Vol. 06, No. 02, pp. 75 –81 (2025)
ISSN: 2708-1818

JOURNAL OF LIFE AND BIO-SCIENCES RESEARCH

www.jlbsr.org

Phenotypic and Genotypic Assessment of Virulence Potential of Multidrug-Resistant *Klebsiella pneumoniae* Isolated from Wounds in Port Harcourt, Nigeria

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Abstract

***Klebsiella pneumoniae* is one of the predominant pathogens found in wounds. Multidrug resistant *K. pneumoniae* poses a challenge to treatment due to complications that may arise. The presence of virulent determinants would pose a further public health risk. This study aimed to assess the phenotypic and genotypic virulence potential of multidrug-resistant *K. pneumoniae* isolated from wounds in Port Harcourt, Nigeria. A total of 200 wound samples were obtained and evaluated for the presence of *K. pneumoniae* using standard microbiological culture techniques and appropriate biochemical tests. Test isolates were assessed for antimicrobial susceptibility and multidrug resistant isolates were identified. Three virulence traits (hemolytic activity, biofilm formation potential and protease production) were determined using standard methods. DNA of test isolates was extracted using the boiling method, and isolates were assessed for the presence of three virulence genes (*entB, mrkD,* and *fimH* genes). Out of 30 *K. pneumoniae* isolates identified and evaluated, 60% were multidrug resistant, 70% (30% beta hemolysis and 40% alpha hemolysis) showed hemolytic activity, 7 isolates (23.3%) were positive for protease production, and 11 (36.7%) exhibiting biofilm forming potential. This study also revealed the presence of the *entB* gene (13.3% occurrence) and the *mkrD* gene (6.7% occurrence), with a co-occurrence of the *entB* and *mkrD* genes in one isolate. The *fimH* gene was not detected in any of the isolates assessed. This study notes a higher occurrence of virulence traits in multidrug resistant (MDR) isolates than in non-MDR isolates.**

***Keywords*:** *Klebsiella pneumoniae, Wounds, Resistance gene, Virulence, Nigeria*
Received: November 02nd, 2025 / Revised: December 12th, 2025/ Accepted: December 20th, 2025 / Online: December 23rd, 2025.

I. INTRODUCTION

Several diseases, including wound, bloodstream, and lung infections, can be caused by the commonly encountered Gram-negative bacteria *Klebsiella pneumoniae*. Multidrug-resistant *K. pneumoniae* wound infections are especially dangerous because they can be challenging to treat and result in life-threatening consequences (Agbagwa and Edje, 2015). Drug-resistant *K. pneumoniae* strains are becoming more common, which has sparked worries throughout the globe as it limits available treatment options and poses a major risk to public health (Zhu *et al*., 2021). In addition to showing increased virulence and resistance to several medications, multidrug-resistant (MDR) *K. pneumoniae* strains often exhibit increased mortality rates and more severe illnesses (Ehwuru *et al*., 2019).

In wounds, especially, the development of infections associated with multidrug resistant organisms has been especially problematic (Prastiyanto *et al*., 2024). Several studies have reported on the presence of drug resistant *K. pneumoniae* in wounds globally, with *K. pneumoniae* often occurring as one of the first two most commonly occurring Gram negative organisms after *Escherichia coli* (Yang *et al*., 2021; Chukwu *et al*., 2022; Prastiyanto *et al*., 2024). Similar reports have been made of *K. pneumoniae* from wounds in Nigeria (Pandukur *et al*., 2020; Akinyemi *et al*., 2021; Ajigbewu *et al*., 2025). One study noted the presence of *K. pneumoniae* as a major risk factor for complications (Yang *et al*., 2021).

This risk from *K. pneumoniae* can be further compounded by the presence of virulence factors in these isolates, with the potential to cause more severe disease conditions. A number of studies from Nigeria explored virulence factors in *K.*

doi: 10.38094/jlbsr602167

pneumoniae as well. However, for some of these studies, isolates were not specifically from wounds and only genotypic methods were used (Ogbolu *et al*., 2022, Akintoyese *et al*., 2025). One study was noted to also carry out phenotypic detection of virulence characteristics (Dada-Adegbola and Abitogun, 2021). None of these studies, however were carried out in the South-South region of Nigeria. This study, therefore, set out to carry out a phenotypic and genotypic assessment of the virulence potential of multidrug-resistant *K. pneumoniae* isolated from wounds in Port Harcourt, Nigeria.

