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ORIGINAL RESEARCH

Genetic Characterization of *Enterobacter hormaechei* Co-Harboring bla_{NDM-1} and *mcr-9* Causing Upper Respiratory Tract Infection

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Purpose: With the spread of multiple drug-resistant bacteria, bla_{NDM-1} and *mcr-9* have been detected in various bacteria worldwide. However, the simultaneous detection of bla_{NDM-1} and *mcr-9* in *Enterobacter hormaechei* has been rarely reported. This study identified an *E. hormaechei* strain carrying both bla_{NDM-1} and *mcr-9*. We investigated the genetic characteristics of these two resistance genes in detail, elucidating various potential mechanisms by which they may be transmitted.

Methods: Bacterial genomic features and possible origins were assessed by whole-genome sequencing (WGS) with Illumina and PacBio platforms and phylogenetic analysis. Subsequent investigations were performed, including antimicrobial susceptibility testing and multilocus sequence typing (MLST).

Results: We isolated an *E. hormaechei* strain DY1901 carrying both bla_{NDM-1} and *mcr-9* from the sputum sample. Susceptibility testing showed that the isolate was multidrug-resistant. Multiple antibiotic resistance genes and virulence genes are widely distributed in DY1901. S1-PFGE, Southern blotting, and plasmid replicon typing showed that DY1901 carried four plasmids. The plasmid carrying *mcr-9* was 259Kb in size and belonged to IncHI2, while the plasmid carrying bla_{NDM-1} was 45Kb in length and belonged to IncX3.

Conclusion: The *E. hormaechei* strain isolated in this study has a broad antibiotic resistance spectrum, posing a challenge to clinical treatment. Plasmids carrying *mcr-9* are fusion plasmids, and those taking NDM are widely disseminated in China, suggesting that we should conduct routine genomic surveillance on such plasmids to curb the spread of drug-resistant bacteria in the region.

Keywords: *E. hormaechei*, New Delhi metallo-β-lactamase, *mcr-9*, whole-genome sequencing, phylogenetic analysis

Introduction

Enterobacter cloacae complex (ECC) comprises the following species: *Enterobacter cloacae, Enterobacter hormaechei, Enterobacter asburiae, Enterobacter kobei Enterobacter ludwigii, Enterobacter nimipressuralis, Enterobacter mori*, etc. ECC is a critical member of Enterobacteriaceae widely encountered in the environment.[1,2] As an opportunistic pathogen, ECC is ubiquitous not only in nature but also in clinical settings and has been associated with various infections, such as bacteremia, respiratory tract infections, wound infections, and urinary tract infections.[3,4]

The prevalence of carbapenemase-resistant Enterobacteriaceae (CRE) has risen since the 2000s.[5] New Delhi Metallo-β-lactamase (NDM) is a type of Metallo-β-lactamase (MBL) able to hydrolyze most β-lactams (including carbapenems).[6,7] Colistin is an antibiotic often referred to as a "last resort" to treat CRE infections.[8] The identification of the first mobile colistin resistance (MCR) gene, *mcr-1*, in 2015 triggered a rash of *mcr* screening reports.[9] Subsequently, ten MCR-family genes and their variants have been described.[10] Among the *mcr*-like genes, *mcr-1* and *mcr-9* are the most widespread. The *mcr-9* gene has been found in 40 countries on six continents.[11]

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In recent years, *mcr* family have continued to emerge in CRE and spread widely among pathogens of animal and human origin,[12] further increasing the public health burden of antimicrobial resistance.[13] This study isolated a strain of *E. hormaechei* carrying both $bla_{\text{NDM-1}}$ and *mcr-9* from the sputum of a patient with an upper respiratory tract infection. Importantly, these antibiotic resistance genes were located on different plasmids, signaling the potential spread of pan-resistant bacteria. However, the genetic characterization of plasmids and bacteria encoding NDM-1 and *mcr-9* remains unclear. In this study, we identified an *E. hormaechei* strain carrying both $bla_{\text{NDM-1}}$ and *mcr-9*. We investigated the genetic characteristics of these two resistance genes in detail, elucidating various potential mechanisms by which they may be transmitted.

