

# Epidemiological Characterization of Colistin and Carbapenem Resistant Enterobacteriaceae in a Tertiary: A Hospital from Anhui Province

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**Purpose:** Antimicrobial resistance, especially carbapenem resistance Enterobacteriaceae and plasmid mediated mobile colistin resistance, is a serious issue worldwide. This study was designed to determine the epidemiological characteristics of plasmid mediated colistin resistance and carbapenem resistant Enterobacteriaceae from tertiary A hospital located in Hefei, China.

**Methods:** Totally, 158 carbapenems resistant Enterobacteriaceae (CRE) were screened for antibiotic susceptibility, *mcr-1*, extended spectrum  $\beta$ -lactamases (ESBLs), metallo- $\beta$ -lactamases (MBLs), and fosfomycin resistance genes using PCR and sequencing. The sequence types were identified by multilocus sequence typing (MLST). Plasmid profiles were determined by PCR based replicon typing (PBRT), and the plasmid sizes were confirmed by southern blotting.

**Results:** The isolates showed high MIC<sub>50</sub> and MIC<sub>90</sub> for all antimicrobials, except tigecycline, meropenem, and colistin. The main Carbapenemase genes were *bla*<sub>KPC-2</sub> (90.5%), *bla*<sub>NDM-1</sub> (3.7%), *bla*<sub>OXA-48</sub> (5.6%) and *fosA3* (14.5%). The *bla*<sub>CTXM-15</sub> found 36.7%, *mcr-1* (3.7%) recorded in six isolates. PBRT revealed *bla*<sub>KPC-2</sub> in *K. pneumoniae* on IncR, IncFII, and IncA/C. *bla*<sub>NDM-1</sub> in *E. coli* on IncFII, whereas in *E. cloacae* noticed on IncHI2 plasmid. *mcr-1* was recorded among IncFIIK, IncFII, and IncF in *E. coli*, *K. pneumoniae*, and *E. cloacae*. Resistance genes (*mcr-1*, *bla*<sub>NDM-1</sub>, *bla*<sub>KPC-2</sub>) harboring plasmids are successfully trans-conjugant to *EC-600*. A high incidence of ST11 was observed in *K. pneumoniae* carbapenem resistant isolates. While in *E. coli*, multiple STs were identified. However, *mcr-1* in ST23 was identified for the first time in Anhui Province. Among *Enterobacter cloacae*, ST270 detected carrying *bla*<sub>NDM-1</sub>. Southern-hybridization confirmed the plasmid sizes 35–150kb.

**Conclusion:** This study indicates the co-carrying of *mcr-1*, *bla*<sub>KPC-2</sub>, and *bla*<sub>NDM-1</sub> among clinical isolates, the prevalence of different Enterobacteriaceae STs is alarming, especially in *E. coli*. Holding such a resistance profile is a threat for humans and animals, which may be transferred between the strains through plasmid transfection. Persistent control actions are immediately necessary to combat this hazard.

**Keywords:** ESBL, MBL, KPC2, *mcr-1*, NDM-1, CTXM-15

## Introduction

The extensive practice of carbapenems in medical treatment and colistin in animal farms has intensified severe community health problems.<sup>1</sup> Carbapenem resistant Enterobacteriaceae (CRE) comprises various types of bacteria, and each one has its mechanism of drug resistance.<sup>2</sup> Counting them, the *K. pneumoniae* carbapenem resistance accounts for about sixty percent, followed by *Escherichia coli* and *Enterobacter cloacae*.<sup>3,4</sup> With carbapenemases, *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> are the maximum predominant

ones in the *K. pneumoniae* and carbapenem resistant *E. coli*, respectively.<sup>5,6</sup> Even the latest research reports indicate the spread of multiple resistance determinants in a single strain.<sup>7,8</sup> Worldwide colistin usage in veterinary farms may increase to 67% by 2030, estimated mostly by BRICS (Brazil, Russia, India, China, and South Africa). Significant and severe farming processes are essential to increase income and animal protein utilization.<sup>9</sup> Colistin is another last therapeutic option to combat infectious diseases caused by gram negative multi-drug resistant bacilli.<sup>10</sup>

