JOPAT Vol 23(2) 1461– 1471, July – December, 2024 Edition.
ISSN2636 – 5448 https://dx.doi.org/10.4314/jopat.v23i2.6

Detection of OMPKs 36/37 Porins and Other Resistance Determinants in Extended Spectrum Beta-Lactamases (ESBLs) -producing *K. pneumoniae*

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Abstract

The rise of β-lactamases and loss of porin by multidrug-resistant *Klebsiella pneumoniae* is an important global issue. This work aimed to reveal how the loss of outer membrane porin (OMP) contributes to resistance in *Klebsiella pneumoniae* to certain antibiotic drugs. In this study, standard microbiological techniques of phenotypic procedures were used to isolate *K. pneumoniae* (P10) from the hospital wastewater sample. A double disk synergy test was used for the phenotypic detection of ESBL. Whole-genome sequencing (WGS) was conducted on the Illumina platform, and the resulting raw read was de novo assembled using RAPT NCBI. The landscape of antibiotic resistance inside the genome was examined using online bioinformatics tools. The biochemical tests identified the isolate to be *K. pneumoniae* with 100% resistance to ceftazidime, cefepime, augmentin, and intermediate resistance to meropenem but was susceptible to gentamicin. The isolate was confirmed as an ESBL-producer by an enlarged inhibitory zone towards Augmentin-clavulanic acid. WGS results showed the isolate harbors bla_{TEM}, and bla_{SHV} with Ompk 36 and Ompk 37 porin loss. The *K. pneumoniae* (P10) isolate exhibited a multidrug-resistant profile characterized by ESBL production, additional resistance genes, and loss of OmpK36/37 porins, collectively conferring resistance to a wide range of antibiotics including carbapenem and posing significant challenges for clinical management and infection control.

Keywords: *Klebsiella pneumoniae*, Outer membrane Porin, Extended Spectrum Beta-Lactamases

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Introduction

One of the main concerns for global health care is the rise in the clinical occurrence of antibiotic-resistant organisms. *Klebsiella pneumoniae* (*K. pneumoniae*), a prominent opportunistic pathogen, that dwells in healthy persons' skin, throat, and intestinal tracts [1], has garnered significant attention due to its ability to produce extended-spectrum beta-lactamases (ESBLs), an enzyme which confer resistance to a wide range of beta-lactam antibiotics, including penicillins and cephalosporins. The global burden of antibiotic-resistance has been significantly increased by the emergence and rapid spread of ESBL-producing strains of *K. pneumoniae* [2]. This poses a critical challenge in clinical settings. They limit treatment options and raise the possibility of unfavorable clinical outcomes, as these bacteria are associated with severe infections such as pneumonia, urinary tract infections, and bloodstream infections, particularly in immunocompromised patients and those undergoing invasive procedures [2, 3].

The pathogenicity of *K. pneumoniae* is further exacerbated by it's ability to acquire resistance genes through horizontal gene transfer, often facilitated by plasmids and integrons, which can harbor multiple resistance determinants [4].

The World Health Organization reports that *K. pneumoniae* is becoming increasingly recognized as a priority antimicrobial-resistant (AMR) pathogen that requires innovative approaches to treatment management strategy [5]. Understanding more about the genetic makeup of these antibiotic-resistant bacteria is essential to perhaps preventing the spread of ESBL isolates.

In addition to producing ESBLs, *K. pneumoniae* uses several additional strategies, such as altering outer membrane porins, to confer resistance to a broad spectrum of antibiotics [6]. Gram-negative bacteria's outer membrane porins (OMPs) are essential parts of the membrane that allow tiny hydrophilic molecules—including antibiotics—to enter the bacterial cell, OMPs provide antibiotics an entry route to reach the periplasm [7,8]. As peptidoglycan and lipopolysaccharide serve as a crucial structural component in safeguarding cell integrity, porins likewise, act as vectors for phages and bacteriocins [6]. The porin channels appear to be the critical entry point for β-lactams, which are typically hydrophilic and charged [9].

The OmpK35 and OmpK36 are the two main porins of *K. pneumoniae*, identical to OmpF and OmpC found in *Escherichia coli* [6]. Several studies have highlighted the pivotal role of these porins in conferring resistance to various antibiotic classes, including β-lactams, carbapenems, and fluoroquinolones. The β-lactams drug class forms the basis of the antibiotic repertoire due to its ability to halt the production of bacterial cell walls. Since porins allow β-lactam antibiotics to penetrate the outer membrane of many types of Gram-negative bacteria, a deficiency or loss of porins leads to antibiotic resistance [10]. This interplay between beta-lactamase production and porin loss is critical, as it not only contributes to the survival of *K. pneumoniae* in hostile environments but also complicates the therapeutic landscape, necessitating the use of last-resort antibiotics like carbapenems [11]. The emergence of a new variant, OmpK37, has been documented, which may also contribute to the antimicrobial resistance profile of *K. pneumoniae* [12].