Materials and Methods

Sample Collection

An *E. hormaechei* strain DY1901 carrying both $bla_{\text{NDM-1}}$ and *mcr-9* was isolated from the sputum of an inpatient in a tertiary hospital in China. A 72-year-old patient was admitted to hospital with left heart failure. The collected sputum was smeared with Mueller-Hinton Agar (OXOID, UK) medium and cultured by suctioning the sputum with a sterile suction device. Bacterial species were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker, Bremen, Germany). In addition, High throughput ANI analysis is used to compare the whole genome sequencing results to reveal distinct species of ECC (Figure S1). As previously described, the isolates were further subjected to PCR to detect the *mcr* and carbapenemase genes (Table S1).[14]

Antibiotic Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by VITEK 2 system.[15] The resistance results of tigecycline and colistin were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST v12.0) guidelines. Susceptibilities against other drugs were determined by the criteria of the Clinical and Laboratory Standards Institute (CLSI v31). All methods were carried out under relevant guidelines and regulations.

Conjugation Assay and Plasmid Characterization

Conjugation experiments used *E. coli* J53 and *E. coli* EC600 as recipient strains. The resulting transformants were selected on BHI agar plates amended with meropenem (two mg/L). PCR and sequencing also screened the presence of $bla_{\text{NDM-1}}$ and *mcr-9*. Plasmid sizes were determined using the S1-nuclease PFGE (S1-PFGE) method.[16] According to the manufacturer's instructions, southern blot hybridizations of plasmid DNA were performed on isolates using DIG-labelled probes (Roche Diagnostics, Germany).

Whole-Genome Sequencing

Whole-genome sequencing (WGS) was performed by Novo Gene Co., Ltd, Beijing, China. Genomic DNA was extracted using a DNA kit (Omega Bio-tek, Norcross, USA). The DNA was subsequently sequenced using Illumina-HiSeq 4000-PE150 (Illumina, San Diego, CA, USA) and PacBio *RS* II platform (Pacific Biosciences, California, USA). Alignment of anti-microbial resistance genes was performed through the ResFinder platform (https://cge.cbs.dtu.dk/services/ResFinder/). Using the website, multilocus sequence typing (MLST) was performed on all isolates (https://cge.cbs.dtu.dk/services/MLST/). The MLST of DY1901 was determined by aligning the housekeeping genes *dnaA, fusA, gyrB, leuS, pyrG, rplB* and *rpoB*. Virulence genes were identified by BLASTN against the VFDB database (http://www.mgc.ac.cn/VFs/main.htm).

Phylogenetic Reconstruction and Analysis

Complete *E. hormaechei* genomes were downloaded from the National Center for Biotechnology Information (NCBI) for phylogenetic analysis. Snippy (rapid haploid variant calling and core genome alignment) was used to compare genomic differences between strains. The alignment file was filtered from variants with elevated densities of base substitutions as putative recombination events by Gubbins version 2.4.1.[17] The filtered core-genome alignment file was used to construct a maximum likelihood tree using FastTree with the GTR+CAT model.

Table 1 Antibiotic Susceptibility Profiles of *Enterobacter hormaechei* Isolate DY1901

Isolate	MIC (mg/L)[a]										
	CO	TZP	CAZ	CRO	CPM	MEM	IPM	AMK	LVX	TGC	SXT
DY1901	0.5(S)	128(R)	64(R)	64(R)	32(R)	16(R)	16(R)	16(S)	8(R)	2(S)	320(R)

Notes: [a]MICs were determined by VITEK 2 system with AST-GN16 panel. Resistance is indicated in bold.
Abbreviations: CO, Colistin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CRO, ceftriaxone; CPM, cefepime; MEM, meropenem; IPM, imipenem; AMK, amikacin; LVX, levofloxacin; TGC, tigecycline, SXT, trimethoprim/sulfamethoxazole.

Results

Antibiotic Resistance Signature of *E. hormaechei*

MALDI-TOF/MS and ANI analysis showed that DY1901 belonged to *E. hormaechei* (Figure S1), and then MIC was determined for *E. hormaechei*. The results of the MIC are shown in Table 1. DY1901 exhibited resistance to piperacillin/tazobactam, ceftazidime, ceftriaxone, cefepime, ertapenem, imipenem, levofloxacin, tetracycline, trimethoprim/sulfamethoxazole, but still susceptible to colistin, amikacin, and tigecycline. DY1901 has a broad drug resistance spectrum and is a multidrug-resistant bacterium.