The rising mobile colistin resistant gene *mcr-1* encodes phosphoethanolamine transferase, vital community health problem,<sup>11</sup> after its initial discovery in china, the global research reports on *mcr-1* propagates. Different Enterobacteriaceae species carrying the *mcr-1* gene on various plasmids have been reported in clinical and veterinary bacterial isolates in Europe, Asia, Africa, South, and North America.<sup>12</sup> Many countries reported the co-occurring of *mcr-1* with many other resistance genes, especially with *bla<sub>KPC-2</sub>* and *bla<sub>NDM-1</sub>* in clinical settings and veterinary livestock.<sup>13</sup> The reports mainly show more than two carbapenem determinants in a single plasmid, thus alarming us for their plasmid nature transmission.

On the other hand, the information on these antimicrobials' susceptibilities on CRE plus colistin are still incomplete. The complete and accurate epidemiological scientific information of each region is critical and essential for future points of view and the proper identification of each resistive determinant. Our study's main aim is to investigate comprehensive and precise epidemiological scientific information of colistin plus carbapenem resistance Enterobacteriaceae.

Overall, in our investigation, 158 carbapenem resistant Enterobacteriaceae (CRE) samples were received from a tertiary A hospital in Hefei, Anhui province, China. To find the epidemiological characteristics of CRE, we performed the antibiotic susceptibility, identification of resistant variants, multilocus sequence typing (MLST), PCR-based replicon typing (PBRT) of resistant determinants, pulse field gel electrophoresis (PFGE), and southern blot hybridization.

## Materials and Methods

### Samples Collection Identification and Study Design

To identify the spread of CRE, especially with mobilized colistin resistance gene *mcr* (1–5) among medical isolates.

We performed a study in a tertiary A hospital, strains collected from March 2018 to April 2019. One hundred fifty-eight samples, including sputum ( $n=95$ ), urine ( $n=43$ ), wound ( $n=12$ ), and blood ( $n=8$ ), were collected from the first affiliated hospital of USTC in Hefei Anhui province, China. The bacterial models include *K. pneumoniae* ( $n=88$ ), *E. coli* ( $n=38$ ), *E. cloacae* ( $n=26$ ), and *Serratia marcescens* ( $n=6$ ). All the isolates were grown on MacConkey agar at 37°C overnight. The next morning the single colony was selected from MacConkey agar plate and grown on Luria-Bertani (LB) broth 10–12 hours or overnight to identify specific bacteria 16s rRNA gene was screened from LB broth and sequenced. Sequencing data were then analyzed by using BLAST ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)) and ([www.ezbiocloud.net](http://www.ezbiocloud.net)).

### Antimicrobial Susceptibility Testing

Seventeen different antibiotics were used for the antimicrobial susceptibility testing. Antibiotics included are amikacin (AMK), ampicillin (AMP), aztreonam (ATM), ceftriaxone (CRO), cefuroxime (CXM), ceftazidime (CAZ), ciprofloxacin (CIP), cefazolin (CFZ), ertapenem (ETP), gentamicin (GEN), imipenem (IPM), levofloxacin (LVX), cefepime (FEP), cefotaxime (CTX), meropenem (MEM), tigecycline (TGC), and colistin (CST). The broth microdilution method (BMDM) was used to test all of them. The clinical and Laboratory Standards Institute (CLSI 2019) recommendations were followed for result interpretation.<sup>13</sup> As the breakpoint value of colistin is missing in CLSI. We consider the European Committee's breakpoint value on Antimicrobials Susceptibility Testing (EUCAST) ([www.eucast.org](http://www.eucast.org)) colistin greater than 2 µg/mL. For carbapenem, the breakpoint value was described as a MIC of  $\geq 4$  µg/mL.

