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Original Article

Co-occurrence of *mcr-3* and *fosA3* in IncP plasmid in ST131 *Escherichia coli*: A novel case

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Abstract

Introduction: Plasmid-mediated colistin resistance genes, especially *mcr-3* combined with the fosfomycin resistance gene *fosA3*, are a grave health concern. Our study was designed to determine the epidemiological characteristics of the combination of *mcr-3* and *fosA3* in Anhui province, China.

Methodology: A total of 127 multi-drug-resistant (MDR) *E. coli* strains were assessed for antibiotic resistance/sensitivity to detect *mcr-3* and *fosA3* using polymerase chain reaction (PCR) and sequencing. The genes of interest were conjugated using EC600, and replicon and sequence types (STs) were identified by PCR-based replicon typing (PBRT) and multilocus sequence typing (MLST). Cluster similarity and genomic relatedness among the positive isolates were confirmed by XbaI PFGE.

Results: The processed *E. coli* isolates were highly resistant to the tested antibiotics; the prevalence of *mcr-3* was 0.78% in the transferable IncP-type plasmid in ST131, whereas *fosA3* prevalence was 38.58% among different transferable plasmids, including IncFIIK, IncFII and IncA/C, and in various STs including ST69, ST1193, ST12, ST46, ST57, ST1196, ST38, ST95, ST131, ST7584 and ST10184. Both were successfully transferred to EC600. The XbaI PFGE cluster exposed similarities among the STs.

Conclusions: Our results show that to control the spread of colistin and fosfomycin resistance genes in human pathogens, the ban on colistin must be continued in animal feeding farms not only in China but around the world; additionally, awareness platforms on the use of colistin must be implemented and strict policies in poultry and pig farms must be maintained. Furthermore, fosfomycin misuse by patients and overuse by physicians must be strictly managed to stop the spread of fosfomycin resistance.

Key words: *MCR-3; FOSA3*; PBRT; MLST; XBAL-PFGE.

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Introduction

Antimicrobial resistance (AMR) has become a major public health problem globally. The massive and inappropriate use of antimicrobial agents in agriculture and medicine is partially or entirely responsible for the increased spread of multi-drug resistance (MDR). Poisoning by MDR pathogens causes more than 70,000 human deaths in the United States (US) each year [1,2]. A group led by Professor Lord Jim O'Neil estimates that by 2050, MDR will cause 10 million deaths worldwide. While the accuracy of this alarming prediction is uncertain, it is recognised that AMR is a massive burden on multiple levels (economic, community, experimental and public health) [3], underlining the importance of a coordinated international response to prevent and control the global spread of AMR [4].

Colistin is a series of non-ribosomally synthesised cationic antimicrobial cyclic peptides (CAMPs) [5] that is widely used in agricultural and medical treatments [6,7]. It was previously thought that the main targets of colistin were negatively charged lipids, specifically, a fraction of lipopolysaccharides (LPS) in the outer leaflets of the outer membranes of bacteria [8]. Despite the risk of nephrotoxicity and neurotoxicity, colistin is still used for the treatment of severe infections caused by MDR pathogens (in particular, carbapenemase-producing *Enterobacteriaceae*) [9-12].

Some infectious bacteria that have developed resistance to colistin include *Klebsiella pneumoniae, Escherichia coli* and *Salmonella enterica*. The chemistry of colistin resistance consistently involves surface modification of bacterial lipid A-centric lipids, including 4-amino-4-deoxy-L-arabinose in *S. enterica*

and *Pseudomonas aeruginosa*, phosphoethanolamine (PEA) adhesion in *Neisseria gonorrhoeae*, *Acinetobacter baumannii* and *Campylobacter jejuni*, and glycine/diglycine alteration in the EI Tor biotype of pandemic *Vibrio cholera* [13-16].

Intrinsic polymyxin resistance is limited to the previously resistant population. The therapeutic utility of colistin as the antibiotic of last resort against carbapenem-resistant superbugs may be influenced by recent developments and the discovery of plasmid-mediated colistin resistance determinants (*mcr-1; mcr-10*) [17,18].

