7%) isolates showed resistance to colistin and were positive for *mcr-1*. The mobile colistin resistance genes, *mcr-2*, *-3*, and *-4*, were not detected in this study. Of the 75 *mcr-1*-positive isolates, all (100%) also carried tetracycline resistance genes, 71 (94.7%) also contained genes associated with β-lactam resistance, 59 (78.7%) contained aminoglycoside resistance genes, and 57 (76%) contained sulfonamide resistance genes. This high prevalence of multidrug resistance among *mcr-1*-positive *E. coli* isolates, including the production of extended-spectrum β-lactamases, is highly concerning. The surveillance findings presented here will be conducive to our understanding of the prevalence and characteristics of multidrug-resistance in *E. coli* in the Tai'an area and will provide a better scientific basis for the clinical treatment of colibacillosis in chickens.

Key words: *Escherichia coli*, colistin, drug resistance gene, *mcr-1*, drug sensitivity test

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INTRODUCTION

The emergence of multidrug-resistant (**MDR**) gram-negative bacteria, in parallel with the lack of new antibacterial agents, has led researchers to recognize the importance of polymyxins (Falagas and Kasiakou, 2005; Giamarellou and Poulakou, 2009). In veterinary use, colistin has been administered as a feed additive in many countries such as China, the United States, the European Union, and Japan to prevent digestive tract disease caused by *Escherichia coli*, *Salmonella*, and other enterobacteria (Liu et al., 2014). However, the transmission of *mcr-1*–mediated colistin resistance between animals and humans poses a threat to human health (Yang et al., 2017). Because colistin is considered a last resort drug to treat severe MDR gram-negative bacterial infections in humans, the mobile colistin resistance determinant *mcr-1* has attracted global attention (Liu et al., 2016). Since first being discovered in 2015 in *E. coli* strain SHP45 isolated from a pig in Shanghai, China (Liu et al., 2016), the plasmid-mediated colistin resistance gene *mcr-1* (for mobile colistin resistance) has been identified in bacteria isolated in many other countries (Falgenhauer et al., 2016; Zhao and Zong, 2016; Trung et al., 2017).

Before the discovery of *mcr-1*, it was widely believed that colistin resistance was mainly caused by chromosomal mutations and clonal spread and that resistance was often unstable and could not be transferred to other

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types of bacteria (Falagas et al., 2010). Although additional *mcr* genes, including *mcr-2*, *mcr-3*, and *mcr-4*, have been identified, *mcr-1* remains the predominant colistin resistance gene in China (Yassin et al., 2017). The distribution of *mcr-1*–containing strains is global, with MDR strains being isolated in Asia, Europe, the United States, and Australia (Ellem et al., 2017; Hernandez et al., 2017; Lindsey et al., 2017; Meinersmann et al., 2017; Wang et al., 2017a,b; Yang et al., 2017; Garza-Ramos et al., 2018; Rau et al., 2018; Creighton et al., 2019; Henig et al., 2019; Macesic et al., 2019;Merida-Vieyra et al., 2019; Moreno et al., 2019). This demonstrates that *mcr-1* genes carried by plasmids have strong transmission capacity.

The complexity and degree of drug resistance amongst *E. coli* strains vary greatly between regions and change over time (Wang et al., 2017a). The occurrence of avian colibacillosis in the Tai'an area of Shandong Province is extremely common. In addition, drug use on farms in this region is not standardized, thereby leading to serious levels of *E. coli* resistance and consequently serious economic losses to the livestock industry. Therefore, the first objective of this study was to obtain an estimate of the prevalence of resistance to common antimicrobial agents among *E. coli* isolates obtained from the livers of sick chickens in Tai'an, China. The second objective was to assess the diversity and distribution of the major β-lactam, aminoglycoside, tetracycline (**TE**), and sulfonamide resistance genes in these *E. coli* isolates. The third objective was to identify any associations between *mcr-1* and other resistance genes in *E. coli* isolated from the sample population. The isolation of *E. coli* from chickens will provide a foundation for the epidemiology of *E. coli* in Tai'an, China. The investigation and monitoring of *E. coli* resistance, especially colistin resistance, are important when assessing the potential economic and public health implications. Such data will provide a scientific basis for the clinical treatment and prevention of *E. coli* in chickens in this region.

MATERIALS AND METHODS

Sample Collection, Isolation, and Identification of E. coli

A total of 400 liver tissue samples were collected from broiler chickens with perihepatitis lesions at different slaughterhouses in the Tai'an area of China (Figure 1). Samples were collected from March to December, 2017. All samples were aseptically obtained from liver tissues and seeded into MacConkey or eosin methylene blue media. Following 3 to 5 rounds of purification, putative *E. coli* isolates were selected based on bacterial colony morphology and confirmed by 16S rRNA gene sequencing and biochemical identification methods.

Colistin Sensitivity Testing

Colistin (polymyxin E) and polymyxin B were resuspended in deionized water to a final concentration of 100 mg/mL before being filtered through a 0.22-μm filter. The resulting antibiotic was stored at −20°C. Because of the poor diffusion of the large colistin molecule using the disk-diffusion method, the *E. coli* isolates were screened for sensitivity to colistin using the broth microdilution method (EUCAST, 2017). Briefly, each of the isolates was cultured in cation-adjusted Mueller-Hinton broth supplemented with colistin (0.02–200 μg/mL) at 37°C for 12 h. Bacterial growth was then determined by measuring the optical density of the cultures at 600 nm. According to the European Committee on Antimicrobial Susceptibility Testing standards (EUCAST, 2017), a strain was judged to be resistant when the minimum inhibitory concentration was >2 μg/mL.

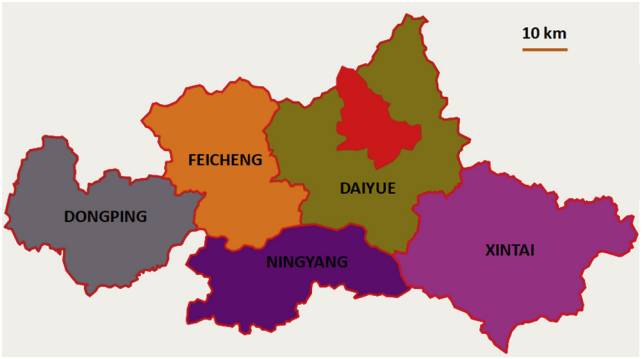

Figure 1. Map of the Tai'an area. A total of 400 liver tissue samples were collected from broiler chickens with perihepatitis lesions at different slaughterhouses in the Tai'an area of China. These areas are Daiyue, Ningyang, Feicheng, Xintai, and Dongping.

Antimicrobial Susceptibility Testing

The *E. coli* isolates were screened for their susceptibility to various other antibiotics using the Kirby-Bauer disk diffusion method as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). The following antibiotics were examined: nalidixic acid, TE, ampicillin, cefotaxime (**CTX**), streptomycin (**S**), ceftriaxone, doxycycline, chloramphenicol (**C**), levofloxacin, sulfamethoxazole/trimethoprim, gentamicin, and amikacin. All these antimicrobial susceptibility testing disks were purchased from Thermo Fisher Scientific (Shanghai, China). The drug sensitivity results were assessed by reference to the American Institute of Clinical Laboratory Standardization (https://clsi.org/standards/) and the European Commission on Antimicrobial Susceptibility Testing (http://www.eucast.org/clinical.breakpoints). *E. coli* strain DH5α (sensitive laboratory strain) was used as a negative control.

Detection of Drug Resistance Genes

Mobile colistin resistance genes *mcr-1*, *mcr-2*, *mcr-3*, and *mcr-4* were detected by PCR as described previously (Liu et al., 2016; Xavier et al., 2016; Carattoli et al., 2017; Yin et al., 2017). The primers and annealing temperature are described in Table 1. Primers used to screen for the presence of genes associated with

Table 1. Primer sequence information.

