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Original Article

Antimicrobial resistance and virulence in *Klebsiella pneumoniae*: a four-month study in Osogbo, Nigeria

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Abstract

Objective: Antimicrobial resistance (AMR) is a growing global health crisis, with *Klebsiella pneumoniae* being a key pathogen due to its multidrug resistance (MDR). This study aimed to investigate the resistance profiles, demographic correlations, and molecular characteristics of MDR *K. pneumoniae* at UNIOSUN Teaching Hospital, Osogbo, Nigeria.

Methods: From January to April 2022, 99 clinical isolates (*K. pneumoniae*) were collected from various specimen types (blood, sputum, urine, wound, stool, and oral cavity). Antibiotic susceptibility was assessed using the Kirby-Bauer disk diffusion method, and virulence genes were analysed using multiplex polymerase chain reaction.

Results: All isolates exhibited resistance to ceftriaxone, cefotaxime, and colistin, with high resistance observed for cefepime and carbapenems (meropenem, imipenem, and ertapenem). Molecular characterization revealed the presence of virulence genes ***K1, K2,*** and ***mrkD*** in 15 isolates, while other tested virulence genes (***fimH, ramA, traT, K3,*** and ***K5***) were not detected. Significant associations were identified between resistance patterns and demographic factors, including age and sex, highlighting potential vulnerabilities in specific populations.

Conclusions: This study underscores the alarming prevalence of MDR *K. pneumoniae* and aligns with global trends of rising AMR. Addressing these challenges requires targeted antimicrobial stewardship programs, infection control measures, public education, and enhanced surveillance systems. Incorporating molecular resistance testing and novel therapeutic agents in future research is crucial to developing effective containment strategies and preserving antibiotic efficacy.

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Introduction

Antimicrobial resistance (AMR) represents one of the greatest challenges to global public health in the 21st century, threatening to undermine decades of progress in treating bacterial infections.[1,2] Among the most concerning pathogens is *Klebsiella pneumoniae* (*K. pneumoniae*), a member of the *Enterobacteriaceae* family that has emerged as a leading cause of multidrug-resistant (MDR) infections worldwide. The rapid evolution of resistance mechanisms in *K. pneumoniae* has significantly reduced the effectiveness of existing antibiotics, complicating treatment and leading to increased morbidity, mortality, and healthcare costs.[3]

K. pneumoniae is a common opportunistic pathogen associated with a wide range of infections, including respiratory tract infections, urinary tract infections (UTIs), bloodstream infections, liver abscesses, meningitis, and surgical wound infections.[4] It is particularly problematic in hospital and community settings, where it ranks as the second most common opportunistic *Enterobacterium*, following *Escherichia coli*, and is a prominent member of the ESKAPE pathogens—a group of bacteria known for their ability to evade antibiotic treatments.[5]

The global rise of AMR in *K. pneumoniae* is particularly pronounced in low- and middle-income countries (LMICs), where the misuse and overuse of antibiotics, combined with inadequate antimicrobial stewardship programs, drive resistance development.[2,6] WHO has classified carbapenem-resistant *K. pneumoniae* as a "critical priority pathogen," underscoring the urgent need for research into its resistance mechanisms and novel therapeutic strategies.[7] In LMICs, the burden of resistance is further exacerbated by the lack of access to advanced diagnostics and newer antibiotics,[8] making it challenging to combat infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE).

The resistance mechanisms in *K. pneumoniae* are diverse, involving the production of extended-spectrum β-lactamases (ESBLs), carbapenemases, and other enzymes that degrade antibiotics. Additionally, genetic adaptations such as efflux pumps, porin mutations, and horizontal transfer of resistance genes through plasmids contribute to its remarkable ability to resist multiple antibiotic classes.[12,13] Carbapenems, often considered the last line of defence for treating severe infections caused by

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ESBL-producing bacteria, are increasingly ineffective due to rising resistance, leaving clinicians with limited treatment options.[9]

Beyond its resistance mechanisms, *K. pneumoniae* is equipped with a range of virulence factors, including capsular polysaccharides (eg, *K1* and K2 serotypes), adhesins, siderophores, and fimbriae, which enhance its ability to evade the host immune system and establish infections.[10] Understanding the interplay between resistance and virulence is critical for designing effective therapeutic and preventive strategies.

