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Antibiotic pressure on the acquisition and loss of antibiotic resistance genes in *Klebsiella pneumoniae*

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Objectives: In this study, we characterize a concurrent disseminated infection with a virulent hypermucoviscous (HMV) *Klebsiella pneumoniae* and an OXA-181-producing XDR *K. pneumoniae* from a patient with recent hospitalization in India. During exposure to meropenem therapy, the highly susceptible HMV *K. pneumoniae* became resistant to carbapenems, consistent with the acquisition of $bla_{\text{OXA-181}}$.

Methods: Twelve *K. pneumoniae* isolates were recovered from the patient and the hospital room environment over a 3 month hospitalization. Phenotypic and molecular studies were completed to characterize the isolates. Oxford Nanopore and Illumina MiSeq WGS were performed to study phylogeny (MLST and SNPs), plasmids and virulence genes and demonstrate changes in the organism's resistome that occurred over time.

Results: WGS revealed that the HMV *K. pneumoniae* belonged to ST23 and harboured an IncH1B virulence plasmid, while the XDR *K. pneumoniae* belonged to ST147 and possessed two MDR plasmids (IncR and IncFII), the $bla_{\text{OXA-181}}$-bearing ColKP3 plasmid and chromosomal mutations conferring the XDR phenotype. Sequential isolates demonstrated plasmid diversification (fusion of the IncR and IncFII plasmids), mobilization of resistance elements (*ompK35* inactivation by IS*Ecp1*-$bla_{\text{CTX-M-15}}$ mobilization, varying numbers of resistance genes on plasmid scaffolds) and chromosomal mutations (mutations in *mgrB*) leading to further antibiotic resistance that coincided with antibiotic pressure. Importantly, the HMV strain in this study was unable to preserve the carbapenem-resistant phenotype without the selective pressure of meropenem.

Conclusions: To the best of our knowledge, we are the first to report a carbapenem-resistant HMV *K. pneumoniae* strain in the USA. Ultimately, this case demonstrates the role of antibiotic pressure in the acquisition and loss of important genetic elements.

Introduction

Klebsiella pneumoniae is responsible for significant morbidity and mortality in hospitalized patients.[1] Two trends in *K. pneumoniae* pathogenicity have been apparent for several years. The first is the emergence of hypervirulent variants of *K. pneumoniae* causing life-threatening, community-acquired and metastatic infections in otherwise healthy patients. This was initially described in and localized to the Asian Pacific Rim. More recently, sporadic cases have been reported worldwide.[2] These variants harbour a virulence plasmid bearing the capsular polysaccharide regulator genes (*rmpA/A2*) and several siderophore gene clusters leading to a hypermucoviscous (HMV) phenotype that can be detected by a positive string test.[3,4] The second trend is the acquisition of MDR plasmids in non-HMV or 'classic' *K. pneumoniae*, exemplified by several hospital outbreaks of carbapenemase-producing *K. pneumoniae* causing high morbidity and mortality.[5,6] Rarely are HMV *K. pneumoniae* isolates also carbapenemase-producing and this occurrence has yet to be observed in the USA.[3] We report the molecular characterization of strains isolated over a 3 month period from a complex case of a concurrent disseminated infection with an HMV *K. pneumoniae* and an OXA-181 XDR *K. pneumoniae* in a patient recently hospitalized in India. We demonstrate the evolution of strains under selective antibiotic pressure.

Patient and methods

Patient—clinical context

The patient is a 44-year-old man with liver cirrhosis who recently returned to the USA after a 2 month visit to India, which included a 7 day hospitalization for injuries related to a fall. Shortly after returning to the USA, he was hospitalized with fevers and right upper quadrant pain and was found to have a highly susceptible *K. pneumoniae* bacteraemia and right-sided pneumonia with an associated uncomplicated parapneumonic effusion causing respiratory distress. His parapneumonic effusion was drained via a chest tube and he was treated with intravenous cefepime for 4 days, intravenous ampicillin/sulbactam for 8 days, oral ciprofloxacin for 4 days and oral amoxicillin/clavulanate for 10 days.

Twenty days after antibiotic cessation, he re-presented with fevers, seizures and respiratory distress. Blood cultures were again positive with a highly susceptible *K. pneumoniae*. CT imaging of his head demonstrated mild hyperattenuated lesions reported as intraparenchymal haemorrhages in the right thalamus and parietal lobe. On hospital day 3, a *K. pneumoniae* isolate that tested intermediate to cefazolin, cefoxitin and tetracycline was isolated from the patient's sputum. On hospital day 8, an XDR *K. pneumoniae* resistant to all tested antibiotics on the standard antimicrobial susceptibility testing (AST) panel was recovered from a further sputum sample. Due to persistent fevers and seizures, repeat brain imaging was performed. A 3.6 cm brain abscess was found in his right thalamus, which was unamenable to drainage. CT imaging of his abdomen demonstrated a 5.4 cm renal abscess, which was drained. Multiple bacterial cultures from blood, sputum, stool and renal abscess sampled during his admission displayed an unusual pattern of antibiotic susceptibilities, fluctuating from highly susceptible to MDR and XDR (Table 1). In addition, some of the *K. pneumoniae* isolates were HMV, as defined by positive string test [Table 1 and Figure S1 (available as Supplementary data at *JAC* Online)]. Due to ongoing clinical deterioration, with XDR *K. pneumoniae* bacteraemia, despite multiple weeks of antibiotic therapy, the patient was discharged to hospice care. A summary of his clinical course, fever curve, antibiotic coverage and bacterial isolates from his second hospitalization is found in Figure 1. Remarkably, at the time of writing this manuscript, he is living at home and remains infection-free and off antibiotics for over 18 months from the time of his transfer to hospice care. Consent from the patient was obtained to present the case report.

Methods

Clinical cultures

Clinical specimens were processed following standard operating procedures based on specimen type and included inoculation onto tryptic soy agar with 5% sheep's blood and MacConkey agar for recovery of Gram-negative organisms. Isolates were identified by MALDI-TOF MS (Bruker Daltonics Inc., Billerica, MA, USA) and AST performed using the BD Phoenix™ Automated Instrument (Becton Dickinson, Sparks, MD, USA). AST for tigecycline and ceftazidime/avibactam was performed using Etests (bioMérieux, Marcy-l'Étoile, France) and AST for colistin was performed using broth macrodilution and results were interpreted based on CLSI or FDA interpretive criteria.[7] Isolates were considered MDR if non-susceptible to $\geq$1 agent in $\geq$3 antibiotic categories and XDR if non-susceptible to $\geq$1 agent in all but $\leq$2 categories.[8]

String test

The string test was considered indicative of an HMV *K. pneumoniae* isolate if the inoculation loop was able to generate a viscous string $\geq$5 mm in length while stretching bacterial colonies away from the agar plate (Figure S1).[2]

Detection of carbapenemase production

All carbapenem-resistant isolates were further tested using the phenotypic modified Carbapenem Inactivation Method (mCIM), to determine whether carbapenem resistance among the clinical isolates was due to carbapenemase production or non-carbapenemase mechanisms.[7,9,10] To determine the genotypes of the carbapenemase-producing isolates, the Check-MDR CT103XL assay (Check-Points, Wageningen, The Netherlands) was performed on isolates positive by the mCIM.[11]

Infection control surveillance cultures

Additional case-finding strategies included a point prevalence survey of hospitalized patients who had epidemiological contact with the index patient. Rectal, wound, urine and/or sputum surveillance cultures were collected, as per the CDC recommendations, from 33 patients.[12] Contact precautions were initiated pending negative surveillance culture results. Additionally, 10 high-touch surfaces from the patient room were sampled using a liquid Amies Elution Swab (Eswab, Copan) and cultured for carbapenem-resistant Enterobacteriaceae (CRE) using the CDC broth enrichment method.[13]

WGS

WGS was performed using both second-generation Illumina MiSeq (Illumina, San Diego, CA, USA) short-read sequencing and third-generation Oxford Nanopore MinION (Oxford, UK) long-read sequencing technologies. For details regarding the sequencing and analysis methods please see the Supplementary Methods and Tables S1 and S2.

Transformant studies

To determine the phenotype conveyed by acquisition of the $bla_{OXA-181}$-bearing ColKP3, plasmid transformant studies were performed as previously described.[14] Briefly, transformants were obtained by electroporation (Gene Pulser, 1.7 kV, 25 μF, 200 ohms) of 3 μL of total plasmid DNA with 50 μL of electrocompetent NEB 5-alpha *Escherichia coli* recipient and selected using agar plates containing 100 mg/L ampicillin. AST was performed on the recipient *E. coli* with and without the plasmid. Confirmation of acquisition of the $bla_{OXA-181}$ gene was performed by the phenotypic mCIM and by the Check-MDR CT103XL assay, as described above.