Gene name	Primer sequences (5′-3′)	Primer size (bp)	Annealing temperature/°C	References
Mcr-1	F-CGGTCAGTCCGTTTGTTC R-CTTGGTCGGTCTGTAGGG	309	55	Liu et al., 2016
Mcr-2	F-TGTTGCTTGTGCCGATTGGA R-AGATGGTATTGTTGGTTGCTG	567	65	Xavier et al., 2016
Mcr-3	F-TTGGCACTGTATTTTGCATTT R-TTAACGAAATTGGCTGGAACA	542	50	Yin et al., 2017
mcr-4	F-ATTGGGATAGTCGCCTTTTT R-TTACAGCCAGAATCATTATCA	487	56	Carattoli et al., 2017
aadA	F-GCAGCGCAATGACATTCTTG R-ATCCTTCGGCGCGATTTTG	282	55	Costa et al., 2008 Wen et al., 2015
aacC2	F-ACCCTACGAGGAGACTCTGAATG R-CCAAGCATCGGCATCTCATA	384		
aacC4	F-ATGACCTTGCGATGCTCTATGA R-CGAATGCCTGGCGTGTTT	486		
aphA3	F-TGACTGGGCACAACAGACAA R-CGGCGATACCGTAAAGCAC	677		
CTX-M	F-AGTGAAAGCGAACCGAATC R-CTGTCACCAATGCTTTACC	365	55	Tian et al., 2013
SHV	F-ATGCGTATATTCGCCTGTG R-CCTCATTCAGTTCCGTTTCC	502		
TEM	F-CAGAAACGCTGGTGAAAGTA R-ACTCCCCGTCGTGTAGATAA	719		
tetA	F-GGCCTCAATTTCCTGACG R-AAGCAGGATGTAGCCTGTGC	372	57	Guillaume et al., 2010
tetB	F-GAGACGCAATCGAATTCGG R-TTTAGTGGCTATTCTTCCTGCC	228		
tetC	F-CTTGAGAGCCTTCAACCCAG R-ATGGTCGTCATCTACCTGCC	418	60	
tetD	F-GGAATATCTCCCGGAAGCGG R-CACATTGGACAGTGCCAGCAG	187		
sul1	F-GTGACGGTGTTCGGCATTCT R-TCCGAGAAGGTGATTGCGCT	779	68	Boerlin et al., 2005
sul2	F-CGGCATCGTCAACATAACCT R-TGTGCGGATGAAGTCAGCTC	721	66	
sul3	F-GAGCAAGATTTTTGGAATCG R-CATCTGCAGCTAACCTAGGGCTTTGGA	880	51	

resistance to TE (*tetA*, *tetB*, *tetC*, and *tetD*), sulfonamides (*sul1*, *sul2*, and *sul3*), aminoglycosides (*aadA*, *aphA3*, *aacC2*, and *aacC4*), and β-lactams ($bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM}) are also shown in Table 1. PCR products were sequenced by Shanghai Bioengineering Co. (Shanghai, China), and the resulting sequences were compared against the GenBank database.

RESULTS

Detection of Colistin Resistance

A total of 130 *E. coli* isolates were recovered from the 400 liver samples from broiler chickens across 5 districts in Tai'an (Table 2). The overall isolation rate was 32.5% (130/400). Susceptibility assays showed that 79 (60.8%) isolates were resistant to colistin E, polymyxin B, or both and that 90.4% of these isolates showed cross-resistance to the 2 antibiotics.

Antibiotic Sensitivity Testing

A column chart (Figure 2) was used to show the rates of resistance of the *E. coli* isolates to the different antibiotics. The lowest rate of resistance was to amikacin (23.1%), whereas all 130 isolates (100%) showed resistance to nalidixic acid. Importantly, all the tested *E. coli* isolates were resistant to at least 3 antibiotics (Figure 3).

Prevalence of Antibiotic Resistance Genes

Various antibiotic resistance genes were identified among the 130 *E. coli* isolates (Table 3), including

Table 2. Results of pathogeny gene screening of chicken *Escherichia coli* isolates.

Sampling area	Sample size	Positive detection rate (%)	Number of *E. coli*
Daiyue	100	36 (36/100)[1]	1-36
Ningyang	80	30 (24/80)	37-60
Feicheng	80	35 (28/80)	61-88
Xintai	80	29 (23/80)	89-111
Dongping	60	32 (19/60)	112-130

[1]Numbers in parentheses are positive/total.

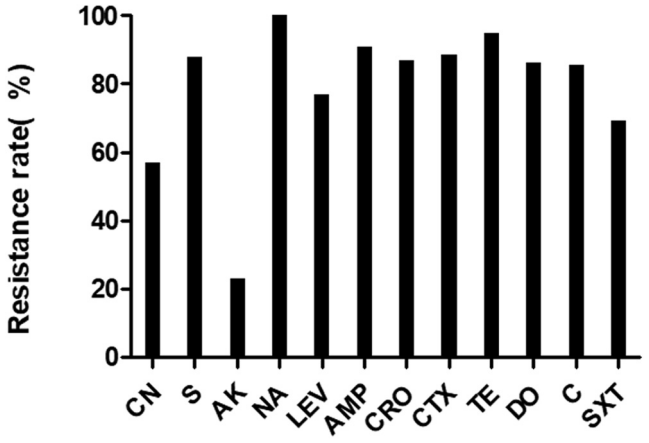

Figure 2. Rates of antibiotic resistance among *Escherichia coli* isolates. The lowest rate of resistance was to amikacin (23.1%), whereas all 130 isolates (100%) showed resistance to nalidixic acid. Abbreviations: AK, amikacin; AMP, ampicillin; C, chloramphenicol; CN, gentamicin; CRO, ceftriaxone; CTX, cefotaxime; DO, doxycycline; LEV, levofloxacin; NA, nalidixic acid; S, streptomycin; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline.

