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Short Communication

Coexistence of $bla_{\text{NDM-5}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-232}}$, $bla_{\text{SHV-182}}$ genes in multidrug-resistant *K. pneumoniae* ST437-carrying OmpK36 and OmpK37 porin mutations: First report in Italy

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ABSTRACT

Objectives: *K. pneumoniae* is a common cause of severe hospital-acquired infections. In the present study, we have characterised the whole-genome of two *K. pneumoniae* ST437 belonging to the clonal complex CC258.

Methods: The whole-genome sequencing was performed by MiSeq Illumina, with a 2 × 300bp paired-end run. ResFinder 4.4.2 was used to detect acquired antimicrobial resistance genes (ARGs) and chromosomal mutations. Mobile genetic elements (plasmids and ISs) were identified by MobileElementFinder v1.0.3. The genome was also assigned to ST using MLST 2.0.9. Virulence factors were detected using the Virulence Factor Database (VFDB).

Results: *K. pneumoniae* KPNAQ_1/23 and KPNAQ_2/23 strains, isolated from urine samples of hospitalised patients, showed resistance to most antibiotics, including ceftazidime-avibactam, ceftolozane-tazobactam, and meropenem-vaborbactam combinations. Both strains were susceptible only to cefiderocol. Multiple mechanisms of resistance were identified. Resistance to β-lactams was due to the presence of NDM-5, OXA-232, CTX-M-15, SHV-182 β-lactamases, and OmpK36 and OmpK37 porin mutations. Resistance to fluoroquinolones was mediated by chromosomal mutations in *acrR, oqxAB* efflux pumps, and the bifunctional gene *aac(6')-Ib-cr.*

Conclusion: The presence of different virulence genes makes these KPNAQ_1/23 and KPNAQ_2/23 high-risk clones.

1. Introduction

The spread of antimicrobial resistant bacteria represents a real threat to public health worldwide. For this reason, in 2018, the World Health Organization (WHO) published a priority list of antibiotic-resistant pathogens, encompassing carbapenem-resistant *Enterobacterales* (CRE), carbapenem-resistant *Acinetobacter baumannii* (CRAB), and carbapenem-resistant *Pseudomonas aeruginosa* (CRPA), for which the development of new antimicrobials is urgent [1]. Among CRE, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a common hospital-acquired pathogen causing severe infection, including pneumonia, bacteraemia, meningitis, liver abscess, and urinary tract infection [2]. *K. pneumoniae* strains could be categorised as 'classical *K. pneumoniae*', including CRKP and multidrug resistant *K. pneumoniae* (MDR_KP), and hypervirulent *K. pneumoniae* (HVKP) [2,3]. In comparison with MDR_KP, the HVKP strains showed a high virulence capability associated with yersiniabactin (*ybt*), salmochelin (*iroB*), siderophore aerobactin (*iucA*), colibactin (*clb*), *rmpADC* virulence factors, and K1, K2, K5, and O loci, which encode the polysaccharide capsule and lipopolysaccharide O antigen, respectively [4–7]. Typically, HVKP strains are susceptible to many drugs, but recent studies report the emergence of HVKP-carrying antimicrobial-resistant plasmids [8,9]. Notwithstanding, MDR_KP clones with hypervirulent lineages have increasingly been reported [3]. Whole-genome sequencing (WGS) and multilocus sequence typing (MLST) of the seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB*) identified several phylogenetic lin-

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eages or clonal complexes (CCs) [10]. The most representative CC of CRKP is CC258, which includes ST11, ST258, ST340, ST437, and ST512 [10]. The main resistant mechanisms in CRKP include the presence of a) encoding genes for NDM-, VIM-, KPC-, and OXA-type β-lactamases; b) expression of efflux pump genes; and c) alterations in outer membrane porins (e.g. mutations in OmpK35, OmpK36, and OmpK37) [11,12].

In the present study, we have characterised two MDR *K. pneumoniae* strains (KPNAQ_1/23 and KPNAQ_2/23), with identical genotypic features, isolated from the urine of hospitalised patients (L'Aquila Hospital, central Italy). The KPNAQ_1/23 and KPNAQ_2/23 harboured different plasmids, $bla_{\text{NDM-5}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-232}}$, $bla_{\text{SHV-182}}$, $bla_{\text{TEM-1B}}$, and other antimicrobial resistance genes (ARGs). In addition, mutations in OmpK36, OmpK37, and *acrR* have been found. This is the first description in Italy of *K. pneumoniae* ST437 lineage.

