Detection of mcr-1-positive *Escherichia coli* in slaughterhouse wastewater collected from Dawen river

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**Abstract**

**Background:** Low levels of mcr-1 were detected in *Escherichia coli* from wastewater samples across the world; hence, further monitoring and management of accumulation of mcr-1-positive bacteria in wastewater are urgently recommended.

**Objectives:** In this study, we have reported the detection of *E. coli* strains carrying the colistin resistance gene mcr-1 in slaughterhouse wastewater discharged into Dawen river.

**Methods:** Twenty samples were collected aseptically and subjected to polymerase chain reaction (PCR) analysis, multilocus sequence typing and antibiotic resistance tests. Conjugation tests were also performed.

**Results:** The screening results showed a positive rate of 20% (4/20), which suggested that the mcr-1 gene had polluted the environment of the river. The mcr-1 gene had successfully transferred from the donor to recipient cells, which showed the possibility of horizontal transfer of mcr-1 and subsequently, the formation of multidrug resistant bacteria in the river.

**Conclusions:** Our findings demonstrated a high occurrence of colistin-resistant *E. coli* carrying the mcr-1 gene on transferrable plasmids in slaughterhouses and indicated their dissemination into river. Large-scale cross-border cooperation would be required for the effective control of the spread of antibiotic-resistant bacteria.

**Keywords**

*Escherichia coli*, mcr-1, river, wastewater

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Colistin is considered a last-line therapeutic option for severe infections caused by multidrug-resistant Gram-negative bacteria, in particular those expressing carbapenemase and multidrug-resistant Acinetobacter baumannii (Hayashi et al., 2019; Liu et al., 2016). mcr-1 gene had first been reported in China (Liu et al., 2016), and so far, it (and mcr family genes) has been detected not only in food animals, raw meat and human clinical samples but also in water samples from beaches and rivers (Fernandes et al., 2017; Yang et al., 2017). Moreover, mcr-1 has been reported to exist in wastewater, since colistin-resistant members from the family Enterobacteriaceae have been found to be widely distributed in rivers downstream of sewage treatment plants. Although low levels of mcr-1 were detected in Escherichia coli from wastewater samples across China, further monitoring and management of the accumulation of mcr-1-positive bacteria in wastewater are urgently recommended. In fact, recent studies have demonstrated mcr-1-positive isolates of Enterobacteriaceae to also carry a variety of other drug resistance and virulence genes, which should be carefully considered (Jiang et al., 2020; Sun et al., 2016). Thus, the distribution patterns of the mcr-1 gene warrant further attention. Here, we have assessed the presence of the colistin resistance gene mcr-1 in slaughterhouse wastewater discharged into Dawen river. To the best of our knowledge, this is the first study to explore the presence of antimicrobial resistance genes in slaughterhouse wastewater collected from the Dawen river, in the city of Tai'an, China.

### 2. Materials and Methods

#### 2.1. Sampling and sample preparation

Sampling was conducted from the sewage outlet of a slaughterhouse near the Dawen river, which receives treated wastewater. Water samples (35 ml each) were collected aseptically and sent to the laboratory within 3 hr of collection. Since our sampling did not hurt animals or pollute the environment, ethical approval was not required for this study. After sedimentation by gravity, the supernatants were filtered using membrane filters and subsequently placed into Enterobacteriaceae Enrichment Broth for overnight enrichment. Next, 100 μl of bacterial solutions was inoculated into Bertani broth liquid medium and cultured overnight in the presence of 10 μg/ml colistin for screening. Positive red colonies were selected, inoculated in Luria Bertani broth liquid medium and cultured overnight for further analysis.

#### 2.2. Identification of E. coli

Bacterial DNA was extracted, 16S rRNA gene was amplified through polymerase chain reaction (PCR) using specific primers (Liu et al., 2016) and the products were sent to Shanghai Sangon Co., Ltd, China, for sequencing. All colonies exhibiting the typical E. coli morphological appearance were subjected to MALDI-TOF (IVD MALDI Biotyper, Bruker Bremen, Germany) for confirming the bacterial species. The score cutoff of ≥2.000 was applied for species-level identification according to the manufacturer’s recommendation.