Despite the global significance of *K. pneumoniae* as a multidrug-resistant pathogen, there is a limited understanding of its epidemiology and resistance mechanisms in LMICs, particularly in sub-Saharan Africa. Nigeria, for instance, faces significant challenges with AMR due to the widespread misuse of antibiotics, insufficient regulatory oversight, and limited availability of novel therapeutic agents.[11] Surveillance efforts in these regions are often constrained by resource limitations, resulting in a lack of comprehensive data on resistance trends and molecular characteristics

This study addresses these gaps by investigating the AMR profiles, molecular characteristics, and virulence gene distribution of *K. pneumoniae* isolates from a tertiary healthcare facility in Nigeria. Specifically, we examine resistance to key antibiotic classes, carbapenem resistance prevalence, and virulence factors linked to capsular serotypes and pathogenicity. Additionally, we explore the associations between resistance patterns and demographic factors, providing valuable insights into the epidemiology of MDR *K. pneumoniae* in a resource-limited setting. By elucidating these critical aspects, the study aims to contribute to developing targeted antimicrobial therapies, effective stewardship programs, and containment strategies tailored to LMICs.

Methods

Ethical considerations

Ethical approval for this study was obtained from the Adeleke University Ethical Review Committee (AUERC) with reference number AUERC/FOS/MCB/10. The study adhered strictly to ethical guidelines for research involving human participants. Informed consent was obtained from all participants prior to specimen collection, and participants were assured of the confidentiality and anonymity of their personal and medical information. All data and specimens collected were securely stored and used solely for this research.

Bacterial collection

UNIOSUN Teaching Hospital, formerly known as LAUTECH Teaching Hospital, is a prominent healthcare institution in Osogbo, Osun State, Southwest Nigeria, serving as a pivotal healthcare hub for the region. Between January and April 2022, a total of 200 clinical specimens were randomly collected from the hospital's Microbiology department. These specimens included blood, sputum, urine, wound, stool, and oral cavity samples. All isolates were patient-unique, ensuring no duplicate samples from the same individual. The selection process was conducted randomly, with no attempts to balance isolates based on the various body sites of origin.

The samples were collected from patients at the study location using sterile swabs. The swab sticks were then placed in sterile coolers with icepacks maintained at 4–8°C upon collection and transported to the laboratory for analysis. Upon arrival at the laboratory, all samples were stored in freezers for long-term preservation until cultivation.

Patient information, including age, gender, admission class, diagnosis, and patient status, was obtained from medical records.

Cultivation of samples and screening for enterobacteriaceae

Each sample was enriched in 225 mL of nutrient broth (Huankai Ltd., Guangzhou, China) and incubated at 37°C for 24 hours. After enrichment, the broth was streaked onto MacConkey agar (Huankai Ltd., Guangzhou, China) and incubated under the same conditions for an additional 24 hours. From the MacConkey agar plates, three pink mucoid colonies were selected and subcultured onto nutrient agar for 24 hours at 37°C.

The bacterial isolates were initially identified using Gram staining, capsule tests, and motility tests based on their morphological characteristics. Biochemical identification was conducted through a series of tests, including catalase, oxidase, citrate, urease, and indole tests. Sugar fermentation tests were performed using glucose, lactose, and sucrose, and Methyl Red/Voges-Proskauer (MR/VP) tests were also included. The identification of likely bacterial species was guided by *Bergey's Manual of Systematic Bacteriology*. Confirmed cultures were preserved in Luria-Bertani broth supplemented with 20% glycerol and stored at −80°C for subsequent analyses.