### Detection of Antibiotic Resistant Genes (ARGs)

The boiling method was performed for the extraction of bacterial DNA templates.<sup>14</sup> Colistin resistant genes for the detection were included *mcr* (1–5), Carbapemases genes for detection were (*bla<sub>KPC</sub>*, *bla<sub>AIM</sub>*, *bla<sub>OXA</sub>*, *bla<sub>DIM</sub>*, *bla<sub>NDM</sub>*, *bla<sub>GIM</sub>*, *bla<sub>SIM</sub>*, *bla<sub>SPM</sub>*, *bla<sub>VIM</sub>*, *bla<sub>IMP</sub>*, and *bla<sub>GES</sub>*). Extended-spectrum genes were included (*bla<sub>CTX</sub>*, *bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, *bla<sub>VEB</sub>*, and *bla<sub>PER</sub>*) according to the protocols defined previously.<sup>15–17</sup> The amplified PCR results were directed for sequencing to

General Biosystems Co., Ltd. (Hefei-China), and the sequencing data was then analyzed and confirmed by using BLAST ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)).

## Multilocus Sequence Typing (MLST)

Carbapenem resistant *K. pneumoniae* STs were identified by MLST, with the screening of seven house-keeping genes comprising (*gapA*, *mdh*, *pgi*, *infB*, *phoE*, *rpoB*, and *tonB*), and were sequenced as defined previously.<sup>18</sup> The sequencing data was analyzed by the Pasteur online database ([www.pasteur.fr/mlst/Kpneumoniae.html](http://www.pasteur.fr/mlst/Kpneumoniae.html)). For the identical and proper alleles sequence type identification of *E. coli*, the seven house-keeping genes used were (*adk*, *gyrB*, *icd*, *mdh*, *fumC*, *purA*, and *recA*), and the data was analyzed by <https://bigsd.b.pasteur.fr/Ecoli/ecoli.html>. The MLST database (<http://pubmlst.org/Ecloacae/>) was used to identify *E. cloacae* sequence types. The essential seven genes of *E. cloacae* screened and sequenced from the *E. cloacae* colony were (*dnaA*, *gyrB*, *leuS*, *fusA*, *pyrG*, *rplB*, and *rpoB*).

## PCR Based Replicon Typing (PBRT)

Among the CRE, the incompatibility of plasmid groups and the plasmid of colistin resistant determinants were identified by performing PCR-based replicon typing (PBRT). KIT 2.0 (DIATHEVA, Italy) was used for the identification of thirty different plasmids; IncHI1, IncHI2, IncI1, IncI2, IncX1, IncX2, IncX3, IncX4, IncL, IncFIIs, IncFIIk, IncFIB-KN, IncFIB-KQ, IncW, IncY, IncP1, IncA/C, IncM, IncN, IncFIA, IncFIC, IncFII, IncB/O, IncT, IncK, IncU, IncR, IncHIB-M, IncFIB-M, and IncFIB were used according to the protocol.<sup>19</sup>

## Conjugation Experiment

To investigate the transferability of resistance determinants, six *mcr-1* resistant strains (2 *K. pneumoniae*, 2 *E. cloacae*, and 2 *E. coli*), six *bla*<sub>NDM-1</sub> (4 *E. cloacae* and 1 *K. pneumoniae* and 1 *E. coli*), and six *bla*<sub>KPC-2</sub> *K. pneumoniae* resistant strains were selected as donors. The recipient bacteria used for conjugation assays was *EC-600* (Nal<sup>R</sup>, Rif<sup>R</sup>), according to the protocol as previously defined.<sup>20</sup> Confirmation of trans-conjugants was done by antimicrobial susceptibility followed by PCR band recognition and finally confirmed by PBRT.

## Pulsed Field Gel Electrophoresis (PFGE) and Southern Hybridization

To find the genomic similarity and identify the position of transmissible *mcr-1*, *bla*<sub>KPC-2</sub>, and *bla*<sub>NDM-1</sub>, isolates were categorized by S1-PFGE and southern hybridization through a specific probe of *mcr-1*, *bla*<sub>KPC-2</sub>, and *bla*<sub>NDM-1</sub>. S1 nuclease was used to digest each genome and then examined through PFGE as described previously.<sup>21</sup> According to the manufacturer's directions, southern hybridization of plasmid DNA was accomplished with a digoxin-labeled *mcr-1*, *bla*<sub>KPC-2</sub>, and *bla*<sub>NDM-1</sub> specific probe (Roche Diagnostics, Mannheim, 32 Germany) as previously described.<sup>22</sup>

