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Antibiotic Resistance, Biofilm formation, Molecular properties and Hypermucoviscosity in Hypervirulent *Klebsiella pneumoniae*

Harsha Moorthi C S[1], Dr. Ramanath Karicheri[2]

[1]Research scholar, Department of Microbiology Index Medical College, Hospital & Research Centre, Indore (MP) Malwanchal University, MP, India
[2]Professor and Head Department of Microbiology Index Medical College, Hospital & Research Centre, Indore (MP) Malwanchal University, MP, India

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ABSTRACT

Klebsiella pneumoniae is a major pathogen behind urinary tract infections (UTIs) and various other health issues and is exhibiting multidrug resistance. This study aimed to isolate, identify, and evaluate the antibiotic resistance and biofilm-forming capacity of *K. pneumoniae* from diverse clinical samples. Out of 190 samples that were processed, 98 were found to be culture-positive. Of which 45% were confirmed as *K. pneumoniae*, predominantly from urine samples. Notably, a higher prevalence of *K. pneumoniae* infection was observed among male patients. Biofilm formation was noted in most of the isolates, which were classified as high biofilm producers, moderate biofilm producers, or weak biofilm producers. Biofilm producers demonstrated increased rates of antibiotic resistance, with the isolates showing resistance to cefuroxime, ceftazidime, gentamicin, meropenem, and imipenem in 77.1%, 74%, 68.4%, 58%, and 55% of cases, respectively. Colistin was evaluated for multiple multidrug-resistant (MDR) strains, and was effective against most of the isolates tested.
Beta-lactamase production was confirmed in the isolates by the double-disc synergy test and 5 ESBL-producing isolates tested positive for the blaCTX-M gene. Agarose gel electrophoresis of PCR products displayed separate amplicons in the 550-to-600 bp range, suggesting the potential presence of blaCTX-M-1. This points to an increased prevalence of biofilm-forming ESBL-producing K. pneumoniae, emphasizing the need to strengthen antibiotic stewardship, infection control practices, and explore alternative therapeutic strategies to address emerging multidrug resistance.

INTRODUCTION

The emergence and spread of antibiotic-resistant bacteria leads challenges to global healthcare systems (WHO 2024). *Klebsiella pneumoniae*, a multi-drug resistant pathogen, is well-known for its ability to form biofilms, and its virulence and diversity of antimicrobial resistance in clinical isolates (Paczosa & Mecsas 2024). *K. pneumoniae* is also considered an ESCAPE pathogen (a group of highly virulent, multidrug resistant nosocomial pathogens) (Santajit S, Indrawattana 2016). Multi-drug resistant (MDR) and extensively drug resistant (XDR) bacteria have emerged from the overuse and misuse of antibiotics resulting in even the most potent drugs being rendered ineffective against these vicious pathogens. Effective management, strict infection prevention, and monitoring drug resistance patterns have become essential roles of health care workers (Ye et al., 2021).

Klebsiella pneumoniae is also known for its ability to form biofilms. Biofilms are complex and structured communities of microorganisms that are covered by a self-produced extracellular polymeric substance matrix comprised of a variety of polysaccharides, proteins, nucleic acids, and other secreted materials. They pose a particular problem in medical implants and indwelling devices, such as stents and catheters, because they hide the bacteria from host defenses and substantially increase resistance to antimicrobial therapy (Guerra et al., 2022).

In addition to biofilm-specific resistance, *K. pneumoniae* frequently evades antibiotics by producing extended-spectrum β-lactamases (ESBLs). The most prevalent β-lactamase enzymes, for example, CTX-M Group 1 β-lactamases, inactivate third-generation cephalosporins. This is a significant mechanism of resistance that could impact treatments of infections caused by these bacteria, with implications for increased hospitalization, failures of treatment, and even increased mortality rates (Muthupandian et al., 2018). The dissemination of CTX-M Group 1 has caused an urgent need for improved infection controls, the development of new antibiotics, and ongoing monitoring of the emergence and spread of resistant bacteria (Chen et al., 2014).

Materials and Methods

This study was conducted from January 2022 to February 2023, in the department of microbiology, Malwanchal University, Indore, Madhya Pradesh, India. The ethical committee approval was obtained with the reference number Mu/Research/EC/Ph.D/2021/197. This study employed an experimental design to investigate high mucoid colonies, biofilm formation, antimicrobial resistance and the presence of *bla CTX-M* gene in *Klebsiella pneumoniae* isolated from various clinical samples.

Inclusion and Exclusion Criteria

The study included *Klebsiella pneumoniae* strains isolated from clinical specimens aseptically received in the diagnostic laboratory of hospitalized patients, exhibiting biofilm-forming capacity. Strains with complete antibiotic susceptibility testing results were included. The strains demonstrating ESBL production were prioritized for molecular detection of *bla CTX-M* gene.

Conversely, samples that did not yield *Klebsiella pneumoniae* isolates were excluded. Samples with suspected contamination or polymicrobial growth were also excluded.

Isolation and Identification

Klebsiella species were isolated from clinical specimens using standard microbiological techniques. Bacterial growth was observed on MacConkey agar and blood agar. Gram staining
and biochemical tests were performed for the

presumptive identification of *Klebsiella* species according to Practical Medical microbiology 14th edition of Collee et al., 1996.

String test

This test were performed to identify hypermucoviscous *K. pneumoniae*, it is a semi quantitative nethod defined as positive if the the inoculam loop or needle is able to generate a viscous string > 5 mm in length from the colonies on agar plate (shon *et al.*, 2013)

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Table 1: The antibiotic discs used for the susceptibility test and the results

Antibiotics	The number of *klebsiella pneumoniae* shown Resistance and sensitivity pattern	
	Resistance (number)	Sensitivity (number)
Amikacin (AMK)	22	16
Amoxicilin – clavulinic acid (AMC)	24	14
Cefepime (FEP)	24	14
Cefoxitin (FOX)	21	17
Ceftriazone (CRO)	22	16
Ciprofloxacin (CIP)	23	15
Imipenem (IMP)	21	17
Meropