Infection and Drug Resistance

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ORIGINAL RESEARCH

Emergence of an Extensive Drug Resistant *Citrobacter portucalensis* Clinical Strain Harboring bla_{SFO-1}, bla_{KPC-2}, and bla_{NDM-1}

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Background: To explore the plasmid characteristics and transfer mechanisms of an extensive drug resistant (XDR) clinical isolate, *Citrobacter portucalensis* L2724hy, co-producing bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2}.

Methods: Species confirmation of L2724hy was achieved through 16S rRNA sequencing and Average Nucleotide Identity (ANI) analysis. Antimicrobial susceptibility testing (AST) employed the agar dilution and micro broth dilution methods. Identification of resistance genes was carried out by PCR and whole-genome sequencing (WGS). Essential resistance gene locations were verified by S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and southern hybridization experiments. Subsequent WGS data analysis delved into drug resistance genes and plasmids.

Results: The confirmation of the strain L2724hy as an extensive drug-resistant Citrobacter portucalensis, resistant to almost all antibiotics tested except polymyxin B and tigecycline, was achieved through 16S rRNA sequencing, ANI analysis and AST results. WGS and subsequent analysis revealed L2724hy carrying bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2} on plasmids of various sizes. The uncommon ESBL gene bla_{SFO-1} coexists with the *fosA3* gene on an IncFII plasmid, featuring the genetic environment IS*26*-*fosA3*-IS*26*-*ampR*-bla_{SFO-1}-IS*26*. The bla_{NDM-1} was found on an IncX3 plasmid, coexisting with bla_{SHV-12}, displaying the sequence IS*5*-IS*3000*-IS*3000*-Tn*2*-bla_{NDM-1}-*ble-trpF-dsbD-cutA-gros-groL*, lacking IS*Aa125*. The bla_{KPC-2} is located on an unclassified plasmid, exhibiting the sequence Tn*2*-*tnpR*-IS*Kpn27*-bla_{KPC-2}-IS*Kpn6*-*korC*. Conjugation assays confirmed the transferability of both bla_{NDM-1} and bla_{KPC-2}.

Conclusion: We discovered the coexistence of bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2} in *C. portucalensis* for the first time, delving into plasmid characteristics and transfer mechanisms. Our finding highlights the importance of vigilant monitoring of drug-resistance genes and insertion elements in uncommon strains.

Keywords: *Citrobacter spp*, XDR, bla_{SFO}, bla_{NDM}, bla_{KPC}, IncFII

Introduction

In the vast microbial landscape, the genus *Citrobacter* stands as a diverse group of bacteria known for its adaptability and ecological ubiquity. Within this genus, the relatively lesser-explored *C. portucalensis* has garnered attention for its potential contributions to various fields, especially in human health. Since its first report in 2017, diverse occurrences of *C. portucalensis* in wastewater, sputum, stool, sludge, poultry and, sea turtles across multiple countries have been discovered.[1–6] In 2021, Chinese researcher Cao et al isolated *C. portucalensis* carrying bla_{NDM-1} from clinical specimens for the first time.[2] Subsequently, in 2022, the coexistence of bla_{KPC-2} and bla_{NDM-1} was reported in an extensively drug-resistant *C. portucalensis* obtained from a hospital in China.[7] Additionally, in 2023,

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researchers isolated *C. portucalensis* carrying bla_{NDM-1} from an endangered marine animal that died from sepsis.[8] This growing body of evidence underscores the increasing clinical and extra-clinical dissemination of drug-resistant *C. portucalensis*, warranting heightened attention to address the associated public health concerns.

In 2017, the World Health Organization (WHO) designated carbapenem-resistant and extended spectrum beta-lactamases (ESBLs)-producing *Enterobacteriaceae* as "extremely important" on its list of antimicrobial resistant "priority pathogens". Numerous antimicrobial resistance genes (ARGs) encoding carbapenemases and ESBLs were discovered. Among these, the bla_{SFO-1}, an uncommon ESBLs gene exhibiting the ability to hydrolyze beta-lactams excluding cephamycins and carbapenems, was first identified on a self-transferring plasmid from *Enterobacter cloacae* isolated from Japan in 1999.[9] The bla_{SFO-1} later manifested in an outbreak of *Enterobacter cloacae* in Spain.[10] Recently, its emergence has been documented in *Enterobacter hormaechei, Escherichia coli and Klebsiella pneumoniae* isolated from China.[11–13] Further investigations are crucial to the understanding of the epidemiology and clinical impact of bla_{SFO-1}. Among the carbapenemase encoding genes, bla_{KPC} stands out as the predominant in *Enterobacteriaceae* bacteria globally.[14] In China, the epidemic of bla_{KPC-2}-carrying bacteria strains is of current concern.[15] Besides bla_{KPC}, the rapid global spread of bla_{NDM} since its discovery in 2008 has posed significant public health challenges as well.

In our study, we identified an extensively drug-resistant clinical isolate of *C. portucalensis* featuring a rare coexistence of bla_{SFO-1}, bla_{KPC-2}, and bla_{NDM-1} genes. This report makes the first documented instance of bla_{SFO-1} in *C. portucalensis*. Additionally, we investigated the plasmid characteristics and transfer mechanisms associated with drug-resistant genes in this particular strain.

Materials and Methods

Strain Isolation and Identification

In September 2020, we obtained a clinical isolate (L2724hy) from the faeces of a 56-year-old outpatient male patient with acute diarrhea at the First Affiliated Hospital of Zhejiang University. Initially, this isolate was mistakenly identified as *C. freundii* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).[16] To confirm its accurate species, we uploaded the 16s rRNA sequence of this strain to BioEZCloud (https://www.ezbiocloud.net/) and analyzed ANI, and the result indicated it to be *C. portucalensis*.[17,18]

Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of L2724hy, transconjugant L2724hy-KPC-EC600, transconjugant L2724hy-NDM-EC600 and *E. coli* EC600 were determined by agar dilution method and micro-broth dilution method.[19] AST results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) 2023 (https://clsi.org), while the breakpoints for tigecycline and colistin were interpreted based on EUCAST (https://www.eucast.org/).