Currently, ten slightly different variants of the *mcr* gene (*mcr-1–mcr-10*) have been discovered in bacteria isolated from animals, food, farms, humans and the environment. As a result, the problem of *mcr* transmission is worsening by the day. The first *mcr-1* gene-carrying plasmid was identified in *E. coli*, *Aeromonas* and *Proteus* of animal and human origin in America, Europe and Asia. *mcr-3* shows 47% and 45% nucleotide sequence identity with *mcr-2* and *mcr-1*, respectively, and *mcr-4* has been identified in *Salmonella* from humans and pigs in Italy, Spain and Belgium. *mcr-5* has been reported in *S. enterica* in Germany*; mcr-6* has 87.9% identity with *mcr-2* and has been reported mainly in Europe; *mcr-7* and *mcr-8* have been reported in China, and both have been detected in *K. pneumoniae; mcr-9* has been detected in a human patient in the US; and *mcr-10* has recently been found in various *Enterobacteriaceae* in several countries [19].

Based on the epidemiological and geographical distribution of *mcr*, *mcr-3* appears to be the second most prevalent variant after *mcr-1*. Phylogenetic analysis shows that *mcr-3* and *mcr-1* are evolutionarily distinct. Currently, *mcr-3* has been found on three continents, i.e., Asia, North America and Europe. Bacterial infections in animals, including pigs, cows and goats, are treated with colistin in Europe, while in many Asian countries, such as China and Japan, colistin is used as a growth promoter, especially in pigs and poultry. Such indiscriminate use of antibiotics has led to the emergence of new colistin resistance variants like *mcr-3* [20].

Fosfomycin, a broad-spectrum antibiotic, entered clinical practice in 1996 and is extremely important in the treatment of humans. Fosfomycin inhibits bacterial cell wall synthesis and, as a reactive antibiotic against gram-negative and gram-positive bacteria, is commonly used to treat lower urinary tract infections. It functions well synergistically with cephalosporin, aminoglycosides and daptomycin. Recently, fosfomycin was proposed for the treatment of MDR bacterial infections. Three types of enzymes, *fosA*, *fosB* and *fosX*, which are primarily responsible for clinical resistance, are encoded both chromosomally and in plasmids. More than ten *fosA* genes have been discovered, of which *fosA3* is the most common variant. It is mainly horizontally distributed and predominantly found in Asia [21,22].

In our study, a total of 127 isolates of colistin- and fosfomycin-resistant strains were collected from the tertiary A hospital in Hefei, China. We performed various techniques for the genomic analysis of these antibiotic resistance genes, including antibiotic susceptibility testing, multilocus sequence typing (MLST) and polymerase chain reaction (PCR)-based replicon typing (PBRT), conjugation, and Xbal pulsed-field gel electrophoresis (Xbal-PFGE).

Methodology

Study design and sample collection and identification

To identify the spread of colistin resistance variants (*mcr-1–mcr-10*), especially in combination with fosfomycin resistance genes (*fosA1–fosA10*) in *E. coli*, we conducted a study at a major teaching hospital in Anhui, China. Bacterial isolates were collected between April 2018 and May 2019. A total of 127 samples, including urine (n = 39), sputum (n = 38), wound (n = 27) and blood (n = 23), were received from The First Affiliated Hospital of the University of Science and Technology of China (USTC). All the isolates were grown on MacConkey agar at 37 °C overnight, and the following day, single colonies were selected and grown on Luria-Bertani (LB) broth overnight for 12–16 hours. To precisely identify the bacteria, the 16s rDNA gene was extracted from the LB broth and sequenced. The sequencing statistics were then analysed using EzBioCloud (www. ezbiocloud.net) and BLAST (www.ncbi.nlm.nih.gov/blast).

Antibiotic resistance and detection of antibiotic resistance genes

The identity of all 127 *E. coli* isolates was confirmed by 16s rDNA sequencing. The boiling method was followed for the extraction of DNA from the *E. coli* strains [23]. The detected genes included the colistin resistance genes *mcr-1–mcr-10* and the fosfomycin resistance genes *fosA1–fosA10*. The tested antibiotics included cefotaxime, ceftriaxone, cefepime, meropenem, aztreonam, fosfomycin, amikacin, tigecycline and colistin. For the interpretation of the results and determination of breakpoint values, the breakpoint values of the European Committee on Antimicrobial Susceptibility Testing (EUCAST;

www.eucast.org) and the recommendations of the Clinical Laboratory Standards Institute (CLSI-2019) were used, following a previously described protocol [24]. The PCR products were sequenced by General Biosystems Co., Ltd. (Hefei, China).

Sequence typing

E. coli multilocus sequence types (STs) were identified using the Pasteur online database (https://bigsdb.pasteur.fr/Ecoli/ecoli.html). The seven housekeeping genes processed for the sequence identification of *E. coli* included *adk, icd, gyrB, fumC, mdh, purA* and *recA* (Table 1) [25].

Restriction enzyme analysis with pulsed-field gel electrophoresis (REA-PFGE)

The Xbal PFGE experimental procedure was carried out according to the PFGE protocol to identify genomic similarity [26]. Bionumerics V8.0 (Applied-Maths, Sint-Martens-Latem, Belgium) was used for the Xbal PFGE gel analysis, the dendrogram was produced based on the unweighted pair-group method with arithmetic mean (UPGMA), and the Dice similarity coefficient was used for the cluster investigation with 1.5% position tolerance.

Replicon typing

The plasmids of the fosfomycin and colistin resistance determinants and their incompatibility groups were identified using the PBRT Kit 2.0 (Diatheva, Italy). This PCR-based replicon typing kit was used to identify more than 29 different incompatible plasmid groups [18].

Transferability of resistance determinants

A conjugation experiment was performed to examine the transferability of the resistance genes of interest. *E. coli* strains bearing two *fosA3* genes and one *mcr-3* gene were randomly selected as donors, while EC-600 (Rif^R-Nal^R) was used as the recipient bacterial isolate. A previously described protocol was followed for the conjugation [18]. Finally, the results were confirmed by antibiotic susceptibility testing and plasmid characterisation using the PBRT Kit.

Statistical analysis

Bionumerics V8.0 (Applied-Maths, Sint-Martens-Latem, Belgium) was used for the chart construction and Xbal PFGE gel analysis, the dendrogram was produced based on UPGMA, and for the cluster investigation, the Dice similarity coefficient was calculated with 1.5% position tolerance.

Ethical approval

The study was approved (approval number 2020KY-191) by the ethical committee of The First Affiliated Hospital of the USTC.

Figure 1. Representing the resistance profile of the antibiotics tested in our study.

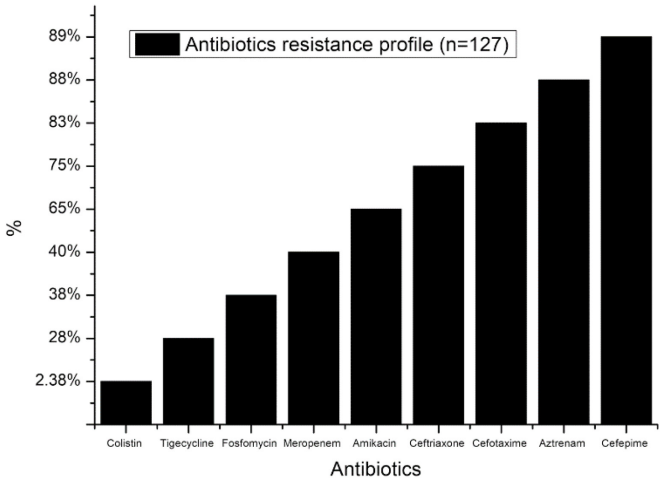


Table 1. *E.coli* MLST primers.

Primer name		DNA sequence (5′-3′)	Temp (°C)	Reference
adK	F	ATTCTGCTTGGCGCTCCGGG	54	[38]
	R	CCGTCAACTTTCGCGTATTT		
fumC	F	TCACAGGTCGCCAGCGCTTC	54	
	R	TCCCGGCAGATAAGCTGTGG		
gyrB	F	TCGGCGACACGGATGACGGC	60	
	R	GTCCATGTAGGCGTTCAGGG		
icd	F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	54	
	R	GGACGCAGCAGGATCTGTT		
mdh	F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	60	
	R	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT		
purA	F	TCGGTAACGGTGTTGTGCTG	54	
	R	CATACGGTAAGCCACGCAGA		
recA	F	CGCATTCGCTTTACCCTGACC	58	
	R	AGCGTGAAGGTAAAACCTGTG		

Results

Antibiotic resistance profile and screening for fosfomycin and colistin resistance determinants