Results

Characterization of the K. pneumoniae clinical and environmental isolates

Table 1 and Figure 1 summarize the timeline of the *K. pneumoniae* isolates acquired during the patient's hospitalization. The *K. pneumoniae* isolates recovered from clinical cultures were highly mucoid and appeared phenotypically similar based on growth characteristics, whereas the AST profiles varied from highly susceptible to resistant to almost all antibiotics tested (Table 1). Isolates that demonstrated susceptibility to most antibiotics tested were also string test positive. It was unknown during the patient's hospitalization whether there were one or multiple strains of *K. pneumoniae* causing the patient's infection or whether the isolates were gaining or losing an MDR plasmid depending on selective pressure from the treatment regimens. Also, we were unable to recover both strains from the same source at the same time. It is unclear if this was due to the HMV and XDR strains causing concurrent but separate infections or whether the similar phenotypic appearance resulted in the isolation of one isolate per culture even though both may have been present. XDR isolates were determined to be positive for the carbapenemase $bla_{OXA-48-like}$ and the ESBL $bla_{CTX-M-15}$ by the Check-MDR CT103XL assay. Of the 10 environmental sites sampled, the countertop of

Table 1. Antibiotic susceptibility profiles and characteristics of the *K. pneumoniae* strains isolated throughout the patient's hospital course

	Isolate #, source and hospital day of isolation						
	#1 blood (day 1)	#2 sputum (day 3); #5 renal abscess (day 32); #6 renal abscess (day 32)	#3 sputum (day 8); #4 sputum (day 24); #10 blood (day 56)	#7 renal abscess (day 39)	#8 and #9 stool surveillance (day 45); #11 and #12 environmental isolates	*E. coli* transformant with ColKP3 plasmid bearing OXA-181	electrocompetent NEB 5-alpha *E. coli* with no plasmid
String test	positive	positive	negative	positive	negative	negative	negative
MLST	ST23	ST23	ST147	ST23	ST147	NA	NA
mCIM	negative	negative	positive	positive	positive	positive	negative
AST results - MIC (mg/L) and breakpoint interpretation or epidemiological cut-off value							
ampicillin/sulbactam	8/4 (S)	8/4 (S)	>16/8 (R)	>16/8 (R)	>16/8 (R)	>16/8 (R)	4/2 (S)
piperacillin/tazobactam	4/4 (S)	4/4 (S)	>64/4 (R)	>64/4 (R)	>64/4 (R)	32/4 (I)	≤2/4 (S)
cefazolin	≤1 (S)	4 (I)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	2 (S)
cefoxitin	≤4 (S)	16 (I)	>16 (R)	>16 (R)	>16 (R)	≤4 (S)	≤4 (S)
ceftriaxone	≤1 (S)	≤1 (S)	>32 (R)	2 (I)	>32 (R)	≤1 (S)	≤1 (S)
ceftazidime	≤0.5 (S)	≤0.5 (S)	>16 (R)	>16 (R)	>16 (R)	≤0.5 (S)	≤0.5 (S)
cefepime	≤1 (S)	≤1 (S)	>16 (R)	≤1 (S)	>16 (R)	≤1 (S)	≤1 (S)
aztreonam	≤2 (S)	≤2 (S)	>16 (R)	≤2 (S)	>16 (R)	≤2 (S)	≤2 (S)
meropenem	≤0.5 (S)	≤0.5 (S)	>8 (R)	4 (R)	>8 (R)	≤0.5 (S)	≤0.5 (S)
ertapenem	≤0.25 (S)	≤0.25 (S)	>1 (R)	>1 (R)	>1 (R)	1 (I)	≤0.25 (S)
imipenem	0.25 (S)	0.25 (S)	>32 (R)	ND	>32 (R)	0.25 (S)	0.25 (S)
trimethoprim/sulfamethoxazole	≤0.5/9.5 (S)	1/19 (S)	>2/38 (R)	>2/38 (R)	>2/38 (R)	≤0.5/9.5 (S)	≤0.5/9.5 (S)
tetracycline	≤2 (S)	8 (I)	>8 (R)	8 (I)	>8 (R)	≤2 (S)	≤2 (S)
gentamicin	≤2 (S)	≤2 (S)	>8 (R)	≤2 (S)	>8 (R)	≤2 (S)	≤2 (S)
tobramycin	≤2 (S)	≤2 (S)	>8 (R)	≤2 (S)	>8 (R)	≤2 (S)	≤2 (S)
amikacin	≤8 (S)	≤8 (S)	>128 (R)	≤8 (S)	>128 (R)	≤8 (S)	≤8 (S)
ciprofloxacin	≤0.5 (S)	1 (S)	>2 (R)	2 (I)	>2 (R)	≤0.5 (S)	≤0.5 (S)
tigecycline	0.5 (S)	2 (S)	2 (S)	2 (S)	2 (S)	0.125 (S)	0.125 (S)
colistin	0.5 (WT)	0.5 (WT)	0.25 (WT)	0.25 (WT)	>16 (NWT)	0.06 (WT)	0.06 (WT)
chloramphenicol	8 (S)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	16 (I)	8 (S)
ceftazidime/avibactam	0.125 (S)	0.125 (S)	2 (S)	0.5 (S)	1 (S)	0.125 (S)	0.125 (S)

S, susceptible; I, intermediate; R, resistant; NWT, non-WT; NA, not applicable; ND, not determined.