S1-PFGE-Southern Hybridization and Conjugation Assays

S1-PFGE was used to identify the size and number of plasmids. The location of the bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2} were determined by S1-PFGE and hybridization experiments. Conjugation assays were conducted using *E. coli* EC600 as recipient and L2724hy as donor.[20] Subsequently, individual colonies of donor and recipient bacteria were carefully selected and suspended LB liquid culture medium at 37 °C for 6 hours. Afterwards, the two suspensions were mixed in a 1:1 ratio (donor: recipient), introduced into 3 mL LB liquid medium, and incubated at 37 °C for 20 hours. An appropriate amount of bacterial solution was spread onto Mueller–Hinton agar (MHA) plates containing rifampicin (200 ug/mL) and meropenem (2 ug/mL). After the incubation at 37°C overnight, a single colony was selected for further cultivation. Moreover, the strain was identified through MALDI-TOF/MS and the presence of drug-resistant genes was confirmed through PCR.

Whole-Genome Sequencing and Data Analysis

We utilized a Qiagen DNA purification kit in the extraction of the whole genomic DNA of strain L2724hy, which was then sequenced via Oxford Nanopore technology to generate raw data SRA. Then, we used Unicyler 0.4.9 to assemble the SRA, resulting in the acquisition of the whole-genome sequence.[21] The prokka v1.14.6 annotation tool was used to annotate the whole-genome sequence.[22] To predict drug-resistant genes, the data were submitted to ResFinder (https://cge.food.dtu.dk/services/ResFinder/), and the identification of insertion sequence and transposons was performed using ISFinder (https://www-is.biotoul.fr/). Plasmid types were confirmed through PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/), and the MLST of the strains was determined via Pubmlst (https://pubmlst.org/). The discovery of transfer-related components was conducted using OriTfinder (https://bioinfo-mml.sjtu.edu.cn/oriTfinder/). The genetic environment surrounding bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2} on the plasmid was presented using Easyfig 2.2.3.[23] Additionally, the comparison of multiple plasmids was carried out using the BLAST Ring Image Generator (BRIG) software.[24]

Results

Strain Isolation and Identification

L2724hy was isolated from the fecal sample of a patient with acute diarrhea and initially identified as *C. freundii* using MALDI-TOF/MS. Subsequent sequencing of the 16S rRNA lead to the reclassification of L2724hy as *C. portucalensis* (99.93% similarity for *C. portucalensis* and 99.86% similarity for C. freundii). Further ANI analysis confirmed L2724hy as *C. portucalensis*, showing a 98.73% ANI to *C. portucalensis* ATCC (CP044098) and a 94.52% ANI to *C. freundii* ATCC (CP033744).

Antimicrobial Susceptibility Testing

As presented in Table 1, the results of antimicrobial susceptibility testing indicated that *C. portucalensis* exhibited resistance to almost all antibiotics, with the exception of tigecycline and polymyxin B. For both transconjugant L2724hy-

Table 1 The MIC Values of L2724hy, L2724hy-KPC-EC600, L2724hy-NDM-EC600 and EC600

Antimicrobials	MIC Values (mg/L)			EC600
	L2724hy	L2724hy-KPC-EC600	L2724hy-NDM-EC600	
Aztreonam	>128 (R)	>128 (R)	>128 (R)	0.125 (S)
Imipenem	16 (R)	4 (R)	4 (R)	0.5 (S)
Meropenem	>32 (R)	4 (R)	4 (R)	0.06 (S)
Ceftriaxone	>128 (R)	64 (R)	128 (R)	0.06 (S)
Cefotaxime	>128 (R)	16 (R)	128 (R)	0.03 (S)
Ceftazidime	>128 (R)	64 (R)	>128 (R)	0.5 (S)
Levofloxacin	>64 (R)	0.5 (S)	0.25 (S)	1 (I)
Ciprofloxacin	>64 (R)	0.25 (S)	0.25 (S)	0.5 (S)
Amikacin	>128 (R)	1 (S)	2 (S)	2 (S)
Gentamicin	>128 (R)	1 (S)	1 (S)	2 (S)
Piperacillin	>128 (R)	>128 (R)	128 (R)	1 (S)
Fosfomycin	>512 (R)	4 (S)	2 (S)	8 (S)
Chloramphenicol	>128 (R)	8 (S)	8 (S)	8 (S)
Trimethoprim/Sulfamethoxazole	>8 (R)	≤0.125 (S)	≤0.125 (S)	≤2.375 (S)
Amoxicillin/Clavulanic acid	>128 (R)	128 (R)	128 (R)	4 (S)
Cefepime	128 (R)	16 (R)	16 (R)	<0.008 (S)
Ceftazidime-avibactam	64 (R)	1 (S)	64 (R)	0.5 (S)
Tigecycline	0.06 (S)	0.06 (S)	≤0.03 (S)	<0.05 (S)
Polymyxin B	2 (I)	2 (I)	2 (I)	0.5 (I)

Note: The quality control strains were *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.
Abbreviations: S, susceptible; R, resistant; I, intermediate.

KPC-EC600 and L2724hy-NDM-EC600, we observed a decrease in the MIC values of carbapenems (imipenem, meropenem), levofloxacin, ciprofloxacin, amikacin, gentamicin, fosfomycin, chloramphenicol, and trimethoprim/sulfamethoxazole (Table 1). Notably, the transconjugant L2724hy-NDM-EC600 was highly resistant to ceftazidime-avibactam (MIC=64 mg/l), as was L2724hy, emphasizing the hydrolysis of ceftazidime-avibactam by NDM-1.

S1-PFGE-Southern Hybridization and Conjugation Experiment

S1-PFGE profiles revealed that L2724hy harbored one chromosome and four plasmids (Figure 1). However, due to overlap or small size, not all plasmids were discernible on the electrophoresis profiles. The presence of bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2} genes on plasmids of different sizes was verified through Southern blotting hybridization. Specifically, the bla_{SFO-1} gene was identified on a ~130 kb plasmid, the bla_{NDM-1} gene was found on a ~54 kb plasmid, and the bla_{KPC-2} gene was located on a ~41 kb plasmid (Figure 1). These results are consistent with the data obtained from whole-genome sequencing.