Antimicrobial Resistance Genes

We provide data on antimicrobial resistance genes in Figure 1. DY1901 carries carbapenem resistance gene $bla_{\text{NDM-1}}$ and colistin resistance gene *mcr-9*. In addition, DY1901 also carries β-lactams, sulfonamides, aminoglycosides, tetracyclines, and other antibiotic resistance genes (Figure 1). We also compared the resistance gene profiles from 30 *E. hormaechei* strains downloaded from NCBI. The results showed that the antibiotic resistance gene carried by 30 *E. hormaechei* strains was dominated by β-lactam resistance genes (93.6%), followed by quinolone resistance genes (Figure 1).

Virulence Genes

The results of the alignment of virulence genes are shown in Figure 1. The virulence genes carried by DY1901 are related to acriflavine resistance B (AcrB), type VI secretion system protein (T6SS), and transcriptional regulator RcsB. The 30 *E. hormaechei* strains downloaded from NCBI mainly encoded AcrB.

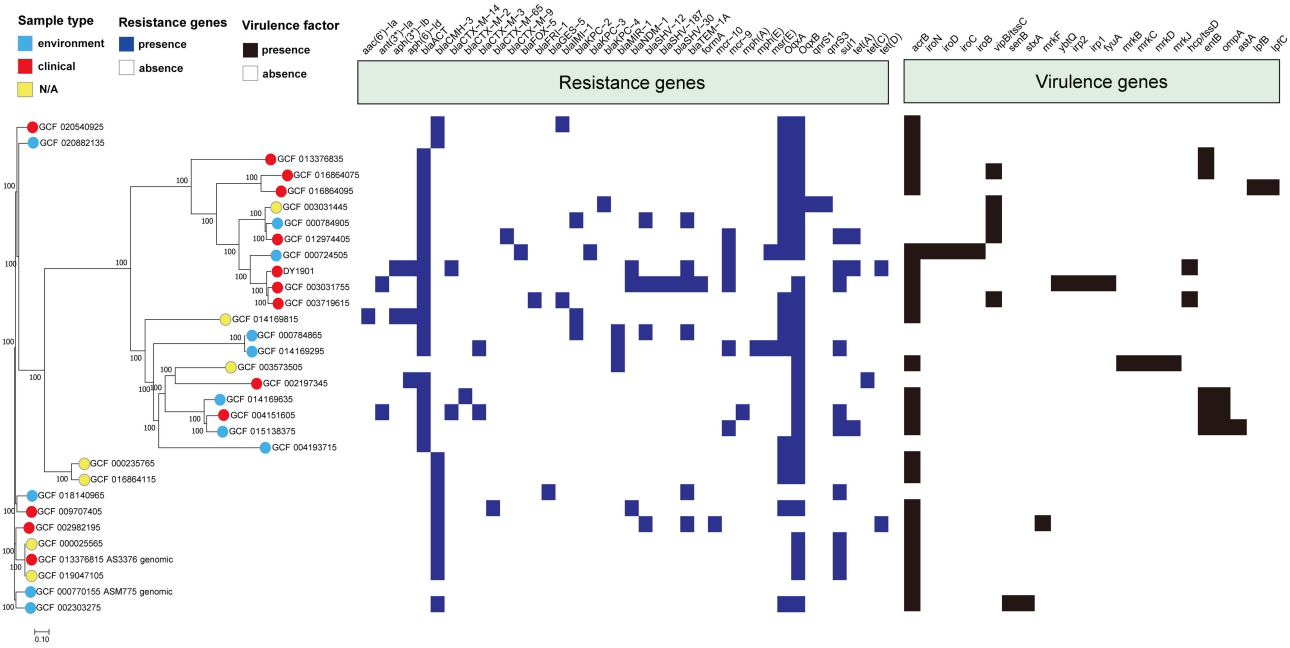

Figure 1 Construction of phylogenetic trees of *E. hormaechei*. The figure includes the sample source of the isolate, antibiotic resistance gene, and the comparison result of the virulence gene. Red dots denote pathogens of clinical origin, blue dots denote pathogens of environmental origin, and yellow dots denote unknown pathogen sources. The aligned antibiotic resistance and virulence genes are indicated by blue and black squares.

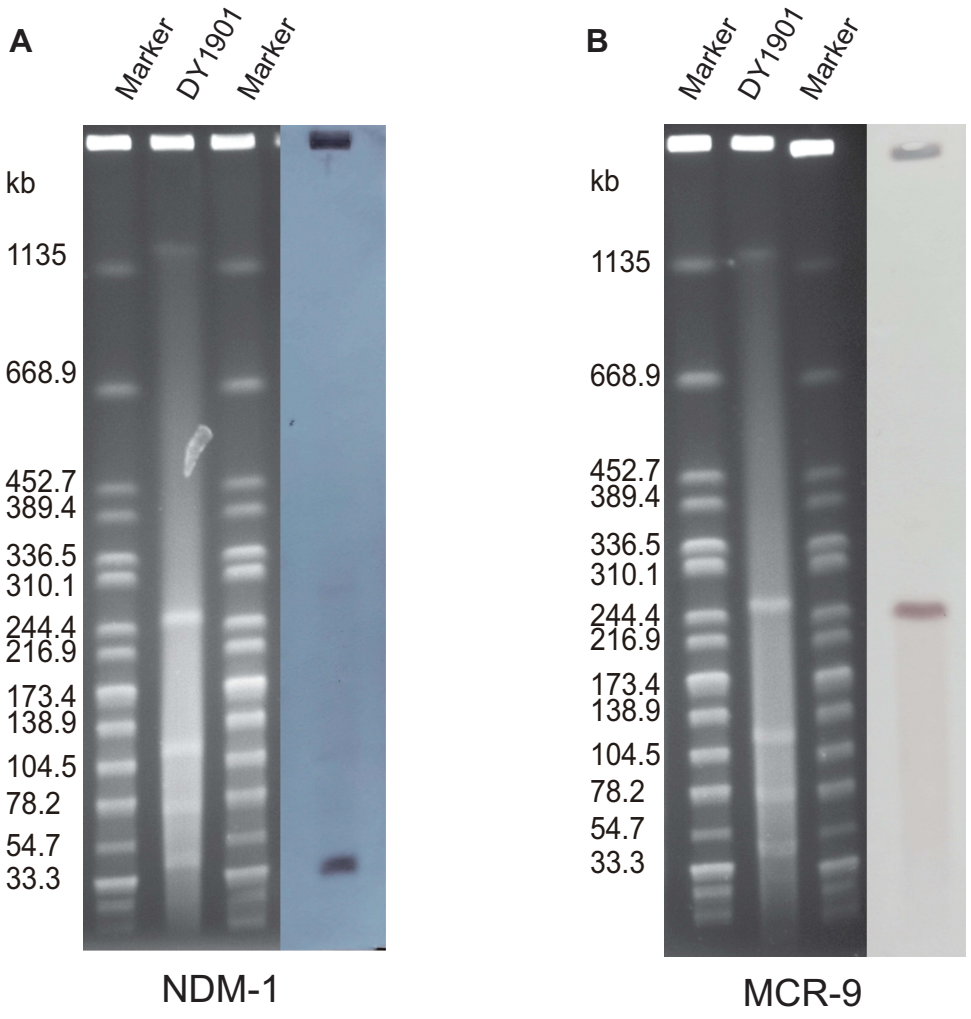

Figure 2 S1 Nuclease-Pulsed Field Gel Electrophoresis (S1-PFGE) and southern blot. The S1-PFGE characterized the number and size of the plasmids of the two isolates. The location of bla_{NDM-1} and *mcr-9* was confirmed by Southern blot. (**A**) is the location and size of bla_{NDM} and (**B**) is the location and size of *mcr-9*.

Molecular Characteristics of *E. hormaechei*

Detailed genomic analysis of *E. hormaechei* indicated that it belonged to ST418. The results of S1-PFGE, Southern blotting, and plasmid replicon typing showed that DY1901 carried four plasmids (Figure 2), the plasmid carrying *mcr-9* was 259Kb in size and belonged to IncHI2, while the plasmid harboring bla_{NDM-1} was 45Kb in length and belonged to IncX3 (Figure 3). After annotating the genes on the plasmid, we found that the *mcr-9* gene is located on the *IS*1R transposon element of pDY1901-mcr. Phylogenetic analysis of all 31 strains showed that DY1901 is closely related to GCF_003031755 and GCF_003339765 from the United States.