Isolates 3, 4 and 8–12 were identified as XDR due to resistance to all antimicrobial categories tested with the exception of polymyxins (colistin; isolates 8, 9, 11 and 12 did acquire resistance and were defined as non-WT) and glycylcyclines (tigecycline). Ceftazidime/avibactam was not included in the definition in accordance with Magiorakos *et al.*[8]

Isolate 7 was identified as MDR as the isolate was resistant to at least one agent in nine categories (a minimum of three categories are required to meet the MDR definition).[8] The AST profile at the time of obtaining the clinical culture was repeated due to the unusual profile (i.e. reduced susceptibility to certain cephalosporins but resistant to the carbapenems). The AST profile was confirmed upon repeat.

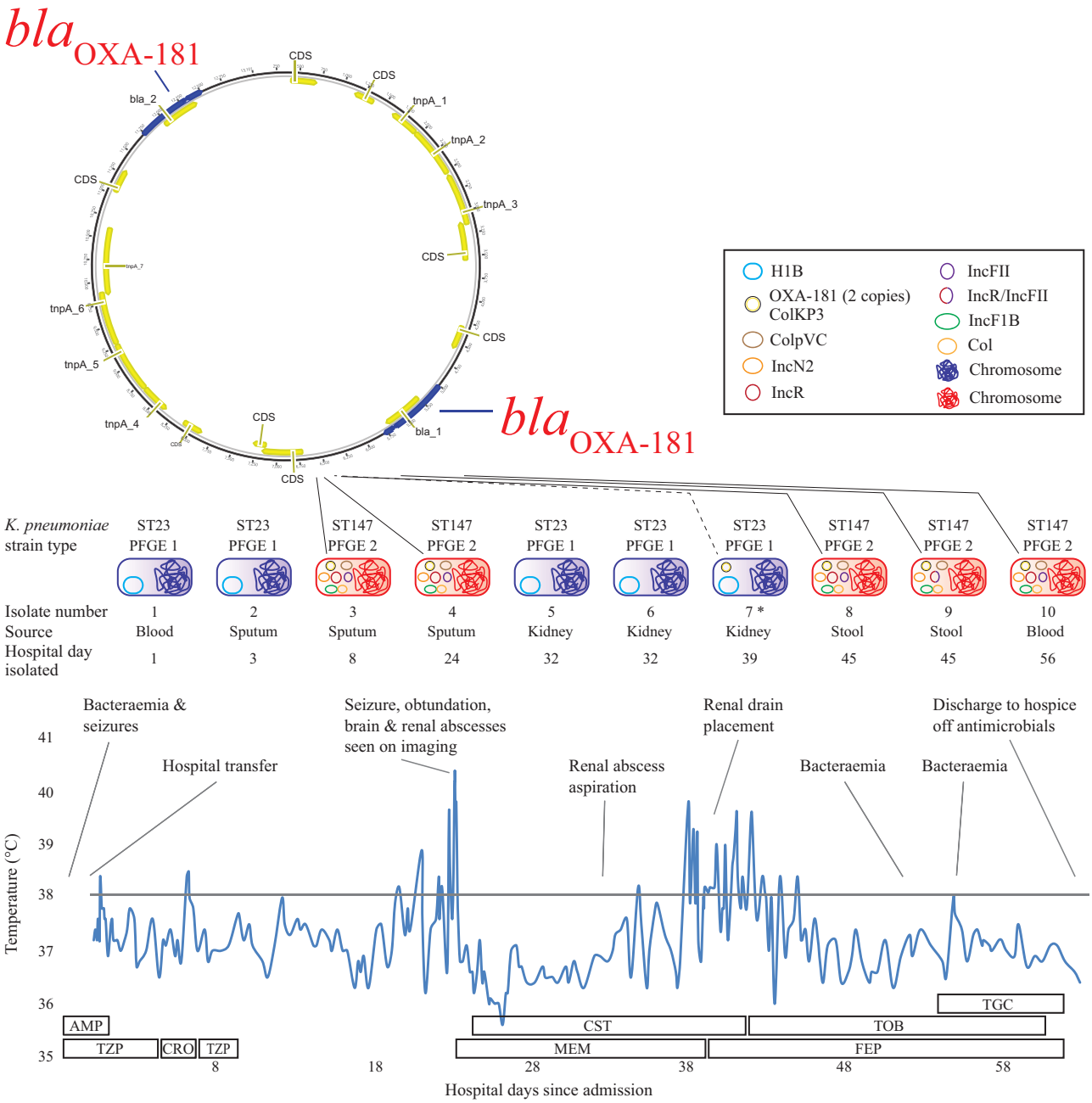

Figure 1. Summary of the patient's second hospital course with a timeline of events, fever curve, Gram-negative antibiotics and timing and location of isolates. *For isolate #7, from the kidney abscess drain (day 39), the AST profile was consistent with acquisition of the $bla_{OXA-181}$-bearing ColKP3 plasmid by the hypervirulent ST23 strain. The AST profile at the time of obtaining the clinical culture was repeated due to the unusual profile (reduced susceptibility to certain extended-generation cephalosporins but resistant to the carbapenems). The AST profile was confirmed upon repeat and the isolate was found to be mCIM positive (positive for carbapenemase production). Unfortunately, by the time WGS was pursued the plasmid was lost *in vitro* and we were unable to demonstrate acquisition of the plasmid. AMP, ampicillin; CRO, ceftriaxone; CST, colistin; FEP, cefepime; MEM, meropenem; TOB, tobramycin; TGC, tigecycline; TZP, piperacillin/tazobactam.

the patient's room was positive for two XDR *K. pneumoniae* strains. Fortunately, no transmission events were identified.

Phylogenetic analysis of strains (MLST and SNP analysis)

WGS analysis revealed that there were two unrelated *K. pneumoniae* strains isolated from the patient (Figure 2): one strain correlating with the HMV *K. pneumoniae* ST23 and one strain correlating with the XDR *K. pneumoniae* ST147. The complete genomes of the HMV *K. pneumoniae* and the XDR *K. pneumoniae* were ~5.4 Mb in size with >99% identity to the HS11286 *K. pneumoniae* reference genome. The XDR *K. pneumoniae* was most closely related to *K. pneumoniae* MS6671, which was isolated from a patient that was hospitalized in the United Arab Emirates and a *K. pneumoniae*