II. Materials And Methods

A. Sample collection and processing

Two hundred wound samples were taken from the wounds of patients from the University of Port Harcourt Teaching Hospital (UPTH). Samples were immediately transported to the laboratory and processed by culturing on the selective Eosin Methylene Blue agar. Following incubation at 37°C for 24 hours, characteristic *K. pneumoniae* colonies were purified by subculturing to Nutrient agar and isolate identities confirmed using biochemical tests such as Catalase, Citrate, Oxidase, Methyl- Red, Voges Proskauer, Urease, Motility, Triple sugar iron test, Starch hydrolysis, Sugar Fermentation (Glucose, Lactose, Sucrose), and indole.

B. Antimicrobial susceptibility test

Antibiotic sensitivity testing measures a bacteria's resistance to antibiotics. A test for antibiotic sensitivity was conducted on Mueller-Hinton agar (MHA) using the following antibiotic discs: Gentamicin (GEN) 10µg, Ampicillin-Cloxacillin (AX) 10µg, Cefuroxime (CXM) 30µg, Nalidixic acid (NA) 30µg, Nitrofurantoin (NIT) 300µg, Ceftriaxone (CRO) 10µg, Ofloxacin (OFX) 5µg, Imipenem (IPM) 10µg, Amoxicillin/clavulanate (AUG) 30µg, Levofloxacin (LEV) 5µg, vancomycin (VAN) 5µg and Cefotaxime (CTX) 30µg.
Antimicrobial susceptibility testing was carried out as previously described using the Kirby Bauer disc diffusion method. In brief, this involved inoculating a bacterial suspension corresponding to a 0.5 McFarland standard onto the surface of a Mueller-Hinton Agar (MHA). This was followed by aseptically placing a bacterial multidisc onto the surface of the inoculated plate. Following a 10-minute pre-incubation, the whole setup was incubated at 37°C. After a 24-hour time frame, plates were examined for zones of inhibition, indicated as clear areas around the antibiotic disc. These were recorded and results interpreted using the Clinical Laboratory Standard Institute guidelines (CLSI, 2014).

C. Phenotypic Detection of selected virulence traits

The presence of three virulence traits in identified isolates was then determined using previously described methods. In brief, biofilm forming potential was determined using both the Congo Red Agar (CRA) method (Taher *et al*., 2016) and the Tube method (TM). The CRA method involved culturing a pure inoculum on the Congo red agar, where organisms with biofilm forming potential produced colonies with black pigmentation. For the tube method (Alghofaili, 2022), biofilm lined on the bottom and wall of the tube was detected. Hemolysis was detected by inoculation to blood agar containing 5% sheep blood (Buxton, 2005), while the protease production was determined by inoculation to Skim milk agar where a positive result was observed as a clear zone surrounding the growth of the organism (Baron and Finegold, 1994; Taher *et al*., 2016).

D. Molecular detection of virulence genes in test isolates

Following DNA extraction using the boiling method as previously described (Otokunefor *et al*., 2019), the presence of three virulence genes (*entB*, *mrkD*, and *fimH* genes) was assayed using previously described primers and protocols (Table 1). Each PCR reaction mixture consists of a 10µl reaction volume made up of 2µl genomic DNA, 0.3µl forward primer, 0.3µl reverse primer, 5.4µl double distilled water, and 2µl FIREpol master mix (solis biodyne) containing 7.5mM $MgC1_2$, 1mM dNTPs, blue dye, yellow dye and 5x reaction buffer containing 0.4M Tris-HCL.
A two percent (2%) agarose gel was used for the electrophoresis. 0.8 gram (0.8g) of agarose powder (CSL-AG100 LE multi-purpose agarose) was dissolved in 40 ml of 1X TAE (TRIS acetate EDTA). The solution was then stained with 9µL ethidium bromide and was allowed to polymerize in the gel electrophoretic cast in which the comb was properly placed. The TAE running buffer was poured into the electrophoresis tank to submerge the polymerized gel. The amplified PCR product of each sample was resolved in the 2% agarose gel at 80v, 250mA for 15 minutes, 4 µL of the products were carefully loaded in the wells, using 100bp molecular weight marker as a control for size. Resolved allelic fragments were visualized using a UV transilluminator.