Discussion

The bacterial natural product colistin is considered the last line of defense against many Gram-negative pathogens,[18] but with the spread of multiple drug-resistant bacteria, bla_{NDM-1} and *mcr-9* have been detected in various bacteria worldwide.[19–21] However, few reports have performed a detailed genomic analysis of *E. hormaechei* with bla_{NDM-1} and *mcr-9*.[22–24] In this study, an *E. hormaechei* strain carrying both bla_{NDM-1} and *mcr-9* was isolated from a patient. The isolate was characterized by genome and phylogenetic analysis using whole-genome sequencing technology.

By comparison, we found that pDY1901-mcr belonged to the IncHI2/IncHI2A plasmid, and no matching plasmid was found by NCBI, indicating that this plasmid may be a new hybrid plasmid. After annotating the genes on the plasmids,

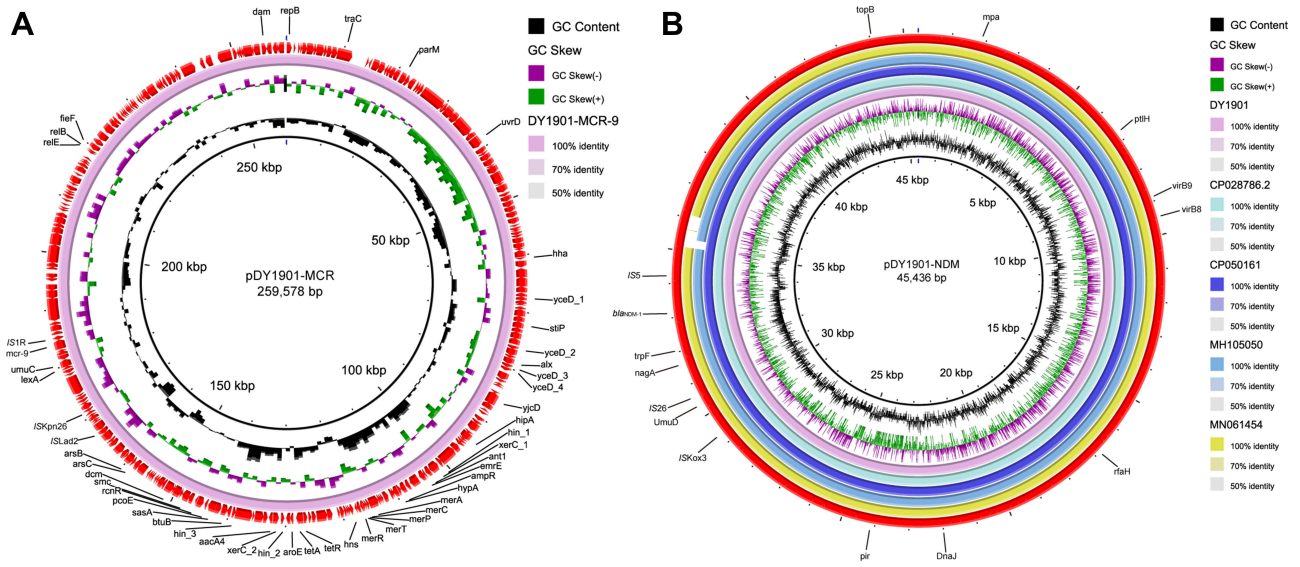

Figure 3 Plasmid profiles of bla_{NDM-1} and *mcr*-9 carrying plasmid from *E. hormaechei* isolate. Sequence comparison of scaffolds (portions of genome sequences reconstructed from WGS data). Arrows indicate the positions and direction of the transcription of genes. The reference plasmid is marked in pink in the inner circle. The color intensity in each ring represents the BLAST match identity. (**A**) is the plasmid construct carrying *mcr-9* and (**B**) is the plasmid construct carrying bla_{NDM}.

we performed a genetic environment analysis of *mcr-9*. It was found that the *mcr-9* gene is located on the *IS*1R transposon element in pDY1901-mcr. IS*1R* transposon is relatively common in *Klebsiella pneumoniae* and *Escherichia coli* and is an essential reason for the spread of drug-resistant bacteria.[25,26] In addition, we found that pDY1901-NDM belongs to the IncX3 plasmid and matched with the NDM-producing *K. pneumoniae* plasmid pCP028786.2, *E. coli* pCP050161, *Salmonella* pMH105050.1, ECC pMN061454.1. It is worth noting that these plasmids were all isolated from China, indicating that such plasmids have spread widely in China. After annotating the genes on the plasmids, we performed a genetic environment analysis of the NDM. The NDM gene was located on the IS*5* transposon element in pDY1901-NDM, and the IS*5* transposon significantly contributed significantly to the plasmid's spread[27] (Figure 3).