SKGH01 posted to the National Center for Biotechnology Information (NCBI) database by Sheikh Khalifa General Hospital in the United Arab Emirates.[15] The HMV *K. pneumoniae* was most closely related to ED23, an ST23 HMV *K. pneumoniae*, which was isolated from patients with primary liver abscess and metastatic meningitis in Taiwan (Figure S2).[16]

SNP analysis using the assembled short-read MiSeq data only or the hybrid MiSeq corrected long-read Nanopore data for all strains revealed that the HMV ST23 strains evolved by up to 4 SNPs/genome, while the XDR ST147 strains evolved by up to 15 SNPs/genome over the patient's hospitalization (Figure 2 and Figure S3). Differences in SNP results were observed between MiSeq and Nanopore assemblies as highlighted in Figure 2.

Plasmid analysis—WGS and transformant studies

WGS revealed up to seven circular plasmids carried by the individual strains. The HMV *K. pneumoniae* strains harboured an IncH1B plasmid (~230 000 bp) with no associated resistance genes but harboured many of the virulence factor genes (as described below; Table S3). The XDR strains harboured up to seven plasmids including $bla_{OXA-181}$-bearing ColKP3 (~13 000 bp), ColpvC (78 000 bp), Col (~3000 bp), IncF1B (~118 000 bp), $bla_{CTX-M-15}$-bearing IncR (~82 000 bp), IncN2 (55 000 bp) and IncFII (~100 000 bp) plasmids. The IncR and IncFII plasmids were identified as MDR plasmids and had variable copy numbers of certain resistance genes among the XDR isolates [*aac(6′)Ib-cr*, *rmtF*, *dfrA14*; Table S4]. Furthermore, plasmid diversification was observed by the fusion of the IncR and IncFII plasmids (~180 000 bp) in isolate #9 where long reads from Nanopore sequencing spanned the fusion areas.

Transformant studies support the likelihood that the HMV strain acquired the ColKP3 plasmid harbouring $bla_{OXA-181}$ from the XDR *K. pneumoniae* strain providing a similar resistance profile to the transformant (isolate #7 and transformant; Table 1). The ColKP3 plasmid bearing $bla_{OXA-181}$ provided elevated MICs of ampicillin/sulbactam, piperacillin/tazobactam, cefazolin and ertapenem.