Antimicrobial sensitivity testing

AST was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar. This method was chosen for its simplicity, practicality, and standardization. A bacterial inoculum of approximately 1–2 × 10^8 CFU/mL was evenly applied to the surface of Mueller-Hinton agar plates.

Six commercially prepared antibiotic disks with fixed concentrations were placed on the inoculated agar surface. The antibiotics tested were Ceftriaxone (5 µg), Cefotaxime (30 µg), Colistin (25 µg), Meropenem (10 µg), Imipenem (10 µg), and Ertapenem (10 µg). The plates were incubated at 35°C for 16–24 hours. After incubation, the growth inhibition zones around each antibiotic disk were measured in millimeters. The diameter of the inhibition zone was used to determine the isolates' susceptibility, considering the antibiotics' diffusion rate through the agar medium. The zone diameters of each drug were interpreted according to CLSI criteria.[12] The classification of isolates as susceptible, intermediate (moderately resistant), or resistant was performed according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints. *E. coli* ATCC 25922 was used as the quality control strain throughout the analysis.

DNA extraction and multiplex PCR

DNA extraction was performed using a commercial kit from Stratec Biomedical Systems (Birkenfeld, Germany). A 1mL volume of bacterial suspension, standardized to .5 McFarland from an overnight culture, was centrifuged, and DNA was extracted from the resulting pellet according to the manufacturer's instructions. The extracted DNA was resuspended in 100µL of TE buffer.

Serotyping primers (*K1, K2, K3*, and *K5*) and virulence-associated genes (*fimH, ramA, traT, mrkD*) were used for the identification and confirmation of *K. pneumoniae* strains. Primers for *K. pneumoniae* 16S–23S ITS were also used for strain

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Table 1. Primers used for serotype and virulence gene identification

Gene	Primer Sequence (5′ → 3′)	Amplicon Size (bp)	Tm (°C)
RmpA	**Forward:** ACTGGGCTACCTCTGCTTCA	535	53
	Reverse: CTTGCATGAGCCATCTTTCA		
fimH-1	**Forward:** GCCAACGTCTACGTTAACCTG	180	43
	Reverse: ATATTTCACGGTGCCTGAAAA		
mrkD	**Forward:** CCACCAACTATTCCCTCGAA	226	43
	Reverse: ATGGAACCCACATCGACATT		
traT	**Forward:** GGTGTGGTGCGATGAGCACAG	288	55
	Reverse: CACGGTTCAGCCATCCCTGAG		
K1	**Forward:** GGTGCTCTTTACATCATTGC	1283	47
	Reverse: GCAATGGCCATTTGCGTTAG		
K2	**Forward:** GGATTATGACAGCCTCTCCT	908	45
	Reverse: CGACTTGGTCCCAACAGTTT		
K5 (Capsular Type)	**Forward:** TGGTAGTGATGCTCGCGA	280	49
	Reverse: CCTGAACCCACCCCAATC		
K3	**Forward:** TAGCAATTGACTTTAGGTG	549	—
	Reverse: AGTGAATCAGCCTTCACCT		

Table 2. Demographic and clinical characteristics of study participants

Category	Subgroup	Frequency (n)	Percentage (%)
Age Group	16–20 years	8	8.08
	20–25 years	7	7.07
	26–30 years	21	21.21
	31–35 years	19	19.19
	36–40 years	22	22.22
	41–45 years	12	12.12
	46–50 years	3	3.03
	51–55 years	3	3.03
	56–60 years	3	3.03
	Unresponsive	1	1.01
Gender	Male	50	50.50
	Female	49	49.49
Admission Ward	Medical	73	73.73
	Surgical	26	26.26
Patient Status	In-patient	44	44.44
	Out-patient	55	55.55
Sample Site	Urine	18	18.18
	Blood	14	14.14
	Sputum	16	16.16
	Stool	15	15.15
	Oral cavity	18	18.18
	Wounds	18	18.18

confirmation, as described previously.[13] The primers used in this study are shown in Table 1.