Bacterial resistance studies showed that DY1901 was resistant to piperacillin/tazobactam, ceftazidime, ceftriaxone, cefepime, ertapenem, imipenem, levofloxacin, tetracycline, trimethoprim/sulfamethoxazole. However, it was sensitive to colistin, amikacin, and tigecycline.[28] The full expression of wild-type *mcr-9* requires additional factors or inducing/derepression conditions, and promoter variation also affects *mcr-9* expression, which may explain why the *E. hormaechei* queried here is sensitive to colistin under the tested conditions.[29] A previous study showed that 48 *E. hormaechei* isolates were collected from a hospital in China from 2000 to 2018. The survey carried out MIC determination on 10 *E. hormaechei* isolates, and the results showed that all 10 *E. hormaechei* isolates were MDR. Similar to this study, *E. hormaechei* was only sensitive to colistin, tigecycline and some carbapenems. In response to the MIC results of DY1901, the patient was treated with tigecycline and *E. hormaechei* was not isolated from the patient's sputum.

As the pathogen continued to be isolated, its resistance spectrum has changed, most notably due to genes encoding β-lactamase, leading to increased resistance to β-lactam antimicrobials.[30] Antibiotic resistance genes can make antibiotics ineffective by changing the antibiotic molecular structure and antibiotic target protein.[31] DY1901 in this study was resistant to ertapenem and imipenem. However, whole-genome sequencing showed that DY1901 carried bla_{NDM-1}, indicating that the genotype was consistent with the bacterial resistance phenotype. In addition, the strain also carried antibiotic resistance genes such as β-lactams, sulfonamides, aminoglycosides, and tetracyclines and had multidrug-resistant phenotypes. Compared with the 30 isolates of *E. hormaechei* downloaded from NCBI, DY1901 lacks quinolone resistance genes, but DY1901 shows resistance to levofloxacin, which may be due to other resistance mechanisms of the pathogen.

Existing reports on *E. hormaechei* indicate that many *E. hormaechei* has a characteristic of strong drug resistance but weak virulence.[32] The virulence genes carried by DY1901 in this study included AcrB, T6SS, and RcsB. T6SS belongs

to the bacterial secretion system and is associated with bacterial colonization, and can help bacteria to transfer effector proteins into host cells or the extracellular matrix for a variety of purposes, including bacterial persistence, adhesion, virulence, invasion, biofilms Formation, and inter-bacterial competition.[33,34] AcrB belongs to the multidrug efflux systems of bacteria, and bacteria are intrinsically resistant to cytotoxic substances through their network of outer membranes and multidrug efflux systems, acting synergistically, thereby reducing the impact of antibiotics on it.[35,36]

Phylogenetic analysis of DY1901 and the complete genomes of *E. hormaechei* in NCBI showed that *E. hormaechei* mainly had three clones, and DY1901 was located in the second clone (Figure 1). From the evolutionary tree, it can be found that DY1901, GCF_003031755, and GCF_003339765 have the closest relationship, so we speculate that DY1901 may have evolved from the strain in the United States. This result reflects the global nature of bacterial resistance from another perspective, which requires extensive attention from worldwide.

Conclusions

We report the detection of an *E. hormaechei* strain carrying both $bla_{\text{NDM-1}}$ and *mcr-9*. This study demonstrated the genetic characterization of plasmids carrying $bla_{\text{NDM-1}}$ and *mcr-9* by whole-genome sequencing. The *E. hormaechei* strain isolated in this study has a broad drug resistance spectrum, posing a challenge to clinical treatment. Plasmids carrying *mcr-9* are fusion plasmids, and those carrying NDM are widely disseminated in China. This suggests that we should conduct routine genomic surveillance on such plasmids to effectively curb the spread of drug-resistant bacteria in the region.

Data Sharing Statement

The whole-genome sequences of the *E. hormaechei* were submitted to GenBank under the following BioProject numbers: PRJNA808678.

Ethical Statement

Written informed consents were obtained from patients. This study was conducted following the Declaration of Helsinki and obtained approval from the Medical Ethics Committee at The First Affiliated Hospital of Zhengzhou University. The ethics permit number is KY-2022-0379.

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Disclosure

The authors report no conflicts of interest in this work.

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