Detection of antibiotic resistance and virulence genes by WGS

The associated antibiotic resistance genes and their location (i.e. chromosomal versus plasmid-mediated) are summarized in Table S4. Plasmid-mediated antimicrobial resistance genes identified among the XDR strains included the β-lactamase genes bla_{TEM-1}, $bla_{CTX-M-15}$ and $bla_{OXA-181}$ and those encoding aminoglycoside [*aac(6′)Ib-cr*, *APH(3′′)Ib*, *APH(6)Id*, *rmtF*], rifampicin (*arr-2*), chloramphenicol (*cat*, *catII*), trimethoprim (*dfrA12*, *dfrA14*) and fluoroquinolone [*aac(6′)Ib-cr*, *qnrB1*] resistance among others. Chromosomally encoded bla_{SHV11} (narrow-spectrum β-lactamase in *K. pneumoniae*), *oqxA/oqxB* (efflux pumps) and *fosA5* (fosfomycin resistance) genes were identified among all HMV and XDR isolates. Chromosomal mutations in *gyrA* and *parC* encoding fluoroquinolone resistance were observed among XDR strains. Mobilization of $bla_{CTX-M-15}$ by IS*Ecp1* resulted in disruption and inactivation of *ompK35* in the XDR strains leading to further carbapenem resistance. Mutations in *ompK36* were observed among both the HMV and XDR strains. Lastly, while under the selective pressure of colistin, the XDR strains developed colistin resistance via a 2 bp insertion of adenine and guanine at position 120 of the *mgrB* gene, a negative regulator of the *phoPQ* two-component regulatory system. Mutations in *mgrB* result in constitutive expression of LPS-modifying genes yielding colistin resistance.

Virulence factor genes are summarized in Table S3. The HMV strains harboured multiple virulence genes for iron-scavenging (aerobactin, salmochelin and yersiniabactin), for the K1 capsular type and for the HMV phenotype (*rmpA/A2*) where the majority were harboured on the H1B plasmid. In contrast, the XDR isolates harboured few virulence factors (yersiniabactin and a type III fimbrial adhesion) that were chromosomally encoded.

Discussion

We report a case of a patient with a concurrent disseminated infection with a highly virulent HMV *Klebsiella pneumoniae* and an OXA-181-producing XDR *K. pneumoniae* with recent hospitalization in India. The co-acquisition of the HMV and XDR strains most likely occurred while the patient was hospitalized in India. This is concerning as HMV isolates have a propensity to cause abscesses and metastatic infections among both healthy and immunocompromised individuals,[2] and isolates harbouring $bla_{OXA-181}$ are often resistant to most available antibiotics.[17] The combination of these two traits is a significant public health concern.

To date, there are sporadic reports in the USA of OXA-48-like carbapenemase-producing Enterobacteriaceae and most have been associated with exposure to the healthcare setting outside the USA, in particular the Middle East or South Asia, as seen in this case.[17] Reports of HMV strains have generally been isolated to Asia but are increasingly reported worldwide.[2,3] Until recently, the majority of HMV *K. pneumoniae* strains have been described as susceptible to most antibiotics, with reports of ESBL-positive and occasional carbapenem-resistant HMV *K. pneumoniae* strains (mostly KPC producers) localized to China with a few cases from India and South America.[3,18–22] Recently, there was a fatal outbreak of ST11, hypervirulent KPC-2-producing *K. pneumoniae* reported from a Chinese hospital.[4] To date, there is only one previous report of two HMV isolates acquiring a $bla_{OXA-48-like}$ gene.[23] To the best of our knowledge, this is the first report of a carbapenem-resistant HMV *K. pneumoniae* in the USA.

In the case of our patient, their highly susceptible HMV *K. pneumoniae* strain became resistant to carbapenems while sparing most of the extended-generation cephalosporins, providing an AST profile consistent with the acquisition of the $bla_{OXA-181}$ gene harboured on the ColKP3 plasmid. Zhang *et al.*[22] published a similar phenomenon of HMV *K. pneumoniae* strains acquiring bla_{KPC-2} under the selective pressure of imipenem exposure. Unfortunately, we were unable to demonstrate acquisition of the plasmid by WGS as the plasmid was lost on further subculture and had reverted to the susceptible phenotype by the time follow-up studies were completed. Transformant studies did support the likelihood that the strain acquired the ColKP3 plasmid harbouring $bla_{OXA-181}$ from the concurrent XDR *K. pneumoniae* strain providing a similar resistance profile to the transformant. Potron *et al.*[24] previously demonstrated the association of $bla_{OXA-181}$ with the small ColKP3 plasmid scaffold and demonstrated the broad host-range specificity of the plasmid through successful transformant studies with *Pseudomonas aeruginosa*.