The *E. coli* strains in this study were collected from different units of the hospital, including the intensive care unit, gerontology, paediatrics, oncology and urinary surgery. A total of 127 *E. coli* isolates were confirmed by 16S rDNA sequencing, and all the strains were highly resistant to the tested antibiotics, with 65% being resistant to amikacin, a high proportion of 88% being resistant to aztreonam, and 60% and 72% being sensitive to meropenem and tigecycline, respectively. The resistance profile is shown in Figure 1. The genes encoding resistance to fosfomycin were assessed to identify *fosA* genes, and 49 out of the 127 *E. coli* strains displayed resistance to fosfomycin with a minimum inhibitory concentration of ≥ 256 µg/mL. The prevalence of fosfomycin resistance noted was 38.58%. The strains showing resistance to colistin with a minimum inhibitory concentration of ≥ 4 µg/mL were processed further to identify *mcr* genes. Of the 127 *E. coli* isolates, only one strain was resistant to colistin (*mcr-3* detected in 0.78%). No other *fosA* genes and *mcr* genes were detected in this study. The primers used for *mcr-3* and *fosA-3* detection are described in Table 2.

Sequence types

The MLST results for the 127 *E. coli* isolates revealed 11 different STs. Of these, ST1196 (14.17%) was the most prevalent ST observed. Other STs detected in our study included ST69 (13.38%), followed by ST57 (12.59%), ST12 (11.81%), ST1193 and ST38 (both 10.23%), ST46 (9.44%), ST95 (7.08%), ST131 (5.51%), and ST10184 (0.78%). In addition, *mcr-3* was detected in ST131, while *fosA3* was detected in different STs (Figure 2).

Xbal PFGE

The Xbal PFGE technique was used for the molecular typing of the *E. coli* isolates. Bionumerics V8.0 was used for the cluster analysis of the Xbal PFGE gel; after the isolates were successfully digested using the Xbal restriction endonuclease, the cluster was exposed, as shown in Figure 3, representing the dissimilarity among the STs of Xbal PFGE.

Figure 2. MLST result of E. coli isolates (n=127), the sequence types identified in our report are presented, and the minimum spanning tree constructed using the genomic sequences of these identified ST's and MLST alleles, while the chart was constructed using the Bionumerics software volume 8.0. ST1196 representing the highest occurrence, while ST10184 was representing the lowest occurrence. The nodes represent the STs, the diameters of the nodes represent the number of isolates, and the length of the branches represents the number of distinct alleles among the seven MLST alleles. The corresponding sequence types are labeled on the nodes.

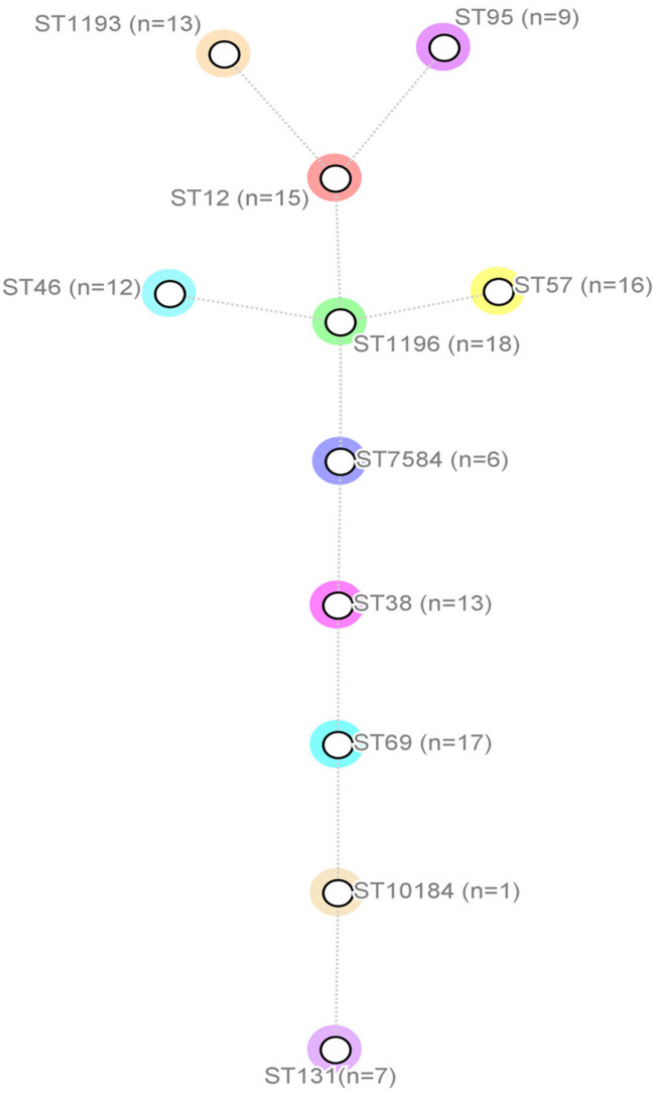


Table 2. Representing the primers of *fosA3* and *mcr-3*.