From the eventually used isolates, 20 isolates were randomly selected for serotyping and virulence gene testing. These isolates were chosen to represent diverse resistance patterns and clinical backgrounds, ensuring a representative sample for analysis. This selection allowed the study to focus on specific genetic traits linked to virulence and capsular types within a manageable sample size.

Two independent Multiplex PCR assays were performed to identify capsular serotypes and capsule-associated genes based on the protocol described previously.[14] Each PCR was conducted in a 25 µL reaction mixture containing 5 pmol of each primer, 2 µL of extracted DNA, and the Master PCR mixture (Yekta Tajhiz Azma®, Iran). Amplified products were subjected to electrophoresis on a 1% agarose gel and stained with CyberSafe stain for visualization. The thermal cycling conditions for both Multiplex PCR assays were identical and followed established protocols.

Statistical analysis

Statistical analyses were conducted using PyCharm (version 2024.1.2) with the scipy.stats library. The chi-square test was employed to examine associations between categorical variables, such as age group, sex, admission class, patient status, and sample type. Observed frequencies were compared to expected frequencies using the chi-square statistic, with a p-value of less than .05 considered statistically significant.

ANOVA was used to investigate differences in mean ages across categorical groups. Age was treated as a continuous variable, while sex, admission class, patient status, and sample type were considered categorical factors. Post hoc tests, including Tukey's HSD, were conducted to determine specific group differences if ANOVA yielded significant results.

Results

Samples recruited for the study

Following selective isolation for *K. pneumoniae*, 101 samples were excluded due to the absence of growth on MacConkey agar, suggesting that these isolates were not part of the *Enterobacteriaceae* family. Consequently, 99 clinical samples that successfully grew on MacConkey agar were included in the study.

Demographic and clinical characteristics

Table 2 summarizes the demographic and clinical characteristics of the study participants, including age and gender distribution, admission details, patient status, and sample site distribution.

Bacterial characterization

All 99 isolates (100%) were identified as capsulated, Gram-negative bacilli, and were nonmotile. All isolates exhibited fermentation of glucose, lactose, and sucrose. They were uniformly positive for MR fermentation but negative for VP fermentation. Additionally, all isolates tested positive for citrate utilization, catalase activity, and urease production, but were negative for indole production and oxidase activity. Using *Bergey's Manual of Systematic*

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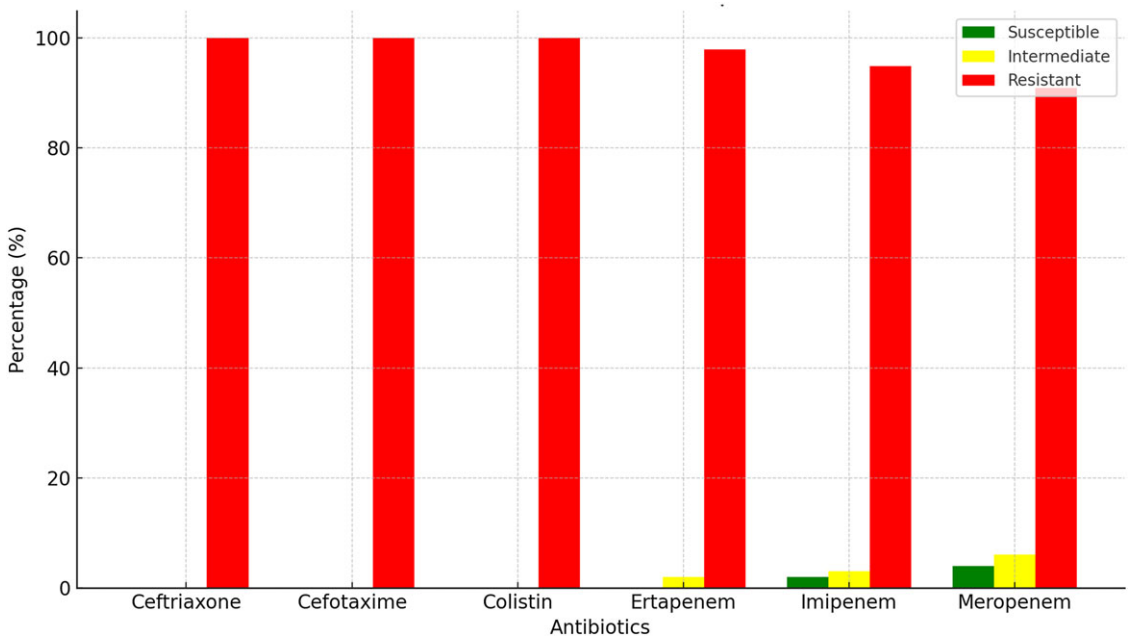