2. Methods

2.1. Strain identification

K. pneumoniae KPNAQ_1/23 and *K. pneumoniae* KPNAQ_2/23 were identified from the urine of two hospitalised patients in San Salvatore Hospital, L'Aquila (central Italy). The two strains were collected in September 2023, 15 days apart, from patients admitted to the internal medicine and rehabilitation medicine wards. The strains were identified on Columbia agar plates (sheep blood 5%; Liofilchem S.r.l., Roseto degli Abruzzi, Italy) by biochemical identification with BD Phoenix M50 (Becton Dickinson Diagnostic System, Sparks, MD, USA). The identification, with a confidence of 99%, was also confirmed by MALDI-TOF MS microflex LT (Bruker Daltonics, Bremen, Germany).

2.2. Antimicrobial susceptibility test

The antimicrobial susceptibility test was carried out on an automatic BD Phoenix M50 system (Becton Dickinson Diagnostic System, Sparks, MD, USA) using a large panel of antibiotics. The minimal inhibitory concentrations (MICs) for ceftazidime-avibactam, ceftolozane-tazobactam, and meropenem-vaborbactam were performed using the E-test method (Test Strip, Liofilchem S.r.l., Roseto degli Abruzzi, Italy). The susceptibility to cefiderocol was performed using Cefiderocol 30 μg Disc (Liofilchem S.r.l., Roseto degli Abruzzi, Italy). The inhibition zone diameter breakpoints were interpreted according to the EUCAST reference standard (≥22 mm is sensible [S], <22 mm is resistant [R]).

2.3. DNA extraction and WGS

Total nucleic acid was extracted from liquid cultures of KPNAQ1/23 and KPNAQ2/23 using a modified protocol of a MagMAX Microbiome Ultra Nucleic Acid Isolation Kit (Applied Biosystems, ThermoFisher Scientific, Monza, Italy) [13]. Short-read sequencing libraries were prepared with an Illumina DNA Prep Kit (Illumina Inc. San Diego, USA) and sequenced on an Illumina MiSeq instrument with a 2 × 300bp paired-end protocol, as previously described [14]. Raw data from sequencing were quality checked using DRAGEN FastQC + MultiQC v3.9.5 (Illumina Inc. San Diego, CA, USA https://basespace.illumina.com/apps/12821810/DRAGEN-FastQC-MultiQC) and assembled with SPAdes Genome Assembler v3.9.0 (Illumina Inc. San Diego, CA, USA https://basespace.illumina.com/apps/3047044/SPAdes-Genome-Assembler?preferredversion).

2.4. Bioinformatics analysis

ResFinder 4.4.2 was used to detect acquired antimicrobial resistance genes (ARGs) and chromosomal mutations (Center for Genomic Epidemiology, DTU, Lyngby, Denmark). Mobile genetic elements (plasmids and ISs) were identified by MobileElementFinder v1.0.3 (Center for Genomic Epidemiology, DTU, Lyngby, Denmark). The genome was also assigned to ST using MLST 2.0.9 (Center for Genomic Epidemiology, DTU, Lyngby, Denmark). Virulence factors were detected using Virulence Factor Database (VFDB) (National Institue of Pathogen Biology, CAMS&PUMC, Bejing, China).

Table 1
Antimicrobial susceptibility of KPNAQ_1/23 and KPNAQ_2/23.

Antibiotic	MIC (mg/L)	Interpretation
Ampicillin	>8	R
Ampicillin-clavulanate	>32/2	R
Piperacillin-tazobactam	>16/4	R
Cefotaxime	>4	R
Ceftazidime	>8	R
Ceftazidime-avibactam[a]	>256	R
Cefepime	>8	R
Cefiderocol	28 mm[b]	S
Cefalexin	>16	R
Ceftolozane-tazobactam[a]	>256	R
Imipenem	>8	R
Meropenem	>8	R
Meropenem-vaborbactam[a]	>256	R
Ertapenem	>1	R
Ciprofloxacin	>1	R
Gentamicin	>4 (2)	R
Tobramycin	>4	R
Fosfomycin	>64	R

[a] The MIC values were determined by the E-test method.
[b] Cefiderocol 30μg Disc. The inhibition zone diameter breakpoints were interpreted according to the EUCAST reference standard (≥22 mm is sensible [S], <22 mm is resistant [R]).