## Results

### Bacterial Isolation and Antimicrobial Susceptibility

The clinical isolates were collected from various departments, including 51/158 (32.27%) high prevalence recorded in the respiratory unit, 37/158 (23.4%) collected from intensive care unit ICU, 23/158 (14.55%) from the urinary surgery department, 17/158 (10.75%) from neurosurgery, 10/158 (6.32%) from gerontology, 9/158 (5.69%) from pediatric, 7/158 (4.43%) from orthopedic and 4/158 (2.53%) from oncology, and the distribution of each bacterial species among the collected samples are listed in the ([Supplemental Figure 1](#)). Overall, 158 different bacterial strains were collected from the First Affiliated Hospital of USTC. All the samples were observed to be carbapenem and colistin non-susceptible by phenotypic approach. The frequency rate of each strain found was *K. pneumoniae* 88/158 (55.7%), *E. coli* 38/158 (24%), *E. cloacae* 26/158 (16.45%), and *S. marcescens* 6/158 (3.79%) ([Supplemental Table 1](#)). The high frequency was observed in sputum 95/158 (60.12%), followed by urine 43/158 (27.21%), wound 12/158 (7.59%), and blood was 8/158 (5.06%) ([Supplemental Figure 2](#)). The antimicrobial sensitivity testing for all collected isolates was performed by broth microdilution method against 17 Antibiotics, in which tigecycline was detected as the most susceptible drug having a sensitivity of 93%. Likewise, the resistivity against the meropenem and colistin were 78.8% and 43.2%, respectively. The sensitivity and resistivity profile for all 17 drugs against collected strain and their MIC values are presented in [Table 1](#).

**Table 1** Representing MICs

Antibiotics	Mic50	Mic90	Range (µg/mL)	%R	%S
Amikacin	>128	>128	1–128	75.9	24.1
Ampicillin	>128	>128	1–128	86.1	13.9
Aztreonam	>128	>128	1–128	92.4	7.6
Ceftazidime	>32	>32	0.25–32	78.9	21.1
Ciprofloxacin	>8	>8	0.06–8	45.3	54.7
Ceftriaxone	>32	>32	0.25–32	84	16
Cefotaxime	>32	>32	0.25–32	86.3	13.7
Cefuroxime	>64	>64	0.5–64	71.4	28.6
Cefazolin	>64	>64	0.5–64	73.8	26.2
Ertapenem	>32	>32	0.5–32	87.5	12.5
Cefepime	>32	>32	0.5–32	91	9
Gentamicin	>128	>128	1–128	77	23
Imipenem	>16	>16	0.125–16	69	31
Levofloxacin	>16	>16	0.125–8	61	39
Meropenem	>16	>16	0.125–16	78.8	21.2
Tigecycline	0.5	1	0.125	7	93
Colistin	4	8	1–128	43.2	56.8

## Prevalence of Resistance Determinants in *K. pneumoniae*, *E. coli*, *E. cloacae*, and *S. marcescens*

The prevalence of *K. pneumoniae* resistant determinants is shown in (Table 2). In 84/88 (95.4%), a high prevalence of serine  $\beta$ -lactamases, producing *K. pneumoniae*

*Carbapenemases bla<sub>KPC-2</sub>* has been observed alone or in combination with *bla<sub>CTX-M-15</sub>* (47.7%), *fosA3* (14.77%), *bla<sub>OXA-48</sub>* (3.4%), and colistin resistant *mcr-1* (2.2%). Overall among the CRE, the prevalence rate of the *bla<sub>KPC-2</sub>* gene was 90.5%.

Concerning the *E. coli* isolates, the prevalence of *bla<sub>KPC-2</sub>* was 89.4%, combined with the ESBL *bla<sub>CTX-M-15</sub>* encoding gene 15.7%. In two *E. coli* isolates, *mcr-1* was caught in 56 and 57 years old male and female urine samples. *fosA3* and *bla<sub>OXA-48</sub>* were observed in four isolates, respectively. The combination of *mcr-1* and *bla<sub>KPC-2</sub>* found 10.56% in *E. coli*.