Figure 1. Antimicrobial resistance patterns of *K. pneumoniae* Isolates *Interpretation based on CLSI standard, 2020*.

Bacteriology, the isolates were conclusively identified as strains of *K. pneumoniae*.

Antibiotic susceptibility

The AMR patterns of *K. pneumoniae* isolates are summarized in Fig. 1. The figure illustrates the percentage of susceptible isolates that exhibited intermediate susceptibility or were resistant to each tested antibiotic. Notably, all isolates (100%) were resistant to ceftriaxone, cefotaxime, and colistin, while varying levels of resistance, intermediate susceptibility, and susceptibility were observed for ertapenem, imipenem, and meropenem.

Serotype and virulence gene identification

Twenty clinical isolates were analyzed for serotype and virulence gene identification using Multiplex PCR. Among these, 15 isolates tested positive for the ***K1*** and ***K2*** serotypes. The ***mrkD*** virulence gene was also present in these 15 isolates.

In contrast, the remaining genes analyzed showed no amplification in the isolates. Specifically, ***fimH, ramA, traT, K3,*** and ***K5*** were negative across all isolates tested. These findings suggest that while the tested isolates predominantly carried the *K1* and *K2* serotypes and the mrkD virulence gene, other virulence and capsular-associated genes were absent in this sample set.

Statistical analysis

The chi-square test revealed significant associations between demographic factors and antibiotic resistance patterns ($p<.05$). Specifically, age group and sex were significantly associated with resistance profiles. Admission class and patient status also showed significant associations. Additionally, significant associations were observed between sample type and sex.

ANOVA indicated a significant difference in mean ages across various categorical groups ($p<.05$). Post-hoc tests revealed specific group differences, suggesting that certain age groups had significantly different resistance patterns compared to others.

Discussion

The emergence and dissemination of AMR in bacterial pathogens substantially threaten global public health. This study's findings highlight the alarming prevalence of MDR *K. pneumoniae* strains in a clinical setting in Nigeria, consistent with global trends.[15,16] By addressing the limited understanding of AMR in LMICs, particularly in Nigeria, this study provides real-world insights into resistance patterns and molecular characteristics of *K. pneumoniae*. The findings also demonstrate the critical role of demographic factors, resistance profiles, and virulence traits in shaping the epidemiology of AMR infections.

Our results revealed that all isolates exhibited resistance to ceftriaxone, cefotaxime, and cefepime. These findings are consistent with other studies in LMICs, where overuse and misuse of third-generation cephalosporins have led to high resistance rates.[17,18] Furthermore, 35% of the isolates were resistant to carbapenems, often considered the last line of defence against severe gram-negative infections. This high level of carbapenem resistance underscores the global spread of CRE[19–21] and aligns with the WHO's prioritization of CRE as a critical public health threat.

The significant associations between demographic factors and resistance patterns provide further insights into the epidemiology of MDR *K. pneumoniae*. The chi-square test revealed that age and sex were significantly associated with resistance profiles, suggesting that specific demographic groups, such as males and older patients, are more vulnerable to MDR infections. These vulnerabilities may be attributed to increased healthcare exposure and comorbidities requiring frequent antibiotic use. Additionally, the analysis revealed significant differences in resistance patterns between isolates collected from different body sites, likely due to variations in local immune responses, antibiotic penetration, and site-specific

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prevalence of resistant strains. The high resistance observed in urinary and wound samples may reflect the selective pressure of antibiotics frequently used to treat these infections.