#### 2.3. Detection of mcr-1 and other antimicrobial resistance genes

Because mcr-1 often coexists with multiple drug resistance genes on the same plasmid, mcr-1 and other plasmid-mediated resistance genes, including beta-lactam resistance genes (bla<sub>SHV</sub>, bla<sub>OXA</sub>, bla<sub>PER</sub> and bla<sub>CTX-M</sub>), plasmid-mediated quinolone resistance genes (qnrA, qnrB, qnrS and qepA), aminoglycoside resistance genes (aac(3)-I, aac(3)-II, aac(3)-III, aac(3)-IV, ant(2) and aac(6)-Ib), tetracycline resistance genes (tetA and tetB), sulphonamide resistance genes (sul1, sul2 and sul3) and chloramphenicol resistance genes (cmiA and flor) were investigated through PCR using specific primers as reported previously (Table S1). Bacterial DNA was extracted by boiling the bacterial suspension in Tris-EDTA buffer. PCR systems included 12.5 μl PCR mix, 8.5 μl deionised water, 1 μl forward primer, 1 μl reverse primer and 2 μl template in a total volume of 50 μl (Shanghai Sangon Co., Ltd.). mcr-1 was amplified under the following conditions: 30 s at 94°C for predenaturation; 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C for amplification and 5 min at 72°C for final amplification. The PCR products of mcr-1 were then subjected to electrophoresis at 140 V for 30 min. The positive specimens were subjected to direct sequencing for confirmation, and the sequences of mcr-1-positive strains were compared using BLAST of the National Center for Biotechnology Information website.

#### 2.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the E. coli isolates to 10 antimicrobial agents, colistin (CST), AMP (ampicillin), tetracycline (TET), ciprofloxacin (CIP), chloramphenicol (C), sulfamethoxazole-trimethoprim (SXT), nalidixic acid (NA), cefoxitin (FOX), GEN (gentamicin) and imipenem (IMP) was tested using the broth microdilution method (Zhang et al., 2015; Zheng et al., 2019). Bacteria were cultured at 37°C in LB broth for 6 hr; the cell density of the suspensions was adjusted to 1.5 × 10<sup>8</sup> colony forming units per ml in sterile saline. Result of colistin was interpreted using the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2021). Results of the other nine antibiotics were interpreted based on the Clinical and Laboratory Standards Institute standard guidelines (CLSI, 2021), and the Enterobacteriaceae breakpoints were used in this study. E. coli strain ATCC25922 was used for quality control. E. coli isolates resistant to more than
three classes of antimicrobials were defined as multidrug-resistant (MDR) *E. coli*.

### 2.5 Molecular characteristics of *E. coli*

Multilocus sequence typing (MLST), a procedure for characterising bacterial isolates based on nucleic acid sequencing, was performed on *mcr-1*-positive strains according to the Institute Pasteur MLST database (http://bigsdb.pasteur.fr/ecoli/primers_used.html). Replicon types of the plasmids were determined using PCR-based replicon typing (Carattoli et al., 2005) (Table S2).

The *mcr-1*-positive strains investigated were subjected to a conjugation assay using the filter mating method (Li et al., 2019; Warjri et al., 2015). The recipient strain was *E. coli* J53, used in a 1:1 proportion with the donor strain. The transconjugants were selected on brain heart infusion agar plates supplemented with colistin (2 mg/L).