Gene	Primer 5′-3′	Annealing temperature	Replicon size	Reference
fosA3 F	GCGTCAAGCCTGGCATTT	56º	282	[39]
fosA3R	GCCGTCAGGGTCGAGAAA			
Mcr-3F	AAATAAAAATTGTTCCGCTTATG	58º	929	[40]
Mcr-3R	AATGGAGATCCCCGTTTTT			

Replicon types

Four different plasmid replicons were detected among the 127 *E. coli* isolates. *E. coli* carried the *fosA3* gene on IncFIIk, IncFII and IncA/C and *mcr-3* combined with *fosA3* in an IncP-type plasmid. The results are detailed in Table 3.

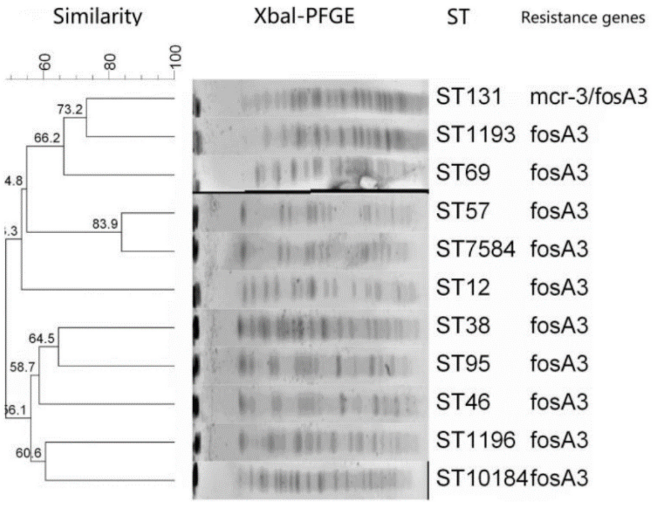
Conjugation

The conjugation experiment was performed to detect the transferability of the plasmids carrying *fosA3* and *mcr-3* resistance determinants. The plasmids of interest were successfully trans-conjugated to EC600 (Rif^R-Nal^R), and the resulting trans-conjugants were checked using PCR-based replicon typing. The results are described in Table 3. To further confirm the accuracy of the transferability, *fosA3*- and *mcr-3*-specific plasmid PCR was performed and the determinants were observed in the trans-conjugants.

Discussion

mcr-3 and its genomic variants have been detected globally since the gene was first discovered in Shandong province in China. In this study, we identified *mcr*-3 in combination with the *fosA3* gene in *E. coli* samples from The First Affiliated Hospital of the USTC. It suggested that the occurrence of the colistin resistance gene *mcr*-3 in Anhui province is under control. This may be a symptom of a wider decline in *mcr* prevalence, which may be partly due to restrictions on the use of colistin in Chinese livestock farms and improved husbandry practices. However, further research is needed. Previously published data indicate a high prevalence of animal *mcr-3* (> 9.5%) in other provinces of China. However, the prevalence of *mcr-3* in our study was relatively low [27]. The low prevalence of *mcr*-3 in our study (Table 3) may be due to sampling times or parameters of colistin resistance different from those in other studies. *mcr-3* was detected both alone and in combination with the fosfomycin resistance gene *fosA3* in our research. PCR-based replicon typing (Table 3) confirmed that the *mcr-3* gene in our study was present in a transferable IncP-type plasmid. Isolated *mcr-3* was processed to detect IS1294 inversion sequences; however, none were

Figure 3. Xbal-PFGE result of *E.coli* producing *fosA3* and *mcr-3* resistant determinants. Bionumerics Volume 8.0 was used to create the Dendrogram for structure analysis, and the ST represents each isolate's sequence type.