The transconjugants were identified as *E. coli* by MALDI-TOF mass spectrometry. PCR confirmed that the transconjugants, namely L2724hy-KPC-EC600 and L2724hy-NDM-EC600, carried the bla_{KPC-2} and bla_{NDM-1} genes, respectively. This indicates the transferability of bla_{NDM-1} and bla_{KPC-2} to *E. coli* EC600.

Genomic Characteristics

The MLST analysis showed that strain L2724hy belongs to ST85. Strain L2724hy has a circular chromosome (5,031,620 bp) and eleven plasmids of various sizes (Table 2). The chromosome contains 5316 coding sequences and 121 RNAs, including 86 tRNAs, 25 rRNAs, and 10 ncRNAs. Its average G+C content is 51.9%. ResFinder identified ten ARGs in

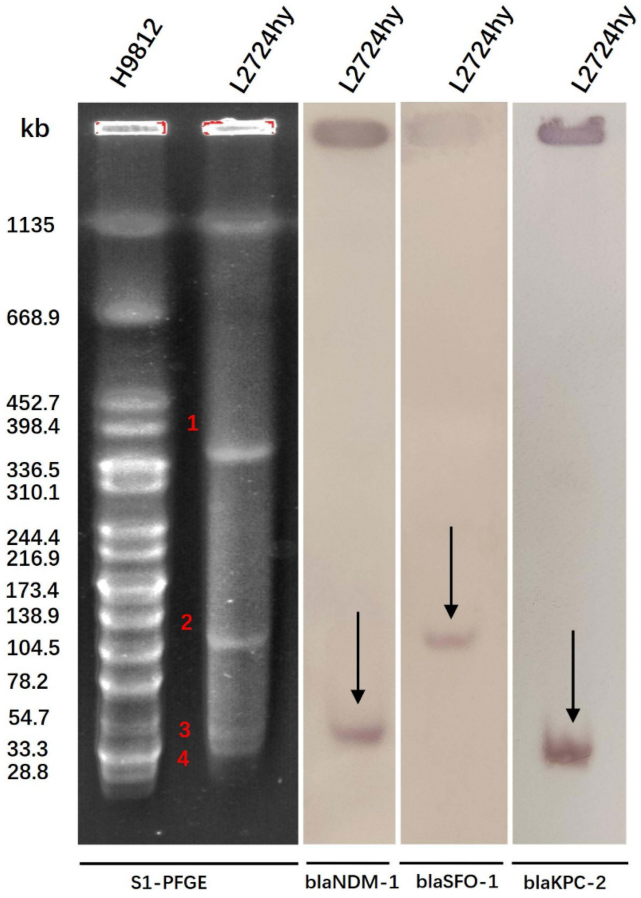

Figure 1 S1-PFGE profiles and southern blotting hybridization for L2724hy. S1-PFGE determines the number and size of plasmids in the strain. Southern blotting hybridization indicates the location of resistance genes bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2}.

Table 2 The Genomic Characteristics of L2724hy

L2724hy	Sizes	Type	G+C%	ARGs
Chromosome	5,031,620 bp	ST85	51.9	*bla*$_{CMY-46}$, *qnrB18*,
p1-L2724hy	365,162 bp	IncH12/IncH12A	46.5	
p2-L2724hy-SFO-1	131,226 bp	IncFII	53.7	*bla*$_{SFO-1}$, *fosA3*
p3-L2724hy-NDM-1	54,000 bp	IncX3	49.2	*bla*$_{SHV-12}$, *bla*$_{NDM-1}$
p4-L2724hy	45,944 bp	IncR	51.9	*mph(A)*
p5-L2724hy-KPC-2	41,244 bp	Undefined	49.1	*bla*$_{KPC-2}$
p6-L2724hy	5,933 bp	Undefined	51.1	*aac(6')-Ib-cr, aac(6')-Ib-Hangzhou*
p7-L2724hy	5,234 bp	Undefined	50.7	
p8-L2724hy	5,040 bp	Undefined	50.0	
p9-L2724hy	2,182 bp	Undefined	50.1	
p10-L2724hy	2,152 bp	Undefined	49.9	
p11-L2724hy	2,013 bp	Undefined	49.3	

strain L2724hy, with *bla*$_{CMY-46}$ and *qnr*B18 located on the chromosome, while the remaining eight were situated on plasmids. The plasmid type of p2-L2724hy-SFO-1 is IncFII, carrying the *bla*$_{SFO-1}$ and *fosA3* genes. Meanwhile, p3-L2724hy-NDM-1 carries two ARGs, bla$_{NDM-1}$ and *bla*$_{SHV-12}$, and is classified as IncX3. p4-L2724hy is an IncR plasmid with *mph* (A), whereas *bla*$_{KPC-2}$ is on an unclassified p5-L2724hy-KPC-2. Another unclassified plasmid, p6-L2724hy, contains two resistance genes, including *aac(6')-Ib-cr and aac(6')-Ib-Hangzhou*.

Our analysis revealed that these distinct drug-resistance genes are consistent with antibiotic phenotypes in L2724hy, which included resistance to carbapenems (*bla*$_{NDM-1}$, *bla*$_{KPC-2}$), cephalosporins (*bla*$_{CMY-46}$, *bla*$_{SFO-1}$, *bla*$_{SHV-12}$, *bla*$_{NDM-1}$, *bla*$_{KPC-2}$), 4-quinolones (*qnrB18*), fosfomycin (*fosA3*), and aminoglycosides (*aac(6')-Ib-cr, aac(6')-Ib-Hangzhou*).