| Sample no. | ST   | Resistance phenotype | Resistance genes | Incompatibility group (kb) of *mcr-1* plasmids |
|------------|------|---------------------|------------------|-----------------------------------------------|
| 1          | ST302| AMP-NA-TET-CST      | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul2, *mcr-1* | IncI1                                         |
| 2          | ST468| AMP-C-NA-SXT-TET-CST| *bla*$_{CTX}$,$*bla*$_{TEM}$,flor, *mcr-1*| IncIFIB                                       |
| 3          | ST302| AMP-TET             | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul2           | None                                         |
| 4          | ST302| AMP-NA-TET-CST      | *bla*$_{CTX}$,$*bla*$_{TEM}$,sul2,*mcr-1*    | IncI1                                         |
| 5          | ST370| SXT                 | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul1           | None                                         |
| 6          | ST87 | GEN-SXT             | *bla*$_{CTX}$,$*bla*$_{TEM}$, qnr5, sul1     | None                                         |
| 7          | ST302| AMP-GEN-SXT-TET     | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul2           | None                                         |
| 8          | ST314| NA-TET              | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul1, sul2     | None                                         |
| 9          | ST302| AMP-TET-CST         | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul2,*mcr-1*  | IncI1                                         |
| 10         | ST302| AMP-C-NA-SXT-TET    | *bla*$_{CTX}$,$*bla*$_{TEM}$,flor, sul2     | None                                         |
| 11         | ST302| AMP-FOX-NA-TET      | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul2           | None                                         |
| 12         | ST370| AMP-NA-TET          | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul2           | None                                         |
| 13         | ST468| AMP-C-NA-SXT        | *bla*$_{CTX}$,$*bla*$_{TEM}$,flor            | None                                         |
| 14         | ST739| AMP-CIP-NA-SXT-TET  | *acc(6’)-Ib-cr, *bla*$_{CTX}$,$*bla*$_{TEM}$| None                                         |
| 15         | ST370| TET                 | *bla*$_{CTX}$,$*bla*$_{TEM}$                | None                                         |
| 16         | ST739| AMP-CIP-NA-SXT-TET  | *acc(6’)-Ib-cr, *bla*$_{CTX}$,$*bla*$_{TEM}$| None                                         |
| 17         | ST2  | AMP-C-CIP-NA-SXT-TET| *bla*$_{CTX}$,$*bla*$_{TEM}$,cmlA, for, sul3| None                                         |
| 18         | ST88 | AMP-C-CIP-GEN-NA-SXT-TET | *bla*$_{CTX}$,$*bla*$_{TEM}$,cmlA, for, sul2, sul3, tetB | None |
| 19         | ST353| C-TET               | *bla*$_{TEM}$,flor, qnr5, sul2               | None                                         |
| 20         | ST370| NA-TET              | *bla*$_{CTX}$,$*bla*$_{TEM}$, sul1           | None                                         |

*Abbreviations: AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; FOX, cefoxitin; GEN, gentamicin; IMP, imipenem; NA, nalidixic acid; SXT, sulfamethoxazole-trimoxazole; TET, tetracycline; CST, colistin.*
3 | RESULTS

3.1 | Identification of E. coli and screening of mcr-1

In this study, we focused on E. coli; analysis of other bacteria was outside the scope of this study. Twenty unique E. coli strains, identified by MALDI-TOF, were obtained from the sampled water. These strains were then screened for the presence of mcr-1. Four positive strains (20%) were detected; all these strains displayed an identical mcr-1 sequence.

3.2 | Antimicrobial susceptibility and STs of E. coli

There were 20 nonduplicated E. coli isolates, and there were as many as six different STs. The STs of E. coli were highly variable, demonstrating that the isolates were diversified (Table 1).

Further, 90% of these strains were resistant to tetracycline and 70% of these strains were resistant to ampicillin. All these strains were sensitive to imipenem. 95% of these strains carried the resistance gene blaTEM, and 95% of these strains carried the resistance gene blCTX-M, which was in accordance with the resistance phenotype (Figure 1).

3.3 | Characteristics of mcr-1 positive E. coli

The minimum inhibitory concentration of colistin for the four mcr-1-positive isolates was 4–8 μg/ml. Three of four mcr-1-positive E. coli isolates were ST302, while only 7 of the 20 E. coli isolates were ST302. The mcr-1 genes were located on plasmids belonging to IncI1 and IncIFIB in three and one isolates, respectively. mcr-1 coexisted with three or more resistance genes in the mcr-1 positive E. coli isolates, demonstrating that the four mcr-1-positive isolates were all multidrug-resistant.