3. Results

3.1. Antimicrobial susceptibility of K. pneumoniae strains

The *K. pneumoniae* KPNAQ_1/23 and *K. pneumoniae* KPNAQ_2/23 clinical strains were isolated from urine samples of hospitalised patients. As showed in Table 1, both strains showed resistance to most antibiotics used in the BD panel. The MICs for ceftazidime-avibactam (>256 mg/L), ceftolozane-tazobactam (>256 mg/L), and meropenem-vaborbactam (>256 mg/L) were determined with an E-test. Both strains were susceptible to cefiderocol, with a zone diameter of 28 mm.

3.2. WGS of KPNAQ1/23 and KPNAQ2/23 strains

Total DNA was extracted from KPNAQ_1/23 and KPNAQ_2/23 in order to carry out WGS. The genome size of both strains was of 5.646.105 bp with a 57% of GC content. MLST indicated that both strains belonged to ST437 (*gapA* 3, *infB* 3, *mdh* 1, *pgi* 1, *phoE* 1, *rpoB* 1, and *tonB* 31), a single-locus variant of ST258 (*gapA* 3, *infB* 3, *mdh* 1, *pgi* 1, *phoE* 1, *rpoB* 1, and *tonB* 79). Indeed, compared with ST258, ST437 showed four mutations in the *tonB* gene (C95T, T118C, C297A, and A327G). Bioinformatic analysis stated that the two *K. pneumoniae* strains showed the identical genetic elements in term of plasmids, ARGs, and chromosomal elements. From this point on, for both strains, we refer to KPNAQ_1-2/23. Chromosomal genes that confer resistance to several classes of antibiotics have been identified in KPNAQ_1-2/23 (Table 2). Specifically, resistance to fluoroquinolones is mediated by seven mutations identified in the transcriptional repressor AcrR (P161R, G164A, F172S, R173G, L195V, F197I, and K201M). AcrR regulates the OqxAB efflux pump. Chromosomal resistance to cephalosporins was due to nine mutations found in OmpK36 (N49S, L59V, G189T, F198Y, F207Y,

Table 2
Whole-genome sequencing of KPNAQ_1/23 and KPNAQ_2/23.

Genotyping of KPNAQ_1/23 and KPNAQ_2/23					
MLST	B-lactam resistance	Macrolide resistance	Quinolone resistance	Rifampicin resistance	Others
ST437	*bla*$_{CTX-M-15}$ *bla*$_{NDM-5}$ *bla*$_{SHV-182}$ *bla*$_{OXA-232}$ *bla*$_{TEM-1B}$	*mph(A)*	*aac(6′)-Ib-cr* *oqxAB*	*ARR-2*	*sul1* *dfrA12* *qacE* *fosA*

Mobile genetic elements	
Plasmids	Insertion sequences (ISs)
Col(BS512) IncFIB(K) IncFII IncFII(K) IncR ColKP3 ColRNAI	ISEc33 IS*102* (IS5 family) IS*Kpn18* (IS3 family) IS*Kpn26* IS*Kpn38* IS*Kpn21* ISEcl1 (IS3 family) IS*903* (IS5 family) IS*Kpn24* (IS*66* family)

Chromosomal mutations	
acrR	P161R, G164A, F172S, R173G, L195V, F197I, K201M
ompK36	A217S
	N49S, L59V, G189T, F198Y, F207Y, T222L, D223G, E232R, N304E
ompK37	I70M, I128M, N230G

Table 3
Virulence factors in KPNAQ_1/23 and KPNAQ_2/23.

Category	Virulence factors	Target genes
Adherence	Type 3 fimbriae	*mrkA,B,C,D,F,H,I,J*
	Type 1 fimbriae	*fimA,B,C,D,E,F,G,H,I,K*
Efflux pumps	AcrAB	*acrA, B*
Iron uptake	Aerobactin	*iutA*
	Ent siderophore	*entA,B,C,D,E,F,S; fepA,B,C,D,G; fes*
	Salmochelin	*iroE,N*
	Yersiniabactin	*fyuA; irp1,2; ybtA,E,P,Q,S,T,U,X*
	Ferrous iron transport *(Shigella)*	*sitC*
	Iron/manganese transport (*Escherichia*)	*sitA,D*
Regulation	RcsAB	*rcsA,B*
Secretion system	T6SS-I	*tssH,L,D,M,A,J,F,GK,I,B,C; ompA; tli1*
	T6SS-II	*clpV*
	T6SS-III	*dotU; icmF; impA,F,G,H,J; ompA; sciN; vgrG*

T222L, D223G, E232R, and N304), while carbapenem resistance depended on an A217S mutation in OmpK36 and an I70M, I128M, and N230G amino acid substitution in OmpK37. In addition, the presence of *fosA* conferred fosfomycin resistance to KPNAQ_1-2/23 strains (Table 2).