(a)

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	HS11286
1		2	31914	31912	4	2	4	31914	31913	31909	31917	31915	30221
2	2		31912	31910	2	4	2	31912	31911	31907	31915	31913	30219
3	31914	31912		2	31910	31914	31910	12	3	7	5	5	29507
4	31912	31910	2		31910	31912	31910	10	1	5	5	3	29505
5	4	2	31910	31910		4	0	31912	31911	31907	31913	31913	30221
6	2	4	31914	31912	4		4	31914	31913	31909	31917	31915	30223
7	4	2	31910	31910	0	4		31912	31911	31907	31913	31913	30221
8	31914	31912	12	10	31912	31914	31912		9	15	15	13	29507
9	31913	31911	3	1	31911	31913	31911	9		6	6	4	29506
10	31909	31907	7	5	31907	31909	31907	15	6		10	8	29504
11	31917	31915	5	5	31913	31917	31913	15	6	10		2	29510
12	31915	31913	5	3	31913	31915	31913	13	4	8	2		29508
HS11286	30221	30219	29507	29505	30221	30223	30221	29507	29506	29504	29510	29508	

(b)

Isolates	1	2	4	6	7	8	9	10	12	HS11286
1		0	31516	2	1	31518	31517	31516	31518	29461
2	0		31516	2	1	31518	31517	31516	31518	29461
4	31516	31516		31518	31517	3	1	3	3	28957
6	2	2	31518		1	31520	31519	31518	31520	29461
7	1	1	31517	1		31519	31518	31517	31519	29460
8	31518	31518	3	31520	31519		2	4	4	28958
9	31517	31517	1	31519	31518	2		4	4	28958
10	31516	31516	3	31518	31517	4	4		4	28956
12	31518	31518	3	31520	31519	4	4	4		28958
HS11286	29461	29461	28957	29461	29460	28958	28958	28956	28958	

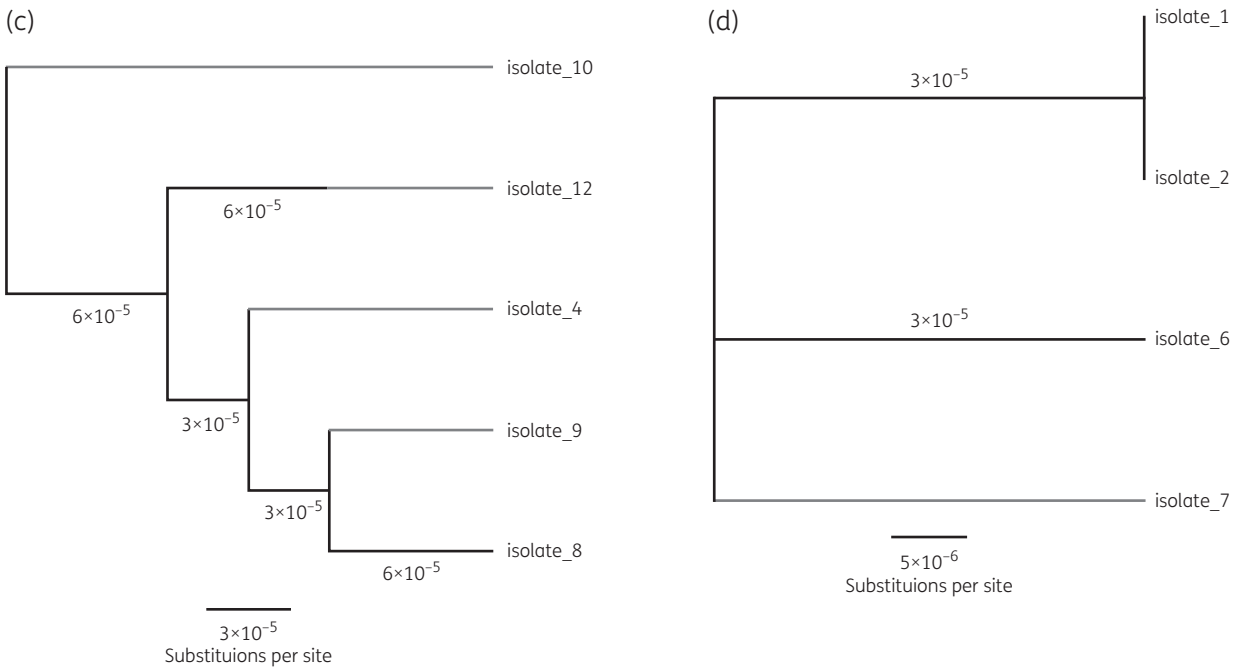

Figure 2. Assessing the genetic relatedness of the *K. pneumoniae* strains by WGS, including SNP analysis using the SPAdes assembled MiSeq short read data (a; number of bp substitutions); SNP analysis using Pilon MiSeq corrected long read Nanopore data (b) with corresponding scaled phylogenetic trees of the core-genome alignment of the XDR OXA-181-producing *K. pneumoniae* (c) and the HMV *K. pneumoniae* (d) strains.