Plasmid Characteristics of bla$_{KPC-2}$, bla$_{NDM-1}$, and bla$_{SFO-1}$

L2724hy carries plasmids of various types, including IncHI2/IncHI2A, IncFII, IncX3, IncR, and unclassified plasmids, as identified by PlasmidFinder. Among these, the IncFII plasmid, designated as p2-L2724hy-SFO-1, has a length of 131,226 bp and carries the resistance genes *bla*$_{SFO-1}$ and *fosA3*. Comparative genomics with three closely related plasmid genomes (CP044029 with 95% coverage and 100% identity, CP042483 with 93% coverage and 100% identity, and CP042519 with 93% coverage and 100% identity) revealed primary variations, most notably the absence of the resistance gene *bla*$_{SFO-1}$ and the transcriptional activator *ampR* (Figure 2a). Additionally, we also identified two strains of *K. pneumoniae* (Genebank: CP114855, and JQ724541) that closely resembled the genetic environment (IS*26*-*ampR*-*bla*$_{SFO-1}$-IS*26*) surrounding *bla*$_{SFO-1}$ in L2724hy (Figure 3a). However, these two strains lack the *fosA3* gene, which is upstream of the IS26-ampR-*bla*$_{SFO-1}$-IS26 sequence in L2724hy. In our study, IS*26*, which is a critical mobile element, is present on both sides of *fosA3*. Based on the OriTfinder results, p2-L2724hy-SFO-1 possesses mobile genetic elements associated with transfer, including an oriT region, type IV coupling protein (T4CP), and various type IV secretion system components (T4SS), which includes *traI, traD, traQ, traN, traC, traV, traA, traJ*, and *traM* (Figures 2a and 4).

p4-L2724hy-NDM-1 was classified as an IncX3 plasmid, with a length of 54,000 bp and carried two resistance genes, *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$ (Figure 2b). The NCBI BLAST search revealed 29 highly similar plasmids to p4-L2724hy-NDM -1, all exhibiting 100% coverage and 100% identity. Specifically, plasmids KU314941, MH234941, and KP987216, originating from distinct strains (*K. pneumoniae, E. coli*, and *C. freundii*) were selected for a comparative analysis of plasmid characteristics. The analysis revealed that these plasmids share similar backbones (Figure 2b), underscoring the widespread of *bla*$_{NDM-1}$ across different species. Upon analyzing the genetic environment of *bla*$_{NDM-1}$ in L2724hy (Figure 3b), a conserved sequence (*ble-trpF-dsbD-cutA-gros-groL*) downstream of *bla*$_{NDM-1}$ on the plasmid was observed. The upstream of *bla*$_{NDM-1}$ comprises an IS*5*-IS*3000*-IS*3000*-Tn*2* sequence, with the notable deletion of IS*Aa125* deviating from the typical *bla*$_{NDM-1}$ structure. Additionally, an IS*26* insertion element surrounding *bla*$_{SHV-12}$ was noted suggesting a potential role in facilitating the dissemination of *bla*$_{SHV-12}$.

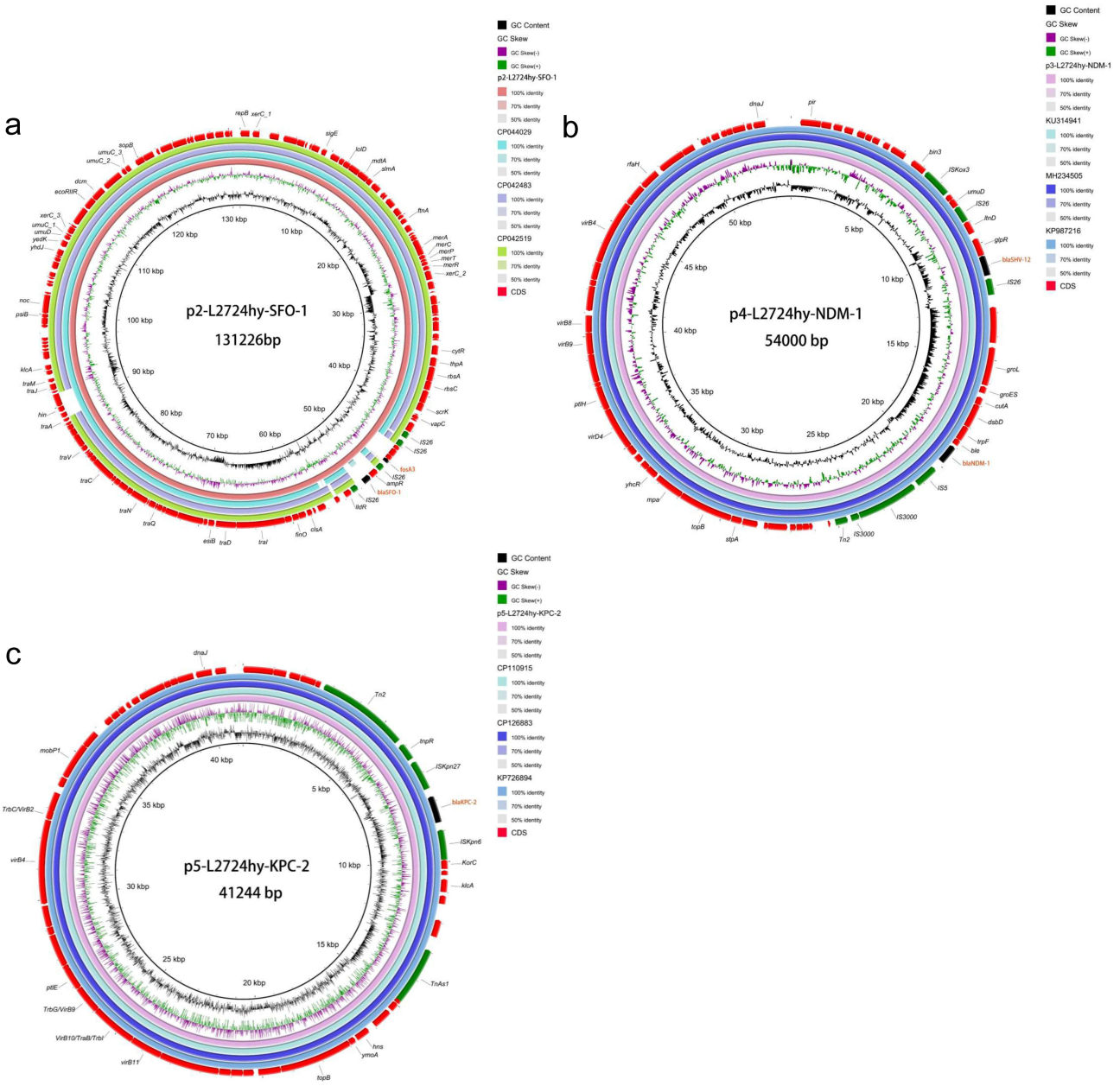

Figure 2 Comparative analysis of plasmids p2-L2724hy-SFO-1, p4-L2724hy-NDM-1, and p5-L2724hy-KPC-2 in L2724hy. The BRIG circle maps show plasmid resistance genes and mobile genetic elements (MGEs). (**a**) plasmid p2-L2724hy-SFO-1 carrying bla_{SFO-1} and *fosA3*. (**b**) plasmid p4-L2724hy-NDM-1 harboring bla_{NDM-1} and bla_{SHV-12}. (**c**) plasmid p5-L2724hy-KPC-2 bearing bla_{KPC-2}.