Regarding *E. cloacae*, the prevalence of *bla<sub>KPC-2</sub>* was detected at 84.6%. *bla<sub>KPC-2</sub>* plus *bla<sub>NDM-1</sub>* saw 15.3%, a combination of *bla<sub>CTX-M-15</sub>* and *bla<sub>KPC-2</sub>* noted 30.70%. Six isolates confirming the presence of *fosA3*, and two isolates have *bla<sub>OXA-48</sub>*. Two *mcr-1* detected in a 77 years old male blood sample and 68 years old female sputum sample. *Enterobacter cloacae* possess the *mcr-1* plus *bla<sub>KPC-2</sub>* remarked 7.6%.

Three isolates of *S. marcescens* carry *bla<sub>KPC-2</sub>* determinant, respectively, and two of them combined with *bla<sub>CTX-M-15</sub>*, genes which are spotted in our article are listed in (Supplemental Table 2).

## Multilocus Sequence Typing (MLST)

A high incidence of ST11 was observed in all isolates of CRKP, while in case of *E. coli* the sequence types noticed were ST69 (*n*=4), ST131 (*n*=3), ST1193 (*n*=3), ST12 (*n*=1), ST46 (*n*=1), ST57 (*n*=1), ST1196 (*n*=1), ST38 (*n*=1), ST95 (*n*=1) and ST23 (*n*=1). Among the *Enterobacter cloacae*, ST270 was detected only, and the result shown in Table 3.

**Table 2** Distribution of Antibiotic Resistance Genes (ARGs)

Resistant Determinants	<i>K. pneumoniae</i> ( <i>n</i> =88)	<i>E. coli</i> ( <i>n</i> =38)	<i>E. cloacae</i> ( <i>n</i> =26)	<i>Serratia marcescens</i> ( <i>n</i> =6)
<i>bla<sub>KPC-2</sub></i> ( <i>n</i> =143)	84	34	22	3
<i>bla<sub>NDM-1</sub></i> ( <i>n</i> =6)	1	1	4	-
<i>bla<sub>OXA-48</sub></i> ( <i>n</i> =9)	3	4	2	-
<i>FosA3</i> ( <i>n</i> =23)	13	4	6	-
<i>mcr-1</i> ( <i>n</i> =6)	2	2	2	-
<i>bla<sub>CTX-M-15</sub></i> ( <i>n</i> =58)	42	6	8	2

**Table 3** Distribution of Plasmid Replicons, STs, and Resistant Genes of Eighteen Bacterial Strains

Sample	Plasmid/PBRT	Trans-Conjugants	Size (kb)	STs	Strain	Resistant Genes
S153	IncFIIk(148bp)	+	70	ST11	<i>K. pneumonia</i>	<i>mcr-1</i>
S39	IncFII (292bp)	+	35	ST11	<i>K. pneumonia</i>	<i>mcr-1</i>
S08	IncFII(292bp)	+	35	ST270	<i>E. cloacae</i>	<i>mcr-1</i>
S05	IncFIIk(631bp)	+	120	ST270	<i>E. cloacae</i>	<i>mcr-1</i>
S319	IncF (683bp)	+	70	ST131	<i>E. coli</i>	<i>mcr-1</i>
S47	IncF(683bp)	+	70	ST23	<i>E. coli</i>	<i>mcr-1</i>
S19	IncFII (288bp)	+	70	ST23	<i>E. coli</i>	<i>bla<sub>NDM-1</sub></i>
S30	IncFII (288bp)	+	70	ST1196	<i>E. coli</i>	<i>bla<sub>NDM-1</sub></i>
S168	IncHI2 (308bp)	+	120	ST270	<i>E. cloacae</i>	<i>bla<sub>NDM-1</sub></i>
S69	IncHI2 (308bp)	+	120	ST270	<i>E. cloacae</i>	<i>bla<sub>NDM-1</sub></i>
S194	IncHI2 (308bp)	+	120	ST270	<i>E. cloacae</i>	<i>bla<sub>NDM-1</sub></i>
S74	IncHI2 (308bp)	+	120	ST270	<i>E. cloacae</i>	<i>bla<sub>NDM-1</sub></i>
S29	IncR(248bp)	+	100	ST11	<i>K. pneumonia</i>	<i>bla<sub>KPC-2</sub></i>
S13	IncFII(288bp)	+	35	ST11	<i>K. pneumonia</i>	<i>bla<sub>KPC-2</sub></i>
S25	IncA/C (418bp)	+	50	ST11	<i>K. pneumonia</i>	<i>bla<sub>KPC-2</sub></i>
S103	IncFII(288bp)	+	35	ST11	<i>K. pneumonia</i>	<i>bla<sub>KPC-2</sub></i>
S137	IncFIIk(631bp)	+	70	ST11	<i>K. pneumonia</i>	<i>bla<sub>KPC-2</sub></i>
S149	IncFII(288bp)	+	35	ST11	<i>K. pneumonia</i>	<i>bla<sub>KPC-2</sub></i>