detected in our study. Several studies have suggested that *mcr-3* is structurally different from other colistin resistance determinants; this may be why other genes, such as *mcr*, did not show combinations in our research. Recently, several published articles reported the spread of *mcr*-3 in environmental samples and hospital wastewater [28]. Similar to *mcr*-1, *mcr*-3 has been reported several times as an extended-spectrum beta-lactamase (ESBL) and metallo-β-lactamase (MBL), especially for $bla_{\text{ctxm-15}}$ and $bla_{\text{ctxm-55}}$ in Asian countries, while $bla_{\text{ndm-1}}$, $bla_{\text{ndm-5}}$ and $bla_{\text{kpc-2}}$ have been reported worldwide [29]. To our knowledge, the co-occurrence of *mcr-3* and *fosA3* was first noted in our clinical isolation report. A Spanish study [30] reported on the epidemiological characterisation of *fosA3,* showing a prevalence of 16.30% across seven different hospitals in Madrid; the STs responsible for *fosA3* (ST69 and ST4038) among the 55 samples examined were also reported. As we tested more samples (n = 127), *fosA3* prevalence was approximately 38.58% (Figure 1) among the different STs in our study, including ST69, which supports the accuracy of our work. *fosA3* has also been previously identified [31] among different transferable plasmids with sizes between 40 and 60 kb. Since the discovery of *mcr-1*, other *mcr*-like genes have

Table 3. Plasmid replicons and their respective resistant determinants, along with the results of conjugation.

Samples	Plasmid/PBRT	Trans-conjugants	Strain	Resistant determinant
S01	IncFIIk/148bp	+	*E.coli*	*fosA3*
S02	IncFII/288bp	+	*E.coli*	*fosA3*
S03	IncA/C/418bp	+	*E.coli*	*fosA3*
S04	IncA/C/418bp	+	*E.coli*	*fosA3*
S05	IncFII/288bp	+	*E.coli*	*fosA3*
S06	IncP/345bp	+	*E.coli*	*mcr-3/fosA3*

been reported globally, mainly pig-derived *mcr-3* and chicken-derived *mcr-7*. *mcr-3* and *mcr-7* are reported to have very similar structures and probably originate from *Aeromonas* species in aquatic environments, supporting the detection of *mcr-3* in environmental and hospital-derived fluids. *mcr-3* and *mcr-1* have been detected globally, mainly in Spain and New Zealand, and have different origins including food, humans, the environment and animals [32-34].

An article published in 2018 reported on the spread of *mcr-3* in China; 0.75% of the tested samples were *mcr-3*-positive; overall, eight positive human and animal samples were identified among the 13 different provinces of China [35]. The prevalence of *mcr-3* (0.78%) was slightly in our study, possibly due to study and sample load variation. The study also reported the prevalence of *mcr-3* in the IncP-type transferable plasmid (Table 3), thus strengthening our study as we also report the duplicate transferable plasmid accounts for the spread of *mcr-3* in Anhui province. On the other hand, the co-occurrence of *fosA3* and *mcr-3* have not been published before.

The genomic characterisation of *mcr-3* revealed its location in many plasmids, usually with sizes over 200 kb, and was reported in a study published by the American Society of Microbiology that detected it in a 261-kb $IncHI_2$-type plasmid [36]. In our investigation, the ST analysis identified ST131 (Figure 2) as responsible for *mcr-3* presence in *E. coli*; other studies have suggested different STs for *mcr-3* dissemination. Furthermore, a swine sample analysis from Vietnam revealed that the spread of *mcr-3* occurred via ST69 and ST1081. The differences between the two regions may have caused the observed variation in STs [37].

Conclusions

In conclusion, to curb the spread of colistin resistance genes among human pathogens, it is necessary to continue the ban on the use of colistin in livestock farms, not only in China but worldwide, maintain vigilance platforms on colistin use, and implement strict policies on poultry and pig farms. *mcr-3* in combination with other resistance determinants or *fosA3* poses an extreme risk; therefore, the excessive and inappropriate use of antibacterial agents should be monitored and addressed to combat such problems in humans in the future, and alternative therapies need to be explored for the management of infections, especially in animal farms and the hospital setting.

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Authors' contributions

MFH, CXF: study design and proposal; MFH, HGE: acquisition of data; MFH, HB: analysis and interpretation of data; MFH, SK, HGU: drafting of the article; MFH, YC: significant modification of the manuscript for intellectual content; CXF, HGE: study supervision.

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