β-lactam resistance genes bla_{TEM} and $bla_{\text{CTX-M}}$; aminoglycoside resistance gene *aphA3*; TE resistance genes *tetA*, *tetB*, and *tetC*; and sulfonamide resistance genes *sul1* and *sul2*. However, *aadA*, *aacC4*, *tetD*, and *sul3* were not detected in this study (Table 3). While 75 of the 130 *E. coli* isolates (57.7%) contained *mcr-1*, none of the isolates contained *mcr-2*, *mcr-3*, or *mcr-4*. Overall, 71 of 75 (94.7%) *mcr-1*–positive isolates contained a β-lactamase resistance gene, 59 of 75 (78.7%) contained an aminoglycoside resistance gene, all 75 (100%) contained a TE resistance gene, and 57 of 75 (76.0%) contained a sulfonamide resistance gene (Figure 4).

DISCUSSION

Before 2015, sporadic cases of colistin-resistant bacterial infections did not attract much attention (Gao et al., 2016). However, the discovery of plasmid-mediated colistin resistance gene *mcr-1* in *E. coli* and *Klebsiella pneumoniae* isolates from Chinese patients and animals represented a transmissible mechanism of colistin resistance (Liu et al., 2016). The location of *mcr-1* on a chromosomally-independent genetic element means that it is more easily replicated and can spread horizontally between different bacteria, forming pan-resistant or even superbug strains. Since their initial discovery in China, *mcr-1*–containing plasmids have been discovered in gram-negative bacteria from many countries worldwide (Malhotra-Kumar et al., 2016; Trung et al., 2017; Cyoia et al., 2019; Brilhante et al., 2019; Vounba et al., 2019).

In addition to *mcr-1*, many of the isolates carried multiple other drug resistance genes, including those encoding extended-spectrum β-lactamases (ESBL), which is highly concerning (Rhouma and Letellier, 2017). ESBL are mainly associated with *E. coli* and *K. pneumoniae*, which often show resistance to multiple antibiotics (Surgers et al., 2019). Antimicrobial resistance poses significant challenges for current clinical care. Continued surveillance of multidrug resistance, including the presence of *mcr-1*, may help pre-empt the spread of *mcr-1* among bacterial pathogens. Modified use of

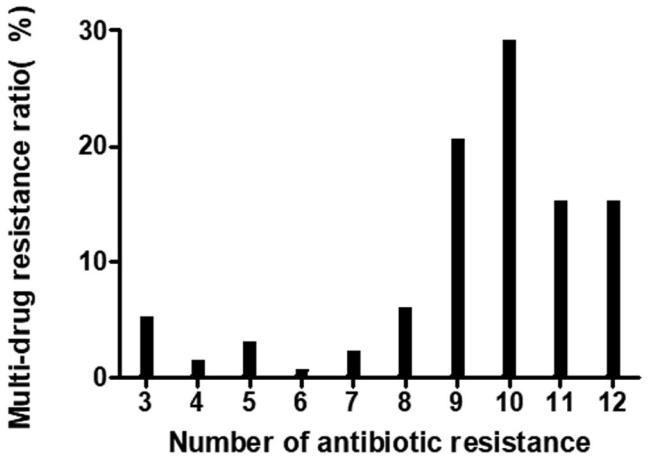

Figure 3. Prevalence of multidrug resistance among 130 pathogenic *Escherichia coli* isolates. All the tested *E. coli* isolates were resistant to at least 3 antibiotics. About 81% (105/130) strains were resistant to 9-12 drugs.

Table 3. Distribution of resistance genes in *Escherichia coli* isolates.