## Plasmid Replicon Typing (PBRT)

Among 158 CRE isolates, seven different types of plasmid replicons were detected. *K. pneumoniae* carrying IncFIIK, IncFII, IncA/C, and IncR were found in *bla<sub>KPC-2</sub>* positive isolates, while *mcr-1* producers among *K. pneumoniae* were detected on IncFIIk and IncFII. *Enterobacter cloacae* were carrying *bla<sub>NDM-1</sub>* on IncHI2, whereas *mcr-1* on IncF and IncFIIK. *E. coli* took *mcr-1* on IncF type replicon however, *bla<sub>NDM-1</sub>* on IncFII. The complete profile is listed in Table 3.

## Conjugation

We performed a conjugation experiment for all resistance genes to detect resistance genes' transformability harboring plasmids (*mcr-1*, *bla<sub>NDM-1</sub>*, *bla<sub>KPC-2</sub>*). Harboring plasmids are successfully trans-conjugant to EC-600 (NaI<sup>R</sup>, Rif<sup>R</sup>). The conjugation was confirmed by PCR-based replicon typing (PBRT) of the trans-conjugants, and the result was mentioned in Table 3. For further conformation, the resistance genes (*mcr-1*, *bla<sub>NDM-1</sub>*, *bla<sub>KPC-2</sub>*) specific

plasmid PCR were performed, and the gene was detected in trans-conjugants.

## SI-PFGE and Southern Blot

SI-PFGE further confirmed that all the strains have several plasmids, varying in size and ranging from 35kb-150kb (Supplemental Figures 3-5). Southern blotting confirmed that the *mcr-1*, *bla<sub>KPC-2</sub>* and *bla<sub>NDM-1</sub>* genes regained from these isolates were located on the given six dissimilar plasmid types, as shown in Table 3.

## Discussions

The present article focuses on the incidence of *mcr-1* combined with other  $\beta$ -lactamases resistance genes, especially with *bla<sub>KPC-2</sub>*, *bla<sub>NDM-1</sub>*, and *bla<sub>CTX-M</sub>* in *E. coli*, *K. pneumoniae*, *Serratia marcescens*, and *Enterobacter cloacae*, and previously stated by many countries.<sup>23-25,26</sup> We specified information on the antibiotics resistance profiles, STs, plasmid replicons profiles, PFGE, and southern blotting of strains having *mcr-1*, *bla<sub>KPC-2</sub>*, and *bla<sub>NDM-1</sub>*.



As far as we know, this report is the initial article associating the carriage of *mcr-1* with *bla*<sub>KPC-2</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>CTX-M</sub> among the CRE in clinical settings from Anhui province, China.

The extensive use of colistin in livestock is mainly attributed to the spread of colistin resistance determined *mcr-1*, threatening the worldwide distribution of colistin efficiency. The rise of *mcr-1*, mostly in *E. coli* and *K. pneumoniae*, is of specific concern. To control the spread of colistin resistance from May 1, 2017, the china government banned colistin in livestock.<sup>27,28</sup> Many reports in china have previously reported the dissemination of *mcr-1* mainly associated with animal origin. Cong Shen reported a study<sup>29</sup> on the prevalence of *mcr-1* from animal origin pigs 308/684 (45%), and the plasmids carrying was reported in IncX4, IncI2, and IncHI2. Another study by X-Zhang reported<sup>30</sup> a very high prevalence of colistin resistance in Jiangsu province, associated with animal origin pigs 303/440 (68.86%), chickens 388/443 (87.5%), and cattle's 30/42 (71.43%). A study conducted by Liu et al<sup>31</sup> on clinical samples in five different china provinces reported 16/1322 (1%) *mcr-1* in human hospitalized patients. In our study, the dissemination of *mcr-1* is 6/158 (3.79%), a little higher than previously reported. The possible reason for the difference between positive isolates in clinical and animal settings is that *mcr-1* mediated colistin resistance started in the animal's origin and successively extend to humans. The use of colistin in hospital settings is infrequent but still the best option of treatment for CRE infections.