Importantly, the HMV strain in this study was unable to preserve the resistance phenotype without the selective pressure of meropenem and it lost the plasmid on further subcultures. Similarly, Huang *et al.*[25] demonstrated the *in vitro* loss of bla_{NDM-1} by *K. pneumoniae* with the removal of selective pressures, which occurred through reduced copy number of the bla_{NDM}-bearing pKPX-1 plasmid or the loss of bla_{NDM-1} via directed repeat-mediated slippage. Another study demonstrated the possible *in vivo* loss of bla_{NDM} in *K. pneumoniae* through a 5 kb deletion on the bla_{NDM-7}-harbouring IncX3 plasmid.[26] These studies suggest that there can be a negative impact on the fitness of *K. pneumoniae* if they harbour certain carbapenemase genes and/or carbapenemase-encoding

plasmids and that acquisition of these genes can be relatively unstable in the absence of selective pressure. The instability may be linked to many factors including the *K. pneumoniae* strains, plasmid incompatibility groups and/or specific carbapenemase genes. Nevertheless, environments allowing for prolonged selective pressures may result in the further evolution of these strains to become fit to harbour carbapenemase genes in the absence of selective pressures. This case provides a strong argument for the judicious use of antibiotics in order to reduce the development and spread of antibiotic resistance. This is further supported by the remarkable recovery of the patient while off antibiotics as they were discharged to hospice care due to the poor prognosis given repeated bloodstream infections.

The XDR phenotype among strains was linked to two MDR plasmids (IncR and IncFII), the $bla_{\text{OXA-181}}$-bearing plasmid (ColKP3) and chromosomal mutations (*ompK35*, *ompK36*, *gyrA*, *parC*, ±*mgrB*). The hypervirulent HMV phenotype was linked to an IncH1B plasmid harbouring many of the associated virulence factor genes including the capsular polysaccharide regulator genes responsible for the HMV phenotype (*rmpA/A2*) and multiple iron-scavenging systems (aerobactin, salmochelin and yersiniabactin) among other chromosomally located virulence factors. In light of the multiple virulence factors associated with this patient's HMV *K. pneumoniae* isolate, it is not surprising that they had an aggressive infection with multi-organ involvement. The concurrent infection with both an HMV isolate and an XDR *K. pneumoniae* can make for a highly virulent combination of epidemiological concern, as was observed in our patient.

WGS was applied to all isolates recovered throughout the 3 month hospital course to demonstrate the changes that occurred in the resistome over time. WGS revealed plasmid diversification (fusion of the IncR and IncFII plasmids), mobilization of resistance elements (*ompK35* inactivation by IS*Ecp1*-$bla_{\text{CTX-M-15}}$ mobilization, varying numbers of resistance genes on plasmid scaffolds) and chromosomal mutations (mutations in *mgrB* leading to colistin resistance) leading to further antibiotic resistance. For the most part, these antibiotic resistance changes could be linked to selective antibiotic pressure (i.e. the likely acquisition of the $bla_{\text{OXA-181}}$ by the HMV strain during exposure to meropenem and chromosomal mutations in *mgrB* during exposure to colistin). This case demonstrates the evolution of the resistome in these strains under selective antibiotic pressure.

Tracing the mutational profile revealed that the isolates varied by up to 15 SNPs in the genome between related strains, while differences in results were observed when interpreting MiSeq versus Nanopore data. As previously suggested by Yang *et al.*,[27] these differences in results and interpretation of SNPs support the need for the development of standards for utilizing SNP analysis for epidemiological and diversification assessments of strains. Furthermore, SNP analysis revealed the potential source of isolates, linking clinical isolates recovered from this patient as the likely source of environmental contamination [i.e. the sputum isolate was most closely related (two SNP differences) to the environmental isolate]. WGS has been demonstrated as a powerful tool in outbreak investigations allowing linking of patients and mapping transmission events.[27,28] We further demonstrate the power of WGS both as an epidemiological tool but also as a tool to track evolution of antibiotic resistance among strains undergoing selective antibiotic pressure.

In summary, we present a complex case of a concurrent infection with an HMV *K. pneumoniae* and an XDR OXA-181-producing *K. pneumoniae* from a patient with prior hospitalization in India. Through WGS, we demonstrate the changes that occurred in the resistome of the isolates were linked to the selective pressures of treatment. Ultimately, this case demonstrates the role of antibiotic pressure in the acquisition and loss of important genetic elements leading to XDR bacteria. Furthermore, we demonstrate the power of WGS as a tool to track antibiotic resistance among strains undergoing selective antibiotic pressure.

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Transparency declarations

P. J. S. and W. T. have received travel funds from Oxford Nanopore to speak at their user group meetings. In addition, W. T. has two patents (US20110226623 A1 and US20120040343 A1) that are licensed to Oxford Nanopore. All other authors: none to declare.

Supplementary data

Figures S1 to S3, Supplementary Methods and Tables S1 to S4 are available as Supplementary data at *JAC* Online.

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