The plasmid p5-L2724hy-KPC-2, carrying bla_{KPC-2} gene, is currently unclassified and 41,244 bp in length. As shown in Figure 2c, the plasmid contains multiple-insertion elements (IS*Kpn27*, IS*Kpn6*), transposons (*Tn2, tnpR, TnAs1*), and transfer-related type IV secretion systems (*virB2, virB4, virB9, virB10*, and *virB11*). The plasmids CP110915, CP126883, and KP726894, are the most similar to p5-L2724hy-KPC-2, all displaying 100% coverage and 100% identity. Upon examination of these highly similar plasmids, it was observed that CP110915 and CP126883 originated from *C. freundii*. As illustrated in Figure 3c, a comparison of the genetic context of the two most similar *C. freundii* plasmids (CP110915, CP126883) with p5-L2724hy-KPC-2 revealed a similar genetic environment for bla_{KPC-2} (Tn*2-tnpR*-IS*Kpn27*-bla_{KPC-2}-IS*Kpn6*-*KorC*) which is entirely consistent with CP110915 from the same region but exhibits a small gap with CP126883 from a different region.

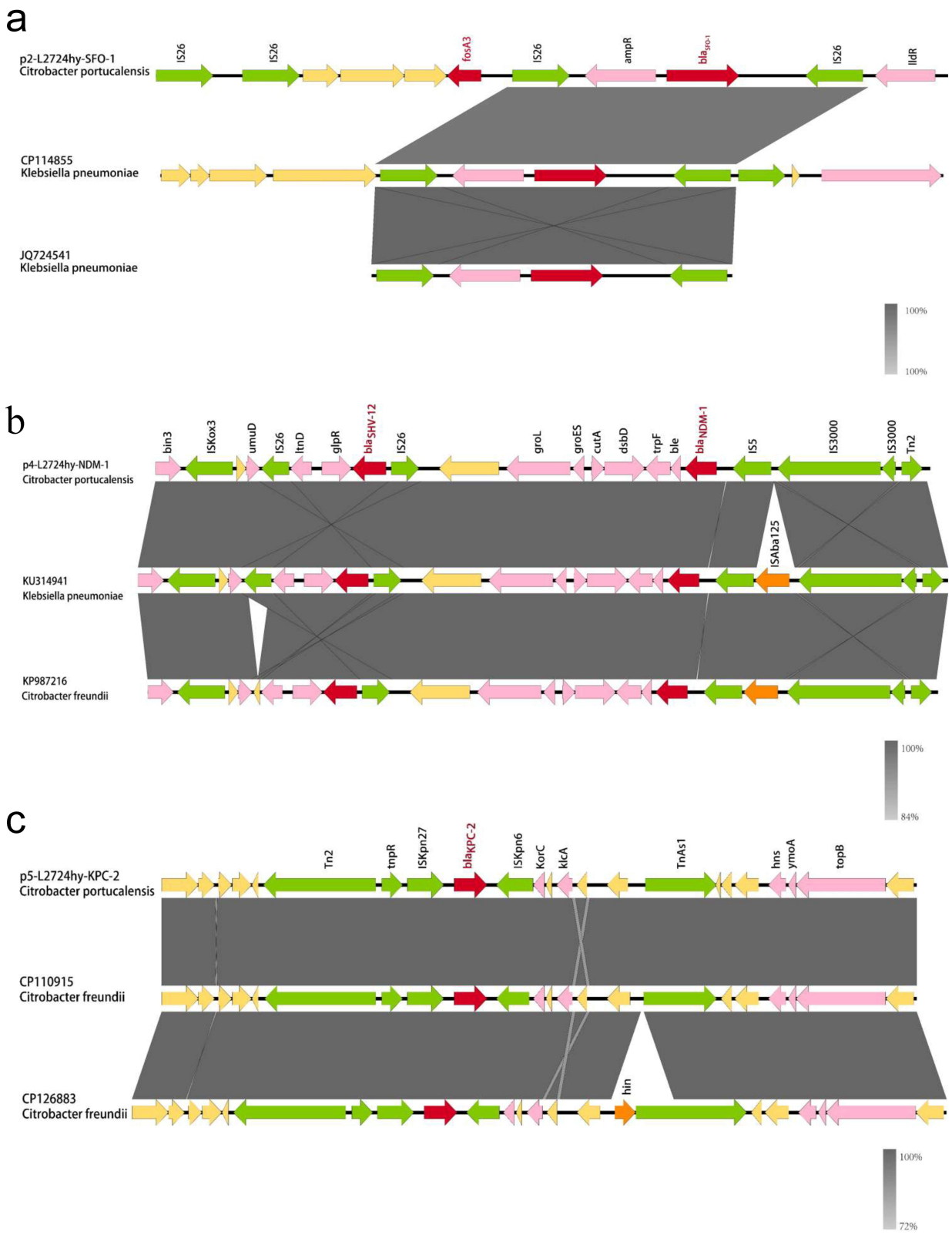

Figure 3 Genetic environment of bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2} in L2724hy. (**a**) bla_{SFO-1} (**b**) bla_{NDM-1} (**c**) bla_{KPC-2}. Red arrows denote drug resistance genes, green arrows denote transposons, pink arrows denote other genes, and yellow arrows represent hypothetical proteins.