In the production of livestock and poultry animals, china is the world's leading country. In 2014 china only produces 56.7 million tons of poultry and 17.5 million tons of pigs, 90% of the production was use internally, and about 10% for export. The prices of veterinary medicines rise from \$20.1 billion in 2011 to \$43 billion in 2019. In the agriculture sector, China is also the world's largest country using colistin.<sup>31</sup> The total requirement for colistin use in agriculture globally in 2015 was 11,942 tons per annum, with total revenue of \$229.5 million and expected to rise 16,500 tons by the end of 2021 with a 4.75% annual growth rate. The top ten companies producing colistin, including eight from China, 73.1%, were produced in Asia, and 28.7% were transferred to Europe. To promote growth and health in fish farms, colistin sulphate combined with other antibiotics in China's use as a food diet<sup>31</sup>. Production of such a high level of colistin is directly proportional to the stress in the veterinary

environment and provides a favorable condition for the strains having *mcr-1*.

The world's topmost antimicrobials are utilized in china<sup>32</sup> The high practice of antimicrobials may activate the rise of antimicrobial resistance AMR. Mainly resistance of  $\beta$ -lactam and colistin resistance reported worldwide.<sup>33–35</sup> Recently, plasmid mediated colistin resistant genes have been extensively exposed.<sup>36–43</sup> We screened 158 MDR Enterobacteriaceae strains isolated from the First Affiliated Hospital of USTC in Anhui province for *mcr-1* to *mcr-5* in human clinical samples. The only *mcr-1* gene was isolated in combination with *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>. Our research project identifies the carriage rate of *mcr-1* (6/158) in humans is not so high but primarily occurred in combination with other resistive genes. The frequency of  $\beta$ -lactamases is very high in our report supporting the data published previously.<sup>44</sup> The frequency rate of *mcr-1* in Enterobacteriaceae among the clinical strains is relatively low in many countries<sup>25</sup> We are reporting the ratio of *mcr-1* (n=6) 3.7%, which is relatively high compared to other provinces. In Changsha, Hunan province, *mcr-1* was noticed in three (2.1%) of the 144 *E. coli* clinical isolates.<sup>45</sup> As compared with an animal origin, especially in pigs, the rate of *mcr-1* is 75%, much higher than clinical findings.<sup>46</sup>

The extreme incidence of *bla*<sub>KPC-2</sub>, *bla*ESBLs, and *bla*<sub>NDM-1</sub>, was reliable with the earlier reports,<sup>47</sup> representing the common co-carrying of antimicrobials resistance genetic factor, which might prime to the development of deadly *K. pneumoniae* toxicities. Amongst them, the relatively extreme occurrence of *bla*ESBL with *bla*<sub>KPC-2</sub> in our finding suggests critical intimidations to public health since several duplicates of *bla*<sub>CTX-M</sub> and *bla*<sub>KPC-2</sub> within plasmids might be joined and circulated into the chromosome, the percentage of *bla*<sub>CTX-M-15</sub> in our project is 36.7% (58/158) which is relatively low as compared to previous reports published from many countries around the globe.<sup>48,49</sup> In that situation, the rise of such isolates would be horizontally and vertically quicker inside clinics. Until now, the co-prevalence of *bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub> was previously noted in *K. pneumoniae*.<sup>50</sup> Primarily, we instigate a high occurrence of *fosA3* (14.7%) plus *bla*<sub>KPC-2</sub>, producing *K. pneumoniae*. *FosA3* has been testified to be chromosomally programmed by medically relevant Gram negative bacteria and enhances inherent fosfomycin resistance, and the high prevalence reported previously.<sup>51</sup> Additionally, the reports showing that the extensive

dissemination of *fosA3* directed that fosfomycin must be vigilantly consumed for handling CRKP diseases.<sup>52</sup>