3.3. ARGs and mobile genetic elements

Among ARGs, in KPNAQ_1-2/23, we have detected the copresence of *bla*$_{NDM-5}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-232}$, *bla*$_{SHV-182}$, and *bla*$_{TEM-1B}$ β-lactamase genes. NDM-5 is an NDM-1V88L variant, OXA-232 is a OXA-48 with T104A, N110D, E168Q, S171A, and R214S substitutions, and SHV-182 is an SHV-11 variant with an S106T mutation. In addition to chromosomal mutations in AcrR, resistance to fluoroquinolones was mediated by *aac(6')-Ib-cr*, a bifunctional gene that confers resistance to aminoglycosides as well. The *dfrA12* gene, an integron-encoded dihydrofolate reductase that confers resistance to trimethoprim, was also identified. Resistance to macrolides and rifampicin was due to the presence of the *mph(A)* and *arr-2* genes, respectively. IncFIB(K), IncFII, IncFII(K), IncR, Col(BS512), ColKP3, and ColRNAI plasmids were identified. We also found IS*Ec33*, IS3 family (IS*Kpn18* and IS*Ecl1*), IS5 family (IS*102* and IS*903*), IS*Kpn26*, IS*Kpn38*, IS*Kpn21*, and IS*Kpn24* (IS66 family) insertion sequence elements.

3.4. Virulence factors

In Table 3, we show the target genes categorised as virulence factors. In particular, KPNAQ_1–2/23 encompassed adhesive fimbriae type 1 and type 3, iron carriers including Yersiniabactin (*fyuA; irp1,2; ybtA,E,P,Q,S,T,U,X*), Aerobactin (*iutA*), Salmochelinn (*iroE,N*), and Enterobactin, ferrous iron/manganese transporters (*sitA,C,D*), efflux pumps (*acrAB*), and T6SS-I/II/III secretion systems (Table 3).

4. Discussion

K. pneumoniae strains analysed in this study showed resistance to most antibiotics tested but were susceptible only to cefiderocol, a new siderophore cephalosporin. The KPNAQ_1-2/23 strains belonged to ST437, which differs from ST258 by four mutations in the *tonB* gene: C95T, T118C, C297A, A327G. *K. pneumoniae* ST437 is a member of CC258, which is the most diffused MDR_KP group worldwide [15]. ST437 is not common in Italy, and this study could be considered the first genomic characterisation of this lineage. It is noteworthy that both strains were isolated in the same period (September 2023) but the two patients were not in contact each other in the hospital wards. The history of the patients before their hospitalisation is unknown.

On the basis of the Kleborate program by Lamm et al., our strains could be included in 'virulence score 1' because, among the selected virulence genes, they carried only *ybt* [12]. As the presence of multiple virulence factors does not justify the inclusion of KPNAQ_1-2/23 strains as hypervirulent, our strains could be considered high-risk MDR_KP. The coexistence in *K. pneumoniae* of different classes of β-lactamases is not atypical. In KPNAQ_1-2/23, we found class A (CTX-M-15, TEM-1B, SHV-182), class B (NDM-5), and class D (OXA-232) β-lactamases. OXA-232, in association with CTX-M-15 and/or NDM-1, was first described in Italy in 2016 and 2019 in *K. pneumoniae* ST16 [16,17]. The $bla_{\text{OXA-232}}$ gene is located on a 6141-bp plasmid carrying the ColKP3 replicon [17,18], the same plasmid found in KPNAQ_1-2/23 strains characterised in the present study. In KPNAQ_1-2/23 strains, mutations in OmpK36 and OmpK37 were found to contribute to the resistance of the ST437 clone to carbapenems and cephalosporins. Resistance to fluoroquinolones is also mediated in our strains by chromosomal mutations in *acrR*. AcrR is the main regulator of AcrAB multidrug efflux pumps, which belong to the RND family and show the widest substrate specificity [19]. Multidrug efflux pumps are intrinsically present in all bacteria and are major contributors to antibiotic resistance and virulence, especially in Gram-negative bacteria. The presence of extended-spectrum β-lactamases (e.g. CTX-M-15), carbapenemases (NDM-5 and OXA-232), and other antibiotic resistance mechanisms in KPNAQ_1-2/23 strains makes therapeutic treatment very complicated.

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