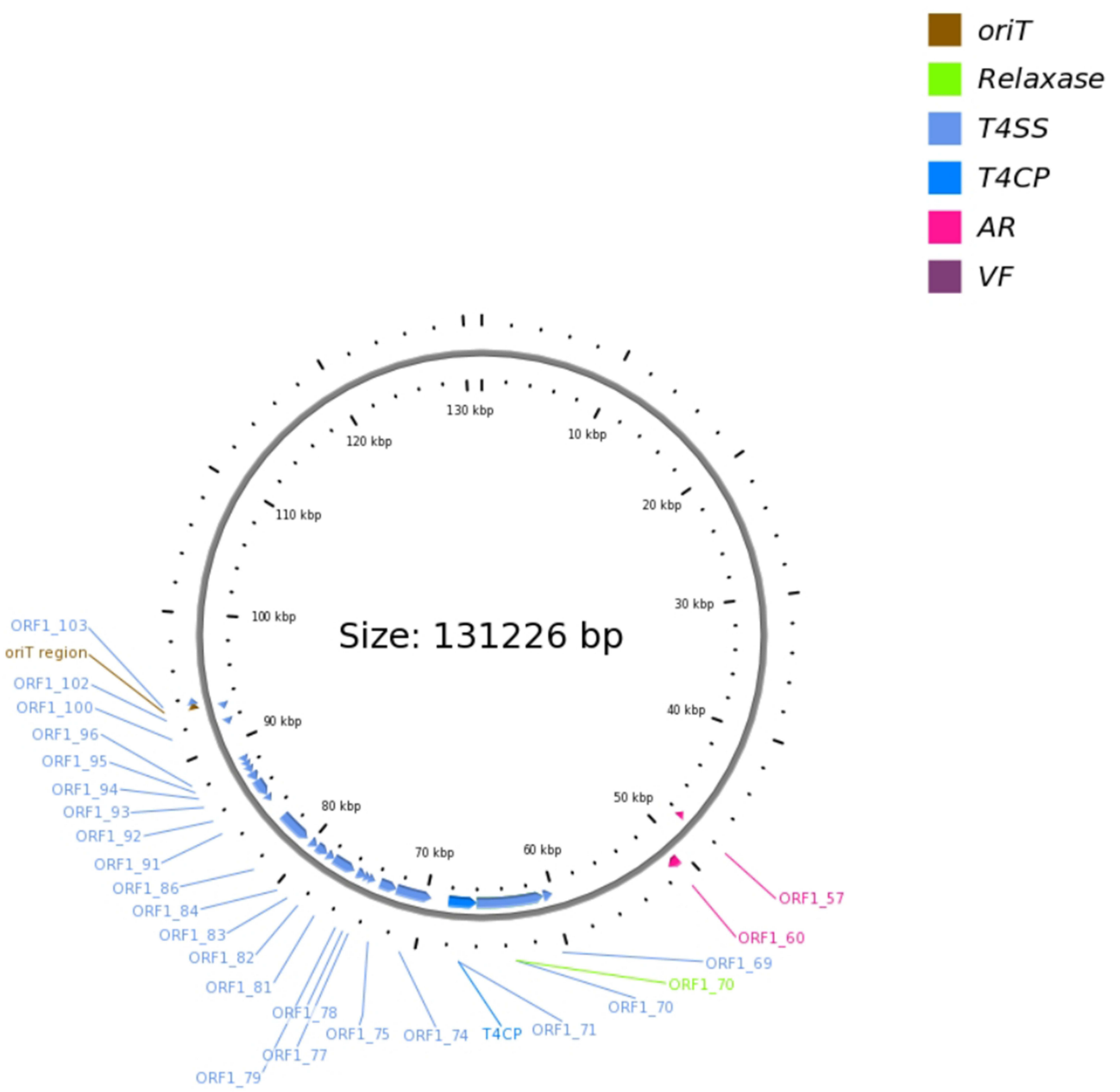

Figure 4 A conjugative plasmid p2-L2724hy-SFO-1. ORF1-57,*fosA3*; ORF1-60, bla_{SFO-1}.
Abbreviation: AR (ARGs), acquired antibiotic resistance determinant genes ;ORF, Open Reading Frame

Discussion

In recent years, the coexistence of ESBLs and carbapenemase genes in *Enterobacteriaceae*, posing challenges to clinical antibiotic treatment.[25] To the best of our knowledge, our study is the first to report the coexistence of bla_{SFO-1} with bla_{NDM-1} and bla_{KPC-2}. Furthermore, there are no reports of the ESBL gene bla_{SFO-1} in *C. portucalensis*.

Since its discovery in 2017, *C. portucalensis* has been found in humans, animals, and environment. However, the misidentification of *C. portucalensis* as *C. freundii* has led to an underestimation of its prevalence in clinical settings.[7,26] In this study, L2724hy *C. portucalensis* was initially misidentified as *C. freundii* using MALDI-TOF/MS, and was later identified as *C. portucalensis* by a combination of 16s rRNA sequencing and ANI analysis. Thus, it is essential to enhance the whole-genome sequencing of less common strains.

Data analysis reveals that the plasmids carrying bla_{SFO-1} include IncA/C, IncHI2, unclassified types.[12,13,27,28] In our study, plasmidFinder revealed that stain L2724hy carries the bla_{SFO-1} gene on an IncFII plasmid that has not been reported before. According to the genetic environment of bla_{SFO-1} (IS*26*-*ampR*-bla_{SFO-1}-IS*26*), the presence of IS*26* on both sides of bla_{SFO-1} suggests a potential correlation with plasmid-mediated horizontal transmission of bla_{SFO-1}, which is consistent with prior research findings.[29] Notably, we also observed that the *fosA3*, is located upstream of IS*26*-*ampR*-bla_{SFO-1}-IS*26* and shares the same IS26 with ampR-bla_{SFO-1}. The *fosA3* has been reported to be closely associated with the transmission of ESBL gene bla_{CTX-M} by the insertion element IS*26*.[30] Therefore, in our study, we believe that IS*26* is a vital insertion element that gradually integrates with *fosA3* and bla_{SFO-1} on the IncFII plasmid, forming a multidrug resistance region, which plays a crucial role in mediating the spread of drug resistance genes. Importantly, this study marks the first instance of bla_{SFO-1} and *fosA3* coexisting on a plasmid.