The outer membrane of *K. pneumoniae* acts as a barrier to antibiotic penetration, influencing the efficacy of antimicrobial agents against their intended cellular targets. As a consequence of this and other resistance mechanisms, *K. pneumoniae* infections are increasingly challenging to manage, even with the use of last-resort antibiotics. The World Health Organization has identified *K. pneumoniae* as one of the critical pathogens in the fight against antimicrobial resistance, underscoring the urgent need for effective surveillance, infection control measures, and novel therapeutic strategies to combat these multidrug-resistant organisms [13]. Understanding the phenotypic and genetic characteristics of ESBL-producing *K. pneumoniae*, particularly the role of outer membrane porins as a resistance mechanism, is essential for developing targeted interventions and improving patients outcomes in the face of this growing threat.

Whole Genome Sequencing (WGS) has emerged as a powerful tool for comprehensive genetic analysis of bacterial pathogens, offering high-resolution insights into resistance determinants and virulence factors. It facilitates a comprehensive understanding of these resistance mechanisms by providing detailed genomic information that can elucidate the genetic basis of resistance, including the identification of specific resistance genes and their associated mobile genetic elements [14]. With the increase in multidrug resistance, employing WGS for the detection of resistance genes such as ompK36/37, is particularly relevant. Therefore, this study employs WGS to detect and focus on the role of OMPs from an ESBL-producing *K. pneumoniae* strain and its drug resistance.

Materials and Methods

Sample collection and Processing

Hospital wastewater samples from a Primary Health Center in Osun State, Nigeria were collected from the source of wastewater discharge point, aseptically using 1 L sterile glass bottles, for the purpose of isolation**.** The sample was immediately transported to the laboratory in an ice box for microbiological analyses within 6 hours of collection.

Bacterial Isolation and Identification

The wastewater sample was filtered through a sterile membrane filter, 0.45 µm pore size, with the help of Millipore filtration unit (Millipore, Merck, South Africa). In duplicates, the trapped bacteria on the membrane filter were aseptically placed on a sterile and labelled MacConkey agar plate. The plate was incubated at 37 °C for 24 hours for bacterial enumeration. Afterwards, the appearance of pink mucoid colonies on MacConkey agar, an indication of lactose fermentation, was assumed *Klebsiella* spp. The presumptive isolates from the water sample were purified by repeated re-streaking and subculturing at 37 °C for 24 hours on nutrient agar.

Gram staining and biochemical tests like indole, methyl red, Voges Proskauer, citrate, motility, and sugar fermentation were carried out following the standard practices outlined in reference to [15] for bacterial identification.

Antibiotic Susceptibility Test

Seven commonly used antibiotics, namely Amoxicillin-clavulanate (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), tetracycline (5 µg), and meropenem (10 µg), were used for the antimicrobial susceptibility testing via the disk-diffusion method. A 0.5 McFarland turbidity culture of the test isolate was evenly spread on Muller Hinton agar, after which the antibiotic discs were aseptically placed over the culture plate and the plate was incubated at 37°C for 18-24 hours. To evaluate the growth inhibitory effect, A millimeter ruler was used to measure the zone of growth inhibition surrounding the disc. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) 2021 guidelines [16].

Phenotypic detection and confirmation of ESBLs

The isolate was screened for ESBL production, using a double disk synergy method, in accordance to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [16]. Discs of ceftazidime (30 µg) and cefotaxime (30 µg) were placed at a 25 mm distance with clavulanic acid (30 µg/10 µg) on Mueller-Hinton agar plates overlaid with 0.5 McFarland bacterial suspension. The plate was incubated overnight (18–24 h) at 37°C. An isolate was identified as an ESBL producers when it showed an increased inhibition zone diameter ≥5 mm for any of the cephalosporin disc in direction to clavulanic acid.

DNA Extraction

The deoxyribonucleic acid (DNA) extraction of a pure 24-hour culture plate was done using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Inqaba Biotec, South Africa) as stated by the manufacturer's instructions. The quantity and quality of the DNA were determined using the Qubit dsDNA (double-stranded DNA) BR assay kit (Invitrogen, USA), and the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, South Africa).