Table 1. Primers used for genotypic detection of virulence genes in test isolates

Gene of interest	Sequences of primer (5'–3')	Size amplicon (bp)	Annealing temperature °C	References
mrkD	CGCTTTTTATCGTCTTAATG GTGATGTAGCGGGTCTCCT	55	880	Ballén *et al*., 2021
entB	ATTTCCTCAACTTCTGGGGC AGCATCGGTGGCGGTGGTCA	60	371	Candan & Aksöz, 2015
fimH	GAAAAAAATAATCCCCCTGTTCA C GTAACCTGGCCTGTGGT	57	850	Ballén *et al*., 2021

III. RESULTS

A. *Biochemical characterization of suspected Klebsiella from various wound sources*

Following biochemical characterization of suspected *Klebsiella* sp., the majority (51.9%, 40/77) were confirmed as *K. pneumoniae*.

B. *Percentage occurrence of resistance in K. pneumoniae test isolates*

The test isolates showed varying levels of resistance to the antibiotics used (Figure 1). The highest level of resistance (42.5%) was against ceftriaxone (CRO) and the lowest (15%) against ampicillin-cloxacillin (AX) and nitrofurantoin (NIT).

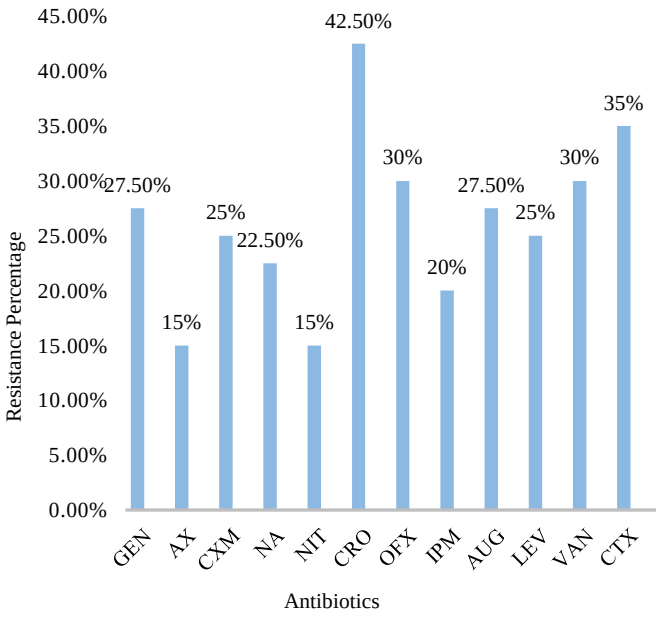

Figure 1: Occurrence distribution of antibiotic resistance in test isolates

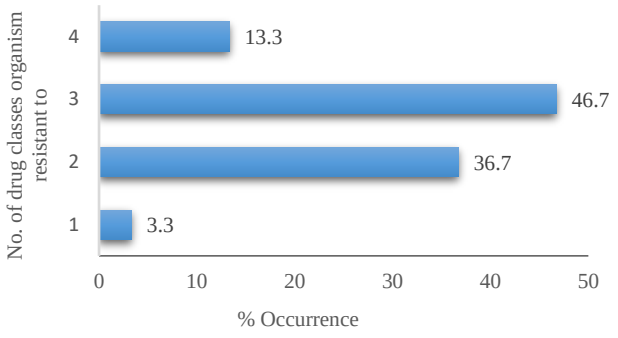

Figure 2. Variation of multidrug and classes

All *K. pneumoniae* isolates were found to exhibit unique antibiogram patterns (Table 2) resulting in 30 patterns in total. The lowest proportion of isolates showed resistance to only 1 drug class (Figure 2). Majority of isolates (60%, 18/30) were found to be multidrug resistant (resistant to 3 or more drug classes), though most of these showed resistance to only 3 drug classes.