NDM-1, a carbapenemase, can hydrolyze nearly all beta-lactam antibiotics, including carbapenemase.[31] The emergence of carbapenemase-producing strains of NDM-1 represents a significant risk to global public health, necessitating a high level of vigilance. In our study, we observed an absence of IS*Aba125* from IS*5* to IS*3000* when comparing it to classical bacteria. According to previous studies, IS*Aba125* is typically involved in the formation of *Tn125*, a complex transposon associated with the plasmid-mediated spread of bla_{NDM-1}.[32,33] Our findings suggest that the lack of IS*Aba125* does not impede the horizontal transfer of bla_{NDM-1}. In the conjugation experiment, we successfully transferred bla_{NDM-1} and bla_{KPC-2} into EC600 for expression, affirming the transferability of the plasmids in our study. As mentioned above, the genetic environment of bla_{KPC-2} in this study aligns entirely that of *C. freundii* (Genebank: CP110915) from the same region, strongly suggesting that bla_{KPC-2} was transmitted from CP110915.

Conclusion

In conclusion, our study reported for the first time the coexistence of bla_{SFO-1}, bla_{NDM-1}, and bla_{KPC-2} in a clinical isolated *C. portucalensis* strain, presenting challenges in multidrug resistance. IS*26* is an essential insertion element that facilitates the propagation of drug-resistant genes on IncFII plasmids. These findings emphasize the urgency of continued monitoring and understanding evolving drug resistance dynamics in uncommon strains.

Nucleotide Sequence Accession Numbers

The nucleotide sequences in L2724hy containing a circular chromosome and eleven plasmids have been submitted to GenBank and assigned accession numbers CP136601-136612, respectively.

Ethical Approval

This study was approved by the clinical research ethics committee of the First Affiliated Hospital, Zhejiang University School of Medicine [number 2020-IIT-591], and was conducted according to the ethical principles of the Declaration of Helsinki. Parental written consent was obtained.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Ribeiro TG, Gonçalves BR, da Silva MS, et al. Citrobacter portucalensis sp. nov. isolated from an aquatic sample. *Int J Syst Evol Microbiol*. 2017;67(9):3513–3517. doi:10.1099/ijsem.0.002154
2. Cao X, Xie H, Huang D, et al. Detection of a clinical carbapenem-resistant Citrobacter portucalensis strain and the dissemination of C. portucalensis in clinical settings. *J Glob Antimicrob Resist*. 2021;27:79–81. doi:10.1016/j.jgar.2021.04.027