Library Preparation

The library preparation of the extracted DNA isolate was carried out on the Nextra illumina library preparation kit (San Deigo CA, USA) following the manufacturer's procedure. Quality control and adapter trimming were performed with bcl-convert1 (v4.1.5), (SeqCenter, 91 43rd Street Suite 250 Pittsburgh, PA 15201 878-227-4915). The adapters in the short-pair-ended reads were trimmed for quality using Trimmomatic v0.36 [17].

Whole Genome Sequencing

Illumina sequencing of the genome isolates was performed on an Illumina NovaSeq 6000 sequencer in one or more multiplexed shared-flow-cell runs, producing 2x151bp paired-end reads. Raw sequence data were quality trimmed using TrimGalore (REF) for the removal of contamination and low-quality sequences.

Bioinformatic analysis

Genome Assembly

The read was assembled using metaSPades with default parameters [18], while CheckM (REF)

was used for assessing the quality of genomes based on completeness and contamination [19].

Genome Annotation

The assembled genome was annotated via the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [20], and Genome taxonomy database tool kits (GTDB tk) [21] was used to assign the taxonomic classification of the isolate.

*De novo assembl*ed genome was used to assign sequence types (STs) by using the Multilocus sequencing typing (MLST) database at the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/MLST/). Gene prediction for resistome, was analyzed by ResFinder 4.1 [22]. Furthermore, the antibiotic resistance mechanisms of the isolate were analyzed using CARD (Comprehensive Antibiotic Resistance Database) [23].

Results and Discussion

Phenotypic analysis of the P10 isolate, including its pink mucoid appearance on MacConkey agar and biochemical test results (Table 1), indicated it to be *K. pneumoniae*. This identification was subsequently confirmed through taxonomic analysis of the WGS data. The genomic features of the P10 isolate are presented in Table 11.

Table 1: Biochemical results of *K. pneumoniae* P10 isolate

Test	Result
Gram Stain	Negative rod
Motility	Positive
Indole	Negative
Citrate	Positive
Methyl Red	Negative
Voges Proskauer	Positive
Glucose	Positive
Lactose	Positive
Maltose	Positive

Table I1: Genetic feature of *K. pneumoniae* P10

Genetic Features	Values
G+C	57%
Contigs	71
Length	5,362,178bp
N50	215,432
tRNA	75

rRNA	13
Completeness	100
ST	163
Accession number	JAWIZY000000000

The distribution of the genome annotations is shown graphically in a circle (Figure 1). The tracks, which are displayed as concentric rings from outermost to innermost, are Position, Contigs/Chromosomes, CDS-forward, CDS-reverse, Non-CDS Features, AMR, GC Content, and GC Skew respectively.

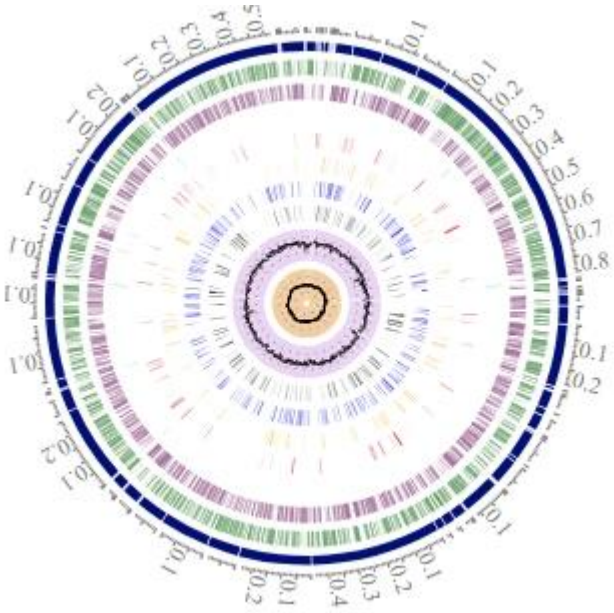

Fig 1: Genomic circular map of *K. pneumoniae* P10

The isolation of *K. pneumoniae* from hospital wastewater has emerged as a significant concern in the context of public health and environmental safety. The recovery of *K. pneumoniae* from this study corroborates with the report of [24] in South Africa, where a prevalence rate of 78.8% for *K. pneumoniae* in hospital effluents, indicating a substantial contamination level by this nosocomial pathogen has been declared.

Detection of antibiotic resistance genes.

Table III: Antibiotic susceptibility profile

Antimicrobial resistance profile

Based on the antibiogram data (Table III), the *K. pneumoniae* isolate showed a resistance phenotype to β-lactams (ceftazidime, cefepime), augmentin, ciprofloxacin, tetracycline, intermediate resistance to meropenem with reduced resistance to aminoglycoside (gentamicin).