Table 2. Antibiogram pattern of antibiotic resistance Klebsiella pneumoniae isolates

S/No	Antibiogram	No. of Isolates	No of drug classes	Multidrug
1	AUG-GEN	1	2	-
2	CRO-CTX	1	1	-
3	CRO-NIT	1	2	-
4	CTX-LEV	1	2	-
5	NIT-OFX	1	2	-
6	ACX-LEV-NIT	1	3	+
7	AUG-CRO-LEV	1	3	+
8	AUG-CRO-OFX	1	3	+
9	AUG-CTX-IPM	1	3	+
10	AUG-CTX-OFX	1	3	+
11	CRO-CTX-GEN	1	2	-
12	CRO-CTX-LEV	1	2	-
13	CRO-CTX-IPM	1	2	-
14	CRO-CXM-OFX	1	2	-
15	CRO-LEV-NA	1	2	-
16	CTX-CXM-GEN	1	2	-
17	CTX-LEV-OFX	1	2	-
18	ACX- CTX-1PM-NIT	1	3	+
19	ACX-AUG-CTX-NA	1	3	+
20	ACX-CRO-CXM-OFX	1	3	+
21	ACX-GEN-IPM-NA	1	4	+
22	AUG-CRO-GEN-IPM	1	4	+
23	AUG-GEN-IPM-LEV	1	4	+
24	CRO-CXM-NA-NIT	1	3	+
25	CRO-GEN-NA-OFX	1	3	+
26	CRO-CTX-GEN-NA	1	3	+
27	CTX-CXM-IPM-OFX	1	3	+
28	CRO-CXM-GEN-NA-LEV	1	3	+
29	CXM-LEV-NA-NIT-OFX	1	3	+
30	CTX-CRO-CXM-GEN-NA-NIT	1	4	+

Out of 30 isolates assessed for hemolytic activity, 70% showed either alpha or beta hemolytic patterns (Figure 3), though partial hemolysis (alpha hemolytic pattern) was observed in the majority. Only 7 isolates (23.3%), however, were positive for protease production and 11 (36.7%) exhibiting biofilm forming potential by both Congo red agar and the tube method (Figures 4 and 5).

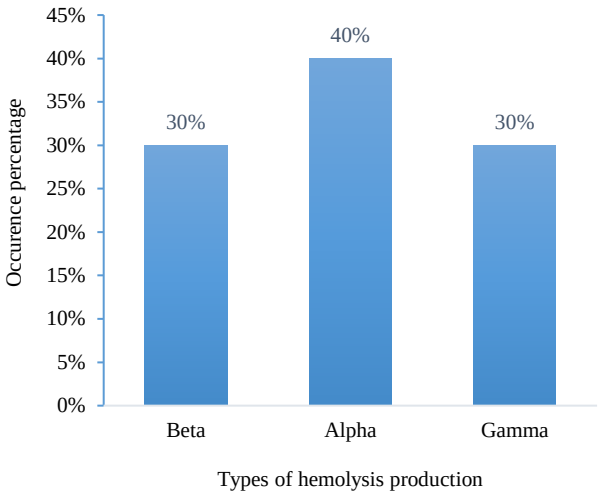

Figure 3. Hemolytic pattern distribution of test isolates

Figure 4. Congo red agar plate with biofilm formation

Figure 5. Tube method formation of biofilm (purple ring in tube indicated positive result

C. Co-occurrence of virulence characteristics

A co-occurrence of virulence characteristics was observed in only 8 isolates, with all three virulence characteristics present only in a single isolate (S45). The majority of the 27 isolates (66.7%) exhibiting at least one virulence trait were also found to be MDR, while only 33.3% of isolates showing no virulence trait were MDR. In general, only 11.1% of MDR isolates exhibited no virulence characteristics, while 33.3% of non-MDR isolates exhibited no virulence characteristics.

D. Molecular assessment of select virulence gene markers

The analysis for three select virulence gene markers showed that 16.7% of isolates had at least one of the genes present. For the *fimH* gene, however, a 0% occurrence was noted among the test isolates; the *entB* gene had a 13.3% occurrence, while the *mkrD* gene showed a 6.7% occurrence. One isolate (S45) had a co-occurrence of both *entB* and *mkrD* genes present (Figures 6 and 7).