Plasmids are extrachromosomal DNA, basically demonstrating the main reservoirs for the horizontal spread of antibiotic resistance between microorganisms.<sup>53</sup> So far, various plasmids have recognized the carrier for the incidence of carbapenemases in *bla*ESBLs and *bla*MBLs.<sup>54</sup> The excessive dominance of IncFII, IncFIik, and IncR plasmid replicons in our report is aware of the importance of realizing antibiotics resistance observation since IncF type plasmids are intensely disseminated carriers for resistant causes in Enterobacteriaceae. In contrast, some studies stated the prevalence of *bla*<sub>ndm-1</sub> on IncFII while the *mcr-1* on IncX4 type plasmid was originating from animal reservoirs.<sup>55,56</sup> Furthermore, the IncR plasmid is a significant reservoir of many antibiotics resistance in Enterobacteriaceae isolates since the preserved IncR backbones contain the multidrug resistant (MDR) sequences.<sup>57</sup> Furthermore, IncFIik plasmids were described to be related to the mainstream of the antimicrobial resistance genes detected more frequently in our study.<sup>58</sup>

The extension of ST11 for the *bla*<sub>KPC-2</sub> carrying *K. pneumoniae* was in the union as according to the earlier articles representing that, ST11 is the main epidemic clone among *K. pneumoniae*.<sup>59</sup> In our finding, to the best of our knowledge, we investigate for the first time the occurrence of multidrug resistant *E. coli* ST23, isolated from the 61 years old female carrying *mcr-1*, *bla*<sub>KPC-2</sub>, *bla*<sub>NDM-1</sub> plus *bla*<sub>CTX-M-15</sub>. Generally, in our study, we experienced *bla*<sub>KPC-2</sub> in different sequence types of *Escherichia coli* mainly with *bla*<sub>CTX-M</sub> or *bla*<sub>NDM-1</sub>, specifically *bla*<sub>KPC-2</sub> plus *bla*<sub>NDM-1</sub> in *E. coli* ST12 and ST57 have not been reported previously from Anhui province. We also report the triple carrying of *K. pneumoniae mcr-1*, *bla*<sub>KPC-2</sub> plus *bla*<sub>CTX-M</sub> in ST11 on IncFIik 70kb plasmid replicon and other *mcr-1* on IncFII 35kb. Both of them are isolated from 61 years old female sputum samples and 80 years old female blood samples. A study done in Brazil on clinical isolates shows *mcr-1* plus *bla*<sub>KPC-2</sub> in sequence type 392.<sup>60</sup>

As we report a high incidence of Multidrug resistance Enterobacteriaceae, we have a valuable suggestion. Awareness is needed while handling the resistance cases of *bla*ESBLs, *bla*MBLs, and colistin. Alertness is needed in hospital settings and food producing animal farms because *mcr-1*, combined with other resistive genes, is a risk to human health. The animals transmit these resistive determinants more frequently and thus might enter to human diet chain very quickly.

## Conclusion

Our study indicates the high incidence of multidrug resistance Enterobacteriaceae, *mcr-1* carrying with ESBL *bla*<sub>CTX-M-15</sub> and *bla*MBLs *bla*<sub>NDM-1</sub>, and *bla*<sub>KPC-2</sub> clinical samples in Anhui China. Holding such a resistance profile is an excellent threat for humans and animals, which may be transferred between the same strains and other strains through plasmid transfusion. Need high vigilance for handling such resistive profiles to control the prevalence of such resistant genes and the future world's life. The spread of *bla*<sub>KPC-2</sub> in different sequence types of *E. coli* and the occurrence of *bla*<sub>NDM-1</sub> in *E. cloacae* is alarming and painful, well organized, and persistent control actions required that are immediately necessary.

## Abbreviations

MLST, Multilocus sequence typing; PFGE, Pulse field gel electrophoresis; PBRT, PCR based replicon typing; MCR, mobilized colistin resistance gene.

## Statement of Ethics

This study has been approved (approval number 2020KY-191) by the ethical committee of the First Affiliated Hospital of the university of science and technology of China (USTC).

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## Disclosure

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