Antibiotic	Susceptible	Intermediate Resistance	Resistance
Cephalosporin	-	-	R
Gentamicin	-	I	-
Ceftazidime	-	-	R
Cefepime	-	-	R
Augmentin	-	-	R
Tetracycline	-	-	R
Meropenem	-	I	-

Key: I- Intermediate, R- Resistant,

The P10 isolate demonstrated ESBL production in the disk synergy test, evidenced by a visible extension of the inhibition zone towards the amoxicillin-clavulanic acid disk.

The widespread occurrence and spread of very resistant *K. pneumoniae* clones have raised serious concerns about world health. The emergence of ESBL strains of *K. pneumoniae* puts a great concern on strains from healthcare system since they can cause serious nosocomial infections. Many studies have highlighted the prevalence and characteristics of *K. pneumoniae* in hospital wastewater, emphasizing its role as a reservoir for multidrug-resistant (MDR) strains that pose risks to both the environment and public health [25].

Antibiotics Resistance Gene

An *in-silico* analysis of the isolate's antibiotic resistance gene and resistance mechanisms was conducted on ResFinder and CARD server. The overall prevalence of ARGs and their associated resistance mechanisms is shown in Table IV. The isolate genome was found to harbor thirteen identified antimicrobial resistance (AMR) genes, conferring resistance to eight classes of antibiotics as listed in Table IV. These include genes for resistance to trimethoprim (*dfrA1*), tetracycline (*tet*), fosfomycin (*fosA*), sulfonamides *(sul2*), fluoroquinolones (*acrR*), aminoglycosides (*aadA*), and beta-lactam antibiotics (bla_{TEM215}, bla_{SHV40}, bla_{SHV56}, bla_{SHV89}, and bla_{SHV144}). Furthermore, the genome of this strain harbors the OmpK36 and OmpK37 genes, which are associated with decreased susceptibility to certain antibiotics. The analysis revealed that the isolate's resistance profile was attributed to both point mutations and acquired resistance mechanisms. The resistance due to point mutation was discovered in our isolate with resistance gene *acrR* to fluoroquinolone, Ompk36 to cephalosporin, and Ompk 37 to carbapenem while other resistances are due to acquired resistance.

Table IV: List of AMR genes identified in genomes of *K. pneumoniae* (P10) isolate

AMR	Gene mechanism	Resistance
fosA	metalloglutathione transferase	Fosfomycin
OqxA	Oqx efflux pump	Amphenicol/ fluoroquinolones
OqxB	Efflux pump	Fluoroquinolones
sul2	Sulphonamide resistance dihydropteroate synthase	Sulphonamides/co-trimoxazole
dfrA	dihydrofolate reductase	Trimethoprim

aac(6)-lb-cr	Acetyltransferase	Aminoglycoside, Fluoroquinolones
acrR	Efflux pump	Fluoroquinolones
tetA	tetracycline efflux	Tetracycline
ompK37	Reduced permeability to antibiotics	Carbapenem
ompK36	Reduced permeability to antibiotics	Cephalosporin
bla_{TEM215}	Enzymes inactivation (b-lactamase)	Cephalosporin
bla_{SHV56}	Enzymes inactivation (β-lactamase)	piperacillin+tazobactam
bla_{SHV85}	Enzymes inactivation (β-lactamase)	Ampicillin
bla_{SHV40}	Enzymes inactivation (β-lactamase)	Aztreonam/cefepime/ceftazidime/cefotaxime
bla_{SHV56}	Enzymes inactivation (b-lactamase)	ampicillin+clavulanic acid
bla_{SHV144}	Enzymes inactivation (β-lactamase)	ampicillin+clavulanic acid, carbapenem

This has revealed that the P10 isolate harbored different resistant determinant genes as shown above. The phenotypic resistance to tetracycline observed in the P10 isolate was genetically supported by the presence of the tetA gene harbored by the isolate, which encodes an efflux pump mechanism. Also, the detection of the aac(6′)-lb-cr gene in P10 isolate is a concerning development in the landscape of antimicrobial resistance. This gene encodes a variant aminoglycoside acetyltransferase that confers resistance not only to aminoglycosides but also to certain fluoroquinolones, representing a unique example of multidrug-resistance. Another resistant gene determinant harbored by the P10 is the OqxAB efflux pump gene, which plays a major role in developing resistance in *Klebsiella pneumoniae* against the antimicrobial's chloramphenicol, quinolones, and tetracyclines [26].