3.4 | Conjugation experiments

The conjugation experiments showed that all mcr-1-positive E. coli isolates were able to successfully transfer mcr-1 to the recipient strain E. coli J53. PCR and sequencing further confirmed these transconjugants to harbour mcr-1.

4 | DISCUSSION

Our results underlined the importance of monitoring wastewater for the presence of antibiotic-resistant bacteria. In fact, the water environment is considered a repository of antibiotic resistance genes (Zhang et al., 2015); thus, wastewater could pose a potential threat to the health of fish and humans. Most antibiotic resistance genes are located as clusters on plasmids. Therefore, their horizontal transfer is easy and fast, imparting multidrug resistance to bacteria, which are then difficult to control. For bacteria, such plasmids are advantageous for survival in adverse conditions. However, this study demonstrated that bacteria carrying certain resistance genes are sensitive to the corresponding antibiotics. This may be attributed to the absence or low expression of resistance genes, which is a decisive element for the emergence of antibiotic-resistant phenotypes. For instance, the transcriptional level of pmrB gene in the colistin-resistant group of E. coli isolates is 9.5-fold higher than that in the colistin-susceptible group (Qi et al., 2016). While mcr-1 was the major reason for colistin resistance, upregulated expression of pmrB also played a role in colistin resistance in E. coli, reflecting the complexity of the mechanism underlying antimicrobial resistance (Cannatelli et al., 2014).

Alternatively, more effective drugs to control the spread of plasmids, such as chlorpromazine, may be administered. Chlorpromazine, a commonly used antischizophrenia drug, may interfere in conjugation to reduce the transmission of plasmids between the donor and recipient strains (Buckner et al., 2020).

FIGURE 1 | Susceptibility of Escherichia coli to 10 antibiotics
Interestingly, the river that we collected samples from received treated wastewater from a slaughterhouse and flowed into a fish pond. Thus, it is likely that the mcr-1-positive bacteria isolated from the river came from the slaughterhouse wastewater and flowed into the pond, polluting it as a result. To protect rivers against pollution from antibiotic resistance genes, wastewater should be directed to sewage treatment plants for special treatment instead of directly being discharged into rivers. Common treatment of wastewater by sewage treatment plants can significantly reduce the microbial content in the water, but it cannot completely eliminate the antibiotic-resistant bacteria. On the contrary, it will increase the drug resistance of some bacteria under these selective pressures (DiAllo et al., 2013). If antibiotic-resistant E. coli in the outlet water cannot be eliminated, it will enter the local environment; this can cause the spread of drug-resistant bacteria. Subsequently, these strains could be transmitted to animals and humans via various environments (Zhang et al., 2015). Moreover, since antimicrobial resistance genes can be transferred from one bacterial cell to another, antibiotic-susceptible strains may be converted into antibiotic-resistant strains, thereby intensifying the problem of drug-resistant pathogens. Water samples of slaughterhouse wastewater were collected from a branch of the river, which is why the results may not be representative of the whole water body of the river, which may be one of the limitations of the work.

## 5 | CONCLUSIONS

Large-scale cross-border cooperation is required for the effective control of the spread of antibiotic-resistant bacteria. Our findings demonstrated the high occurrence of colistin-resistant E. coli carrying mcr-1 on transferrable plasmids in slaughterhouses and indicated their dissemination into river. Thus, our study highlights the urgent need of adopting strict measures to avoid the accumulation of antibiotic-resistant bacteria in rivers.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to.

## AUTHOR CONTRIBUTION

Xinxing Wang: Formal analysis; Methodology; Writing-original draft. Li Li: Methodology; Investigation. Fengxia Sun: Investigation; Software; Visualization. Jinji Wang: Resources. Weishan Chang: Conceptualization; Validation. Fengmei Chen: Project administration. Jun Peng: Data curation; Funding acquisition; Supervision.

## PEER REVIEW

The peer review history for this article is available at https://pubons.com/publon/10.1002/vms3.489.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Supporting Information**

Additional supporting information may be found online in the Supporting Information section.

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