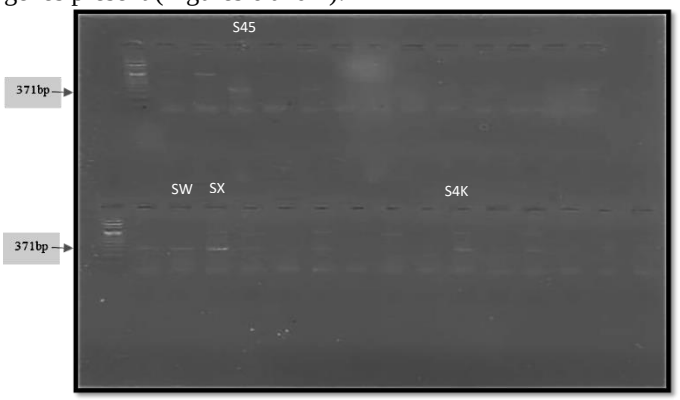

Figure 6. *entB* DNA product (371 bp) from typical isolates of *K. pneumoniae*

Figure 7. *mrkD* DNA product (880bp) from typical isolates of *K. pneumoniae*

IV. DISCUSSION

Klebsiella pneumoniae is one of the most common bacteria associated with wound infections (Puca *et al.*, 2021). In this study, a 20% occurrence (40 isolates) was identified as *K. pneumoniae* from 200 samples, which is quite similar to reports from previous studies. Prastiyanto and colleagues recently noted a 21.5% occurrence of *K. pneumoniae* in wound infections in Indonesian patients (Prastiyanto *et al.*, 2024), while Doko *et al*. (2024) reported a 20.9% occurrence from wound surfaces. These were slightly different from the 11.1% occurrence observed by Ajigbewu and colleagues from chronic wounds in Nigeria fairly recently (Ajigbewu *et al.*, 2025) and 28% occurrence reported from burn patients in

Pakistan (Qaisar *et al*., 2023). A much lower occurrence of 6.7% was reported by Pandurkar and colleagues from diabetic wounds (Pandukur *et al*., 2020)

In this study, resistance rates of the organisms ranged from 15% to 42.5%. A similar range was observed in a Pakistan study, which, though they noted rates ranging from 1.5% to 97.3%, reported the majority (16/18, 88.9%) below 50% (Prastiyanto *et al*., 2024). It was, however, much lower than rates observed recently by Akintoyese and colleagues (Akintoyese *et al*., 2025), who noted all rates above 80%. Doko *et al*. (2024) reported rates ranging from 0% to 90.7%, though the majority had rates less than 50%. All these variations might be a function of a number of factors such as isolate source, the nature of the wound, and the treatment history of the patient.

The fact that the highest rate of resistance was against ceftriaxone, a third-generation cephalosporin, is worrisome due to the extended spectrum of this class of antibiotics and their clinical efficacy especially against antibiotic-resistant Gram-negative bacteria (Hamadalneel *et al*., 2024; Gebremeskel *et al*., 2023; Fatima *et al*., 2021). Even higher rates of above 80% have been recently reported (Mijović *et al*., 2022; Nwafia *et al*., 2019). A recent study actually noted the rising occurrence of resistance against third-generation cephalosporins, with rates evolving from 58.9% to 71.5% between 2020 and 2024 (Bwanali *et al*., 2025). This highlights a need to advocate stewardship regarding third-generation cephalosporins specifically.

This study reported a high rate of multidrug resistance (60%), similar to reports by previous studies (Mike-Ogburia *et al*., 2025; Ameshe *et al*., 2022; Otokunefor *et al*., 2018) showing MDR rates ranging from 90.2% to 70%. This high rate of resistance detected can be attributed to the overuse of antibiotics (Aktar *et al*., 2014; Isaiah *et al*., 2025). The high level of multidrug resistance in this study could be due to an interplay of other resistance mechanisms co-expressed by the isolates, such as extended spectrum beta lactamases and quinolone resistance genes. Furthermore, prior antibiotic use in hospitals or through auto-medication, overuse of antibiotics in livestock and fish farming, poor infection control in health care facilities, and poor hygiene and sanitation exacerbate multidrug resistance. In contrast to this study, the fact that resistance to three or more antibiotic classes was seen in over 90% of cases highlights the danger of multidrug-resistant *K. pneumoniae* in wound infections.