The WGS results further confirmed that P10 isolate was an ESBL producer as the genome harbors bla_{TEM} and different variants of bla_{SHV} (Table 1V). Bla_{TEM} and bla_{SHV} harbored by P10 isolate [9] have earlier been reported to belong to class A beta-lactamase producer [27]. Gram-negative bacteria that exhibit β-lactam resistance have been linked to several factors, including the presence of hydrolyzing enzymes, changes to the outer membrane's permeability, modifications to antibiotic target and increased expression of the multidrug efflux pump [28]. Among the frequently recognized fundamental mechanisms of antimicrobial resistance are the enzymatic degradation of antibiotics and the decrease in membrane permeability [29]. The discovery of ESBL-producing *K. pneumoniae* in wastewater can facilitate the transfer of resistance genes to other bacteria, compounding the public health threat posed by these pathogens [30].

OmpK35 and OmpK36 are two porins found on the outer membrane of *K. pneumoniae*, these outer membrane proteins (OMPs) play crucial roles in the permeability of the bacterial cell wall to antibiotics, thereby influencing resistance profiles. In this study, two porins loss (OmpK 36 and OmpK37) was reported which supported the phenotypic intermediate resistance of the isolate to meropenem, a carbapenem antibiotic. Martı´nez-Martınez *et al*. [31] have reported that meropenem can exert a selective pressure that favors the acquisition of carbapenem resistance. Another previous report has also indicated that porin loss has more relevance for increased resistance to meropenem than to imipenem [32]. The detection of these porins and other resistance genes in hospital wastewater is of concern, as it reflects the environmental persistence and potential spread of resistant strains.

This study has revealed mutations in the OmpK37 gene of the isolates, resulting in outer membrane protein deletions (Table IV). Studies have shown that mutations in these porin-coding genes and subsequent loss of porins impair antibiotic penetration, leading to increased resistance to beta-lactam antibiotics, including cephalosporins and carbapenems, which are critical in treating infections caused by *K. pneumoniae* [33]. This effect becomes

particularly significant when combined with the overproduction of cephalosporinases like ESBL or AmpC enzymes, potentially leading to clinically relevant levels of resistance.

Research indicates that the presence of OmpK36 and OmpK37 is often correlated with the virulence and resistance mechanisms of *K. pneumoniae*. The absence or reduced expression of OmpK36 and OmpK37 has been implicated in the resistance of *K. pneumoniae* to carbapenems, as these porins are essential for the uptake of beta-lactams [34].

OmpK36 plays a crucial role in *K. pneumoniae* antibiotic susceptibility, with mutations potentially reducing antibiotic influx and enhancing resistance [35]. Deletions of OmpK36 and OmpK37 are commonly observed in carbapenem-resistant *K. pneumoniae* strains, particularly among ESBL producers [35]. The findings from this study align with this pattern, as P10 isolate exhibits both phenotypic resistance to meropenem a carbapenem and ESBL production, suggesting a potential link to porin alterations. This result also corroborates those of Khalifa *et al.* [36], who found that ESBL-producing bacteria develop antibiotic resistance as a result of porin depletion.

The loss of porins has been shown to enhance resistance to non-β-lactam antibiotics, including fluoroquinolones, in ESBL-producing organisms [35, 37]. This phenomenon may explain why our P10 isolate, an ESBL producer, also harbors the fluoroquinolone resistance gene acrR. The combination of porin loss and specific resistance genes likely contributes to the isolate's broader antimicrobial resistance profile.

Moreover, the detection of these proteins in hospital wastewater raises concerns about the environmental dissemination of resistant strains. Hospital wastewater is known to harbor high concentrations of antibiotics and resistant bacteria, which can contribute to the spread of resistance genes in the environment [38].

Conclusion

This study demonstrates the value of genomic analysis in unraveling the complex mechanisms of antibiotic-resistance genes in ESBL-producing *K. pneumoniae* (P10). The isolate genome possesses ARGs which confer resistance to eight classes of antibiotics including beta-lactam, fluoroquinolone and aminoglycosides. Mutations in detected porin-coding genes OmpK36/37 likely plays a crucial role in reducing membrane permeability, thereby enhancing resistance to cephalosporins and carbapenems respectively. The uncovering of OMPK36/37 together with other ARGs in this isolate from hospital wastewater is particularly concerning, as it suggests a potential route for environmental dissemination of antibiotic resistance. This finding underscores the urgent need for improved wastewater treatment strategies and requirement of more stringent antibiotic stewardship practices in healthcare settings.

Acknowledgment

The authors acknowledge Helix Biogen Institute, Ogbomosho for their technical assistance in carrying out WGS of the isolate.

Declaration of competing interest

The authors declare that they have no known competing financial or personal interest.

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