CASE SERIES

Nanopore Genomic Analysis of Multidrug-resistant *Klebsiella pneumoniae* from Critically Ill Patients: A Case Study

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Abstract

Aim and background: To genomically characterize pathogenic multidrug-resistant (MDR) *Klebsiella pneumoniae* from clinical samples from patients in intensive care units, and study its diversity, resistance determinants, resistance patterns, and ARGs from critically ill patients of a semi-rural hospital in a low- and middle-income country (LMIC) with basic bioinformatics expertise. Whole genome sequencing (WGS) for MDR clinical pathogens is a promising approach in critically ill patients where treatment success is essential for patient survival.

Materials and methods: MDR *K. pneumoniae* isolated by conventional microbiological methods in a single hospital in India were subjected to whole genome sequencing to retrospectively analyze three randomly chosen, VITEK-confirmed clinical isolates. Two were AmpC, β-lactamase, and carbapenemase producers, and one was a carbapenemase-producing *K. pneumoniae* clinical pathogen isolated from patients in the ICU. DNA was extracted from these isolates and subjected to WGS. Genomic data were analyzed using the EPI2ME cloud platform.

Results: WGS identified the clinical isolates as *Klebsiella pneumoniae* spp. pneumoniae with cases 1 and 2 as AmpC, β-lactamase, and carbapenemase producers, and case 3—in addition—was a carbapenemase producer with NDM-1, CTXM-15, and OXA-232. Hence, WGS provided a better understanding of AMR mechanisms for improved treatment options.

Conclusion: Three cases of *K. pneumoniae* isolated from critically ill patients were subjected to whole-genome sequencing to detect and confirm AmpC and carbapenemase production along with their causative antibiotic resistance genes, giving critical information about treatment failures. This was our preliminary step toward making high-end diagnostic genomics accessible for critical patient care in a tertiary care hospital.

Keywords: Case report, *Klebsiella pneumoniae*, Multidrug resistance, Nanopore technology, Whole genome sequencing.

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Introduction

The emergence of antimicrobial resistance (AMR) has created "superbugs" that make treating basic infections difficult (and in some cases impossible) and surgery risky. Emergence of resistance in microorganisms is a continuous phenomenon; its amplification and spread are the result of human behavior.[1] *Klebsiella pneumoniae*, classified as an ESCAPE organism, is an important pathogen in this era of antimicrobial resistance, with resistance to multiple classes of antibiotics.[2,3] It is a pathogen capable of causing severe and fatal disease along with other important carbapenemase producers such as *Escherichia coli*.[4] It is a part of the normal flora of the gut microbiome of healthy humans and animals, in addition to being an opportunistic healthcare-associated pathogen. It accounts for about one-third of all Gram-negative infections.[5]

Extended-spectrum cephalosporins in the early 1980s were a major addition in the fight against β-lactamase-mediated AMR. Hydrolytic enzymes called extended spectrum β-lactamases (ESBLs), which destroy cephalosporins have led to a major drawback in therapeutic options. These plasmid-mediated enzymes that confer bacterial resistance to penicillin are commonly found in *Klebsiella* spp. and *E. coli*.[6]

AmpC enzymes, which also hydrolyze cephalosporins and penicillin, are found in *E. coli* and *K. pneumoniae*. AmpC genes are encoded by chromosomes or plasmids; the plasmid-mediated pathogens in particular can be transmitted horizontally in the hospital, leading to healthcare-associated infections (HAIs).[7]

Along with *E. coli*, *K. pneumoniae* has rising rates of non-susceptibility against four major antibiotic classes, namely third-generation cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems.[8] Acquired carbapenemases such as New Delhi metallo-β-lactamases (NDM) and carbapenem-hydrolyzing oxacillinases such as OXA-48 and OXA-48-like are a cause for concern. Rampant use of antibiotics has led to carbapenem resistance, which has now spread all over the world.

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Klebsiella pneumoniae has now become resistant to several frontline antibiotics, limiting the treatment of both nosocomial and community-acquired infections.[9] They acquire antibiotic resistance through extended spectrum β-lactamase (ESBL) genes (e.g., bla_{TEM}, bla_{CTX-M} and bla_{OXA}, which confer resistance to cephalosporin and monobactam antibiotics, and genes encoding for the five major carbapenemases (e.g., bla_{NDM}, bla_{VIM} and bla_{KPC}), causing resistance to carbapenems. Carbapenemase-producing *K. pneumoniae* in patients leads to higher incidence of mortality due to lack of treatment options.[10] Both ESBL and carbapenemase genes are often located on self-transmissible plasmids, contributing to their easy spread.[11,12] Plasmid-mediated AmpC genes cause HAIs in hospital settings as they spread through *K. pneumoniae* and *E. coli*.[7]

The concept of antimicrobial surveillance started well over two decades ago in Europe.[13] Since then, they have surveillance systems in place, and strategies have been formulated and followed to rein in this silent pandemic.[14] Strategies include regular monitoring of pathogens, their infections, and susceptibility patterns.