3. Chang H, Mishra R, Cen C, et al. Metagenomic analyses expand bacterial and functional profiling biomarkers for colorectal cancer in a Hainan Cohort, China. *Curr Microbiol*. 2021;78(2):705–712. doi:10.1007/s00284-020-02299-3
4. Gupta RK, Singh AK, Bajaj A, Khardenavis AA, Purohit HJ. Phylogenomic analysis of Citrobacter sp. strain AAK_AS5 and its metabolic capabilities to support nitrogen removal behavior. *J Basic Microbiol*. 2023;63(3–4):359–376. doi:10.1002/jobm.202200323
5. Hasan MS, Sultana M, Hossain MA. Complete genome arrangement revealed the emergence of a poultry origin superbug Citrobacter portucalensis strain NR-12. *J Glob Antimicrob Resist*. 2019;18:126–129. doi:10.1016/j.jgar.2019.05.031
6. Thomas SG, Abajorga M, Glover MA, et al. Aeromonas hydrophila RIT668 and Citrobacter portucalensis RIT669-potential zoonotic pathogens isolated from spotted turtles. *Microorganisms*. 2020;8(11):1805. doi:10.3390/microorganisms8111805
7. Luo X, Yu L, Feng J, et al. Emergence of Extensively Drug-Resistant ST170 Citrobacter portucalensis with Plasmids pK218-KPC, pK218-NDM, and pK218-SHV from a Tertiary Hospital, China. *Microbiol Spectr*. 2022;10(5):e0251022. doi:10.1128/spectrum.02510-22
8. Sellera FP, Fuentes-Castillo D, Fuga B, Goldberg DW, Kolesnikovas CKM, Lincopan N. New Delhi metallo-β-lactamase-1-producing Citrobacter portucalensis belonging to the novel ST264 causing fatal sepsis in a vulnerable migratory sea turtle. *One Health*. 2023;17:100590. doi:10.1016/j.onehlt.2023.100590
9. Matsumoto Y, Inoue M. Characterization of SFO-1, a plasmid-mediated inducible class A beta-lactamase from Enterobacter cloacae. *Antimicrob Agents Chemother*. 1999;43(2):307–313. doi:10.1128/AAC.43.2.307
10. Fernández A, Pereira MJ, Suárez JM, et al. Emergence in Spain of a multidrug-resistant Enterobacter cloacae clinical isolate producing SFO-1 extended-spectrum beta-lactamase. *J Clin Microbiol*. 2011;49(3):822–828. doi:10.1128/JCM.01872-10
11. Qiao J, Ge H, Xu H, et al. Detection of IMP-4 and SFO-1 co-producing ST51 Enterobacter hormaechei clinical isolates. *Front Cell Infect Microbiol*. 2022;12:998578. doi:10.3389/fcimb.2022.998578
12. Zhao JY, Zhu YQ, Li YN, et al. Coexistence of SFO-1 and NDM-1 β-lactamase genes and fosfomycin resistance gene fosA3 in an Escherichia coli clinical isolate. *FEMS Microbiol Lett*. 2015;362(1):1–7. doi:10.1093/femsle/fnu018
13. Zhou K, Yu W, Shen P, et al. A novel Tn1696-like composite transposon (Tn6404) harboring bla (IMP-4) in a Klebsiella pneumoniae isolate carrying a rare ESBL gene bla (SFO-1). *Sci Rep*. 2017;7(1):17321. doi:10.1038/s41598-017-17641-2
14. Hobson CA, Pierrat G, Tenaillon O, et al. Klebsiella pneumoniae Carbapenemase variants resistant to ceftazidime-avibactam: an evolutionary overview. *Antimicrob Agents Chemother*. 2022;66(9):e0044722. doi:10.1128/aac.00447-22
15. Han R *et al*. (2020). Dissemination of Carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) Among Carbapenem-Resistant Enterobacteriaceae Isolated From Adult and Children Patients in China. Front. Cell. Infect. Microbiol., 10 10.3389/fcimb.2020.00314
16. Chen C, Xu H, Liu R, et al. Emergence of neonatal sepsis caused by MCR-9- and NDM-1-co-producing Enterobacter hormaechei in China. *Front Cell Infect Microbiol*. 2022;12:879409. doi:10.3389/fcimb.2022.879409
17. Sánchez-Pérez S, Comas-Basté O, Duelo A, Veciana-Nogués M Teresa, Berlanga M, Latorre-Moratalla M Luz and Vidal-Carou M Carmen. (2022). Intestinal Dysbiosis in Patients with Histamine Intolerance. Nutrients, 14(9), 1774 10.3390/nu14091774
18. Jin Y, Xu H, Yao Q, et al. Confirmation of the need for reclassification of Neisseria mucosa and Neisseria sicca using average nucleotide identity blast and phylogenetic analysis of whole-genome sequencing: hinted by clinical misclassification of a Neisseria mucosa strain. *Front Microbiol*. 2021;12:780183. doi:10.3389/fmicb.2021.780183
19. Liu R, Xu H, Zhao J, et al. Emergence of mcr-8.2-harboring hypervirulent ST412 Klebsiella pneumoniae strain from pediatric sepsis: a comparative genomic survey. *Virulence*. 2023;14(1):233–245. doi:10.1080/21505594.2022.2158980
20. Xu H, Wang X, Yu X, et al. First detection and genomics analysis of KPC-2-producing Citrobacter isolates from river sediments. *Environ Pollut*. 2018;235:931–937. doi:10.1016/j.envpol.2017.12.084
21. Wick RR, Judd LM, Gorrie CL, Holt KE, Phillippy AM. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol*. 2017;13(6):e1005595. doi:10.1371/journal.pcbi.1005595
22. Huang J, Zhu J, Gong D, Wu L, Zhu Y, Hu L. Whole genome sequence of EC16, a bla(NDM-5)-, bla(CTX-M-55)-, and fosA3-coproducing Escherichia coli ST167 clinical isolate from China. *J Glob Antimicrob Resist*. 2022;29:296–298. doi:10.1016/j.jgar.2022.04.001
23. Liu S, Xu H, Guo X, et al. Emergence and genetic characterization of plasmid-encoded VIM-2-producing pseudomonas stutzeri with novel integron in1998 isolated from cerebrospinal fluid. *Infect Drug Resist*. 2021;14:3415–3424. doi:10.2147/IDR.S320294
24. Zheng B, Xu H, Lv T, et al. Stool samples of acute diarrhea inpatients as a reservoir of ST11 hypervirulent KPC-2-producing Klebsiella pneumoniae. *mSystems*. 2020;5(3). doi:10.1128/mSystems.00498-20
25. Seman A, Mihret A, Sebre S, Awoke T, Yeshitela B, Yitayew B, Aseffa A, Asrat D and Abebe T. (2022). Prevalence and Molecular Characterization of Extended Spectrum β-Lactamase and Carbapenemase-Producing Enterobacteriaceae Isolates from Bloodstream Infection Suspected Patients in Addis Ababa, Ethiopia. IDR, Volume 15 1367–1382. 10.2147/IDR.S349566
26. Wang L, Li Z, Xiao N, et al. Genetic characterization of bla (NDM-1)-carrying Citrobacter portucalensis sequence type 328 and citrobacter freundii sequence type 98. *Infect Drug Resist*. 2022;15:2235–2242. doi:10.2147/IDR.S361761
27. Ai W, Zhou Y, Wang B, et al. Corrigendum: first report of coexistence of bla (SFO-1) and bla (NDM-1) β-lactamase genes as well as colistin resistance gene mcr-9 in a transferrable plasmid of a clinical isolate of Enterobacter hormaechei. *Front Microbiol*. 2021;12:741628. doi:10.3389/fmicb.2021.741628
28. Guo Q, Wang P, Ma Y, Yang Y, Ye X, Wang M. Co-production of SFO-1 and DHA-1 β-lactamases and 16S rRNA methylase ArmA in clinical isolates of Klebsiella pneumoniae. *J Antimicrob Chemother*. 2012;67(10):2361–2366. doi:10.1093/jac/dks244
29. Zhou K, Zhou Y, Zhang C, et al. Dissemination of a 'rare' extended-spectrum β-lactamase gene bla(SFO-1) mediated by epidemic clones of carbapenemase-producing Enterobacter hormaechei in China. *Int J Antimicrob Agents*. 2020;56(3):106079. doi:10.1016/j.ijantimicag.2020.106079
30. Lee S, Park Y, Yu J K, Jung S, Kim Y, Jeong S H and Arakawa Y. (2012). Prevalence of acquired fosfomycin resistance among extended-spectrum -lactamase-producing Escherichia coli and Klebsiella pneumoniae clinical isolates in Korea and IS26-composite transposon surrounding fosA3. Journal of Antimicrobial Chemotherapy, 67(12), 2843–2847. 10.1093/jac/dks319
31. Bose P, Rangnekar A, Desikan P. NDM-beta-lactamase-1: where do we stand? *Indian J Med Res*. 2022;155(2):243–252. doi:10.4103/ijmr.IJMR_685_19
32. Alattraqchi AG, Mohd Rani F, A Rahman NI, et al. Complete genome sequencing of Acinetobacter baumannii AC1633 and Acinetobacter nosocomialis AC1530 unveils a large multidrug-resistant plasmid encoding the NDM-1 and OXA-58 carbapenemases. *mSphere*. 2021;6(1). doi:10.1128/mSphere.01076-20
33. Corrêa LL, Kraychete GB, Rezende AM, et al. NDM-1-encoding plasmid in Acinetobacter chengduensis isolated from coastal water. *Infect Genet Evol*. 2021;93:104926. doi:10.1016/j.meegid.2021.104926