Drug resistance gene type	Detected drug resistance gene	Positive rate (%)
β-Lactams	*TEM*	90.0% (117/130)[1]
	SHV	3.1% (4/130)
	CTX-M	53.8% (70/130)[2]
	aadA	
Aminoglycosides	*aadC2*	20.8% (27/130)
	aphA3	55.4% (72/130)
	aacC4	
Tetracyclines	*tetA*	82.3% (107/130)
	tetB	13.8% (18/130)
	tetC	98.4% (128/130)
	tetD	
Sulfonamides	*sul1*	63.8% (83/130)
	sul2	41.5% (54/130)
	sul3	

[1]Numbers in parentheses are positive/total.
[2]Negative result.

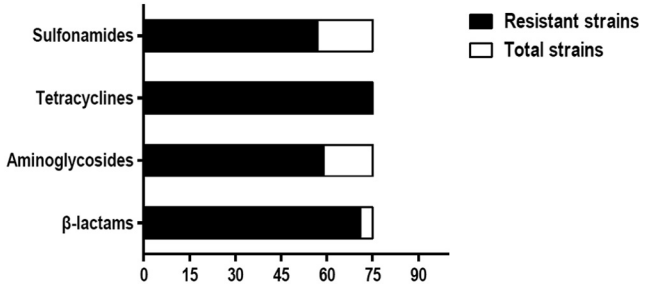

Figure 4. Antibiotic resistance genes found to coexist with *mcr-1*. Overall, 94.7% (71/75) of the *mcr-1*–positive isolates contained a β-lactamase resistance gene, 78.7% (59/75) contained an aminoglycoside resistance gene, 100% (75/75) contained a tetracycline resistance gene, and 76.0% (57/75) contained a sulfonamide resistance gene.

antimicrobial agents and public health interventions, coupled with novel antimicrobial strategies, may help mitigate the effect of MDR organisms in the future. Multiple reports have confirmed that *mcr-1* can coexist with other resistance genes on the same plasmid, including bla_{NDM}, bla_{CTX-M}, bla_{TEM}, bla_{CMY}, *fosA*, *qnrS*, *floR*, and *oqxAB* (Sun et al., 2016; Bi et al., 2017; Lai et al., 2017; Li et al., 2017; Faccone et al., 2019). In the present study, we analyzed the resistance of the 130 *E. coli* isolates to antibiotics belonging to 6 different classes. The results showed that all the isolates showed resistance to at least 3 classes of antibiotics, indicating multidrug resistance, and that 20 of the isolates were resistant to all 12 tested antibiotics. The coexistence of *mcr-1* and other drug resistance genes makes the treatment of infections caused by these isolates more difficult (Wang et al., 2017a,b). Furthermore, if these resistance genes are located on the same plasmid, they can be cotransferred with *mcr-1*. The spread of such MDR plasmids poses a major threat to public health (Malhotra-Kumar et al., 2016).

At present, different committees have reported different polymyxin sensitivity breakpoints (Nation et al., 2015; EUCAST, 2017). As recommended by the SENTRY monitoring program (JMI Laboratories, North Liberty, IA; Gales et al., 2006), we used a minimum inhibitory concentration value of $\geq$2 μg/mL to indicate resistance of the *E. coli* isolates to polymyxin B. Using this criterion, we found that 60.8% of the *E. coli* isolates were resistant to polymyxin, which was a significantly higher rate than that determined by the SENTRY program (24.0%). The antibiotic susceptibility profiles of *mcr-1*–positive *E. coli* isolates were also tested for polymyxin B and colistin susceptibility by the ETEST (BioMérieux, Marcy l'Etoile, France), and as observed in the present study, cross-resistance to the 2 antibiotics was noted (La et al., 2019). In recent years, with the intensification of many types of farming, the problem of drug resistance among pathogenic *E. coli* strains has become increasingly significant (Stoesser et al., 2017). As such, appropriate prevention and control measures need to be implemented. Therefore, it is necessary to conduct epidemiological investigations and monitoring of resistance genes in *E. coli* to provide a scientific basis for the control and clinical treatment of resistant strains.

In conclusion, we showed that all colistin resistance among *E. coli* isolates from broiler chickens in Tai'an, China, can be attributed to *mcr-1*, with none of the isolates containing the *mcr-2*, *mcr-3*, or *mcr-4* genes. The coexistence of *mcr-1* and ESBL-encoding genes, including bla_{TEM} and bla_{CTX-M}, along with the extremely high rates of multidrug resistance among the colistin-resistant *E. coli* isolates is a major concern. These results suggested that *mcr-1*–positive *E. coli* isolates are likely to carry other resistance genes.

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