Molecular characterization of the isolates showed that all tested isolates harbored the virulence genes ***K1, K2***, and ***mrkD***, which are associated with capsule formation and adherence, enhancing the pathogen's ability to evade host immune responses and persist in the host.[22,23] These findings align with reports from similar studies, which have identified these virulence factors as critical contributors to the success of MDR *K. pneumoniae* in clinical settings.[24–26] However, this study did not detect other tested genes, including ***fimH, ramA, traT, K3,*** and ***K5***, suggesting a potential variation in virulence profiles among isolates.

To provide additional clinical context, recent guidance from the Infectious Diseases Society of America (IDSA) highlights the use of novel therapeutic agents, such as meropenem-vaborbactam, ceftazidime-avibactam, imipenem-cilastatin-relebactam, and cefiderocol, for managing infections caused by AMR gram-negative bacteria, including *K. pneumonia*.[27] These agents represent critical advancements in the treatment of CRE and other resistant infections. However, due to resource constraints and the limited availability of these agents in LMICs, this study did not include them in its resistance testing. Future studies should prioritize evaluating the efficacy of these agents in resource-limited settings to inform treatment guidelines better and improve clinical outcomes.

Comprehensive prevention strategies are essential to combat the spread of MDR *K. pneumoniae* and other AMR pathogens. Key strategies include antimicrobial stewardship programs (ASPs) to optimize antibiotic use, infection control measures such as strict hand hygiene and isolation precautions, and enhanced surveillance to track resistance patterns and detect outbreaks.[28] Additionally, public education on the dangers of antibiotic misuse, regulated use of antibiotics in veterinary and agricultural practices, and investment in the research and development of new antibiotics and vaccines are critical. International collaboration and regulatory measures to control the sale and use of antibiotics are also needed to support these efforts.

This study has several limitations. AST was conducted exclusively using the disk diffusion method, which, although well-standardized and practical, provides limited quantitative insights compared to methods such as broth microdilution or automated systems. This study did not include molecular or genetic testing to identify specific resistance genes, such as common β-lactamase genes (eg, *CTX-M, AmpC*) or carbapenemase genes (eg, *NDM, OXA*). These genetic markers are critical for understanding resistance mechanisms and could provide valuable insights into the spread of resistance within *K. pneumoniae*. The absence of molecular characterization limits the ability to assess the full genetic basis of resistance and its clinical implications. Future studies should incorporate molecular resistance testing to provide a more comprehensive understanding of the genetic drivers of AMR in *K. pneumoniae*.

The study is further limited by its single-center design and relatively small sample size, which may restrict the generalizability of the findings. Additionally, the absence of testing for newer therapeutic agents recommended by the IDSA guidelines limits the clinical applicability of this study. Expanding testing capabilities to include novel agents and adopting molecular resistance analysis would enhance the depth and relevance of future research.

By addressing these challenges comprehensively, we can mitigate the impact of AMR and safeguard the efficacy of antibiotics for future generations. This study emphasizes the urgent need for targeted interventions, such as antimicrobial stewardship programs and the adoption of IDSA-recommended therapies, to improve clinical outcomes. Expanding testing capabilities and incorporating newer agents in LMICs is critical to developing effective strategies to combat MDR *K. pneumoniae* and other resistant pathogens.

Conclusion

This study reveals a concerning prevalence of multidrug-resistant *K. pneumoniae* strains in a clinical setting in Nigeria. The findings highlight significant associations between demographic factors and resistance patterns, emphasizing the complexity of managing these infections. There is an urgent need for comprehensive antimicrobial stewardship programs, robust infection control measures, and continued research into novel therapeutic strategies and vaccines. Enhanced surveillance, public education, and international collaboration are essential to mitigate the spread of AMR. By addressing these challenges through targeted and coordinated efforts, we can preserve the effectiveness of antibiotics and protect public health.

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