A study by Karimi *et al*. (2021) reported that 74.5% of *K. pneumoniae* isolates from clinical samples were positive for biofilm formation, while Nirwati *et al*. (2019) found that only 26.95% of *K. pneumoniae* isolates from respiratory tract infections showed strong biofilm formation using the Congo red agar method. Liu *et al*. (2024) noted that most infections are established by the presence of biofilms in the host. All of these indicate an effect of source on biofilm forming potential, which is probably reflected in the 37% occurrence of biofilm forming potential observed in this present study.

Beta hemolysis (complete hemolysis) was observed in 30% (9) *K. pneumoniae* isolates, 40% (12) isolates were alpha hemolytic (partial hemolysis), while 30% (9) isolates were non- hemolytic (gamma hemolysis). A study by Hullur *et al*. (2022) found that only 4.66% of *K. pneumoniae* isolates from different clinical samples were hemolytic, while Imtiaz *et al*. (2021) showed 8% hemolytic activity. The production of hemolysin among Gram-negative bacteria is indicative of other virulence and toxigenic factors (Hullur *et al*., 2022). The assessment of bacterial hemolytic activity is a vital criterion in determining virulence, and it serves as a determinant of virulence in bacterial pathogenesis.

The genotypic assessment of virulence potential was performed by assessing the isolates for the presence of *entB*, *mkrD*, and *fimH* virulence genes. The *entB* gene product (371 bp) was amplified within S4K, S45, SW, and SX isolates (13.3%). This contrasted the findings of Soltan *et al*. (2018), which revealed that the *entB* gene was the most prevalent gene, 58 (95.1%), among 16 determined virulence genes, followed by *mrkD* 17 (27.9%) and *fimH* 11 (18%) genes.

The *fimH* gene encodes the FimH protein, a type 1 fimbriae adhesive subunit and has been widely detected in *K. pneumoniae* by a number of researchers with detection levels up to 96% (Stahlhut *et al*., 2009; Schroll *et al*., 2010; Ferreira *et al*., 2019; Stahlhut *et al*., 2010), Pourmohammad-Hosseini *et al*., 2023; Swedan and Aldakhily, 2024; Abeni *et al*., 2024). The source of these isolates varied, ranging from environmental isolates to human isolates such as liver isolates, urinary tract isolates, and bloodstream isolates. Unlike these previous studies, however, there was a 0% occurrence of the *fimH* gene in the present study. A similar result was noted recently in a study carried out in Osun State, Nigeria (Akintoyese *et al*., 2025), which reported a 0% occurrence also of *fimH* in clinical isolates collected from a variety of clinical specimens. This indicates that biofilm formation in the isolates in this study was not *fimH* dependent.

The genetic factor known as *mrkD,* or multiple-resistance keeping determinant, is present in the *K. pneumoniae* genome and is responsible for the bacteria's resistance to several drugs. This study revealed the presence of *mrkD* in just two isolates, S45 and S61 (6.6%), which is relatively similar to the low rates reported by Wang *et al*. (2019) and Wasfi *et al*. (2012). Unlike other studies that amplified the *mrkD* gene in rates ranging from 65% to 96% (Akintoyese *et al*., 2025; Swedan and Aldakhily, 2024; Ferreira *et al*., 2019; Shakib *et al*., 2018; Ali *et al*., 2022), in *K. pneumoniae* isolates from samples of bloodstream infections and urinary tract infections.

V. Conclusion

This study provides a comprehensive report of virulence traits and antimicrobial resistance (AMR) in the test isolates and note a higher occurrence of virulence traits in multidrug resistant (MDR) isolates than in non-MDR isolates. The diversity of isolates as indicated by the variety of antibiograms could be indicative of a lack of spread of a single clone but rather represent multiple evolutions of AMR.

Additionally, this study provides crucial information on the occurrence of specific virulence genes.

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