This has not been the case in developing countries due to various challenges like low diagnostic capabilities or nonhomogeneous methods, which hamper data collection and monitoring, ultimately resulting in poor surveillance of disease trends and formulating treatment guidelines. A uniform, reliable phenotypic drug susceptibility test in diagnostic laboratories is a must to trace resistance patterns of pathogens as a part of any surveillance efforts.

High-end genomic tools such as next-generation sequencing (NGS) can help in diagnostics, surveillance, as well as clinical management of critically ill patients. Though these are the norm in developed countries, their use is limited in LMICs, which is unfortunate considering the ability of this technology to pinpoint actual mechanisms at play at the genetic level of the microorganisms and in turn impact treatment outcomes for patients.

We chose long-read sequencing in this study to see what it can bring to the table in terms of pathogen dynamics and additional information on antimicrobial resistance patterns in a semi-rural hospital in India, where antimicrobial resistance is a rising problem, adding to the conundrum of other healthcare challenges. We used whole-genome sequencing (WGS) to identify determinants of antimicrobial resistance in *K. pneumoniae* clinical isolates from patients admitted to RajaRajeswari Medical College and Hospital (RRMCH). The isolates were sequenced and analyzed at the in-house sequencing facility of the hospital's central research wing.

Materials and Methods

Study Design, Location, and Sample Collection

This was a preliminary study to characterize pathogens isolated from clinical specimens using WGS. This was conducted at RajaRajeswari Medical College and Hospital (RRMCH), a tertiary hospital catering to a semi-rural population of Bangalore, South India. Specimens were collected from critically ill patients admitted to medical and surgical departments of the hospital between 2021 and 2023 after obtaining approval from the Institutional Ethics Committee and informed patient consent. Sociodemographic data and clinical details of the study participants were recorded in a designated record.

Laboratory Methods and Data Analysis

Patient clinical samples were collected as a part of routine clinical care and transported to the Microbiology Laboratory at RRMCH for analysis by conventional microbiology culture and sensitivity by Kirby-Bauer disc diffusion method as described.[15] VITEK 2 Systems (v.9.02) was used for confirmation of antimicrobial susceptibility tests (AST) retrospectively prior to genome sequencing. At the Molecular Medicine Division of the Central Research Lab, RRMCH, the three isolates were stored at–80°C prior to DNA extraction done using the QIAamp DNA kit (Qiagen GmbH, Germany) as per manufacturer's instructions. DNA quantity and quality were assessed by NanoDrop, Qubit, and gel electrophoresis. Double-stranded DNA libraries with high molecular weight DNA were prepared and sequenced on the nanopore platform.

Sequencing Quality Control and Assembly

All Nanopore fast5 reads were base-called with 100× coverage. All isolates' Nanopore reads (original and subsampled) were de novo assembled using Flye (v2.8) (with plasmids flag), Unicycler (v0.4.8) (hybrid settings), and Canu (v2.1.1) (default). The best assembly per isolate was selected, with a preference for Flye > Unicycler > Canu when the assemblies were comparable.[16]

DNA Library Preparation and Genome Sequencing

DNA repair and end-prep were done by NEBNext Ultra II End repair/dA-tailing module and NEBNext Quick Ligation module FFPE DNA repair mix (NEBNext® Companion module NEB, UK) along with ligation sequencing gDNA kit-SQK-LSK110 from Oxford Nanopore Technologies (ONT), UK per manufacturer's protocol. Additional purification was done using Kapa pure beads (Kapa Biosystems, Roche, AG), along with ligation sequencing per manufacturer's protocol. The prepared library was sequenced using MinION flow cells (FLO-MIN106D R9 version, ONT) on the MinION1kb device (ONT). Sequencing data were collected after samples passed quality control and were base-called and aligned online through the MinKNOW software v.3.6.5 (ONT).[17]

Antimicrobial Resistance Gene (ARG) Identification

Antimicrobial resistance genes (ARGs) were identified using ResFinder (version 2.1.22) tools available online from the Centre for Genomic Epidemiology (CGE) at the Technical University of Denmark and determined using hybrid assemblies and tools from cgview v.1.7 accessed on 18/1/2025 (http://cge.cbs.dtu.dk/services/).[17] Antimicrobial resistance genes (ARGs) were analyzed using the Comprehensive Antibiotic Resistance Database (CARD) (date: 30 Oct 2023).[18]

Species Identification

In addition to Kraken 2,[19] species identification was done against reference Klebsiella species (NCBI) and identified as *K. pneumoniae* spp. pneumoniae and deposited at GenBank, National Centre for Biotechnology Information (NCBI), USA.[20]

Ethics

Ethical approval for the study was obtained from the Institutional Ethics Committee, RRMCH. RRMCH-IEC/65/2021.

Results

Demography of Study Participants

The participants of the study were between 18 and 75 years of age. They were inpatients admitted to the hospital. Causes included emergency visits, surgery, and sepsis. Length of stay ranged between 48 hours and 60 days. One participant discharged himself against medical advice, the second died after 30 days of hospitalization, and the third recovered postsurgery as shown in Table 1.

Antimicrobial Resistance Pattern Using Conventional Microbiology

Antimicrobial sensitivity (AST) was tested by Kirby-Bauer's disk diffusion method. It was retrospectively confirmed by VITEK AST against a panel of twenty-one antimicrobial agents prior to genome sequencing. All three isolates were identified as *K. pneumoniae* spp. pneumoniae. MIC was recorded. Resistance was seen against β-lactam (BL) and β-lactam inhibitor (BLI) group of drugs, except in patient 1, whose sputum sample showed intermediate resistance, and patient 2, whose urine sample was sensitive to cefoperazone-sulbactam on VITEK. Resistance was also seen against carbapenems in all three patients. In addition, notable resistance was also found against third-generation, fourth-generation cephalosporins, quinolone, and fluoroquinolone groups of drugs in all three clinical isolates. Sensitivity was seen only with tigecycline, colistin, and fosfomycin in urine isolates. The antibiotic sensitivity pattern is shown in Table 2.

Antimicrobial Genes

- Long-read sequencing was able to identify all the antimicrobial genes conferring resistance to different antibiotic groups as presented in Table 3. They were present either in the plasmid or

Table 1: Sociodemography of patients associated with three *K. pneumoniae* isolates from in-patients of RRMCH

ID	*Age*	*Ward*	*Gender*	*Diagnosis*	*Source of sample*	*Length of stay (days)*	*Patient outcome*
Case 1	33	MICU	M	Lower lobe pneumonia with septic Shock with MODS	Sputum	8	Discharged against medical Advice
Case 2	72	Neurosurgery	M	Vestibular schwannoma	Urine	31	Death
Case 3	35	Surgery	M	Adenocarcinoma of ascending colon	Urine	7	Recovery

Table 2: Phenotypic antimicrobial resistance profile of *K. pneumoniae* isolates by Kirby-Bauer's disk diffusion method and VITEK

	P1 sputum		*P2 urine*		*P3 urine*	
Antimicrobial	*Kirby-Bauer*	*Vitek*	*Kirby-Bauer*	*Vitek*	*Kirby-Bauer*	*Vitek*
			β-lactams (BL)			
Ampicillin	R	R	R		R	
Cephalosporins						
Cefuroxime	R	R		R		R
Cefuroxime axetil	–	R		R		R
Ceftriaxone	–	R	R	R	R	R
Cefepime	–	R		R		R
Carbapenems						
Imipenem	R	R	R	R	R	R
Meropenem	R	R	R	R	S	R
Ertapenem	R	R	R	R	R	R
Monobactam						
Aztreonam			R		R	
			β-lactam inhibitors (BLI)			
Amoxicillin + Clavulanic acid	R	S	R	I	R	R
Piperacillin + Tazobactam	R	R	R	R	R	R
Cefoperazone + sulbactam	–	I		S	R	R
			Aminoglycosides			
Amikacin	R	S	R	R	R	I
Gentamicin	–	S	–	R	R	R
			Quinolones			
Nalidixic acid	–	R		–		–
			Fluoroquinolones			
Ciprofloxacin	R	I		R	R	R
Nitrofurantoin	–	I	R		R	
Norfloxacin			R		R	
			Others			
Fosfomycin (urine)				S		S
Tigecycline	–	S		S		R
Colistin	–	I	–	I	–	R
Cotrimoxazole	R	S	R	R		R

R, resistant; S, sensitive; I, intermediate.

Table 3: ARGs of clinical importance

Resistance to antibiotics	*Case 1 (Sputum) genes identified*	*Case 2 (Urine) genes identified*	*Case 3 (Urine) genes identified*
Beta-lactams (BL)	Beta-lactamase genes		
Ampicillin	*acrB*	*acrB*	$bla_{TEM-240}$
Cephalosporins			
Cefuroxime			
Cefuroxime axteil			
Ceftriaxone	*kpnG*	*kpnF*	
Cefepime	*kpnG*	*kpnF*	
Carbapenems	Carbapenemase genes		
Imipenem	*lptD*	*lptD*	*lptD*
Meropenem	bla_{NDM-1}	Mex-B	bla_{KPC}
Ertapenem	*kpnG*	*kpnG*	*kpnG*
Beta-lactam inhibitors (BLI)	BLI inhibitor genes		
Amoxicillin/clavulanic acid	*H. pylori* pbp1 mutants conferring resistance to amoxicillin		
Piperacillin/tazobactam	*kpnG*	*kpnG*	*kpnG*
Cefoperazone/sulbactam			
Aminoglycosides	Aminoglycoside genes		
Amikacin	*rmtB*	*rmtB*	*rmtB*
Gentamicin	*kpnG*	*acrD*	*kpnG*
Quinolones			
Nalidixic acid		*emrR*	*TolC*
Fluoroquinolones	Fluoroquinolone genes		
Ciprofloxacin	*oqxB*	*oqxB*	*oqxB*
Nitrofurantoin	*oqxB*	*oqxB*	*oqxB*
Others			
Colistin	ArnT	ArnT	eptB
Fosfomycin		FosA2	FosA6
Tigecycline	*oqxB*	*oqxB*	*oqxB*
Trimethoprim/Sulfamethoxazole	*oqxB*	*oqxB*	*oqxB*

the chromosome. Extended spectrum β-lactamase (ESBL) genes belonging to all three groups TEM, SHV, and CTX-M were found in the plasmids of all three isolates along with carbapenemase resistance genes. Isolates from case 1 and case 2 had AmpC producer genes located on the chromosome.

- ARGs causing resistance to BL-BLI group of antibiotics: acrB was responsible for resistance to ampicillin in Cases 1 and 2. Case 3, however, had $bla_{TEM-240}$, a β-lactamase gene which in addition caused resistance to piperacillin, most cephalosporins, and monobactams. Ceftriaxone and cefepime resistance were due to *kpn*G, a protein homolog found in case 1, and *kpn*F in case 2. Resistance to piperacillin-tazobactam, a BLI in all three cases, was caused by *kpn*G.
- ARGs causing resistance to carbapenem drugs: *lpt*D and *kpn*G, responsible for resistance to imipenem and ertapenem, respectively, were found in all three cases. Carbapenemase gene bla_{NDM-1} led to meropenem resistance in case 1 and Mex-B in case 2. bla_{KPC} in case 3 and OXA-48-like carbapenemase genes $bla_{OXA-181}$ and $bla_{OXA-232}$ were detected in the third case from urine.
- ARGs against aminoglycosides: *rtmb* was responsible for resistance to amikacin in all three cases. Gentamicin resistance in cases 1 and 3 was due to *kpn*G, whereas in case 2 it was due to *acr*D.
- ARGs of quinolones and fluoroquinolones: *oqx*B rendered ciprofloxacin and nitrofurantoin ineffective in all three cases. Nalidixic acid resistance was due to emrR in case 2 and TolC in case 3.
- Other ARGs: Colistin resistance was seen in all three cases, but was caused by ArnT in cases 1 and 2, whereas *ept*B was the reason in case 3. FosA2 and FosA6 found on plasmid conferred resistance to fosfomycin in cases 2 and 3. Tigecycline resistance was seen in case 1 due to the presence of *oxq*B, which was incidentally also found in cases 2 and 3. *oxq*B caused resistance to trimethoprim-sulfamethoxazole in all three cases.[18]

Discussion

The present study aimed to investigate the diversity and resistance determinants of *K. pneumoniae* isolates collected from three critically ill patients at a tertiary hospital in Southern India and to compare antimicrobial resistance patterns from long-read sequencing data with phenotypic results of conventional microbiological tests. All the cases were admitted to the ICU with long stays and were vulnerable to *K. pneumoniae* infections, which are also a major cause of hospital-acquired infections such as sepsis, pneumonia, bloodstream infections, and infections in the

newborn. The pathogen can resist several antibiotics, leading to fewer treatment options and sometimes death. The emergence of cephalosporin-hydrolyzing enzymes has hampered their use as a lifesaving therapeutic option for patients. As a healthcare-associated infection, it has the potential to spread into the community as well. Resistance in *K. pneumoniae* includes the use of restricted antibiotic treatment (carbapenem antibiotics), which has spread to all regions of the world. AMR has led to treatment failures in about 50% of patients with *K. pneumoniae* infections in some countries, especially with carbapenem antibiotics.[21] *K. pneumoniae* is a common cause of nosocomial and community-acquired infections in newborns, the elderly, and immunocompromised patients.[22] It has acquired AmpC genes encoded via the plasmid or chromosome, which can also be transmitted horizontally in a healthcare facility.[23] Also, isolates with inducible AmpC expression may initially test as susceptible to third-generation cephalosporins, which can complicate treatment decisions, especially for Enterobacterales. *Enterobacter cloacae, K. aerogenes*, and *Citrobacter freundii* should be regarded as harboring inducible AmpC, and third-generation cephalosporins should be avoided regardless of susceptibility results.[24]

All three clinical isolates showed resistance to BL-BLI group antibiotics, carbapenems, monobactams, aminoglycosides, fluoroquinolones, and restricted antibiotics by conventional Kirby-Bauer's disk diffusion antibiotic susceptibility panel testing. Hence, we chose to genetically determine true antimicrobial resistance causative factors.

Humans serve as the primary reservoir for *K. pneumoniae*. In the general community, 5–38% of individuals carry the organism in their stool, and 1–6% in their nasopharynx. The main reservoirs of infection are the patient's gastrointestinal tract and the hands of hospital personnel, which can lead to a nosocomial outbreak.

Case 1 was a retroviral and tuberculosis-positive patient admitted to the medical ICU with sepsis in multiorgan dysfunction, with lower lobe pneumonia as a focus. His sputum sample yielded MDR *K. pneumoniae* on culture. He was started on meropenem, which was not effective due to the presence of the ARG $bla_{\text{NDM-1}}$, a carbapenemase gene that also leads to resistance against doxycycline. The patient did not respond to trimethoprim/sulfamethoxazole due to the presence of *oqxB*, which resulted in resistance due to an efflux pump mechanism as shown in Table 3.

Case 2 was a 72-year-old patient who developed sepsis following bronchopneumonia after undergoing surgery for a left-sided cerebellopontine angle tumor with a vestibular schwannoma and ischemic heart disease. A urine sample sent for culture grew MDR *K. pneumoniae*. The patient was initially started on ceftriaxone, which did not work due to the presence of *kpnG*, which pumps ceftriaxone out of the cell, and amikacin wasn't effective due to *rtm*B, which prevents binding of amikacin to the bacteria and AmpC.[24] The patient was given piperacillin-tazobactam, which was again ineffective due to the gene *kpnG*. Levofloxacin did not work due to *oqx*B, which directed pumping of the antibiotic out of the cell. Meropenem was ineffective due to MexB, which transports meropenem out of the cell. As all these antibiotics were ineffective, the patient was started on colistin, which failed due to ArnT, which alters the target of colistin, thus making it ineffective. Colistin was discontinued after 2 days due to the patient's declining renal function. He could not be nebulized with colistin due to bronchopneumonia.[25] The patient did not survive sepsis and post-op brainstem oedema. Crucial information regarding ARGs could have helped this patient's management of sepsis and possibly his outcome.

Case 3 was a 33-year-old diabetic patient with ascending colon adenocarcinoma who developed a post-hemicolectomy surgical site infection. MDR *K. pneumoniae* was isolated from the patient's urine by conventional culture identification. The isolate *K. pneumoniae* spp. pneumoniae was a β-lactamase producer with the gene $bla_{\text{TEM-240}}$ and a carbapenemase producer with bla_{KPC}, *kpnG*, and *lpt*D genes, and $bla_{\text{OXA-232}}$ and $bla_{\text{OXA-181}}$ genes were also found on plasmids known for their ability to hydrolyze carbapenems. The patient was treated with linezolid and clindamycin, which were not appropriate. This patient had acquired additional genes for colistin resistance (*ept*B) and fosfomycin resistance (*fos*A6).

All three clinical isolates were sensitive to cefoperazone-sulbactam, which was not used in any of these cases, usage of which might have led to better patient outcomes.

This case series makes a point toward the utilization of genomic tools in patient care to give a better understanding of treatment options for clinicians. This was our endeavor toward making high-end diagnostic genomics accessible for patient care in a tertiary care hospital.

Conclusion

Three cases with MDR *K. pneumoniae* isolated from their clinical samples showed a wide variety of ARGs, all of which might have contributed to treatment failures in these patients. With the silent pandemic of global AMR looming large, it becomes imperative to utilize genomic tools wherever available in order to get a better understanding of circulating MDRs. This would, in turn, translate into better choices for clinicians in treating such patients in the ICU, and improved antimicrobial prescription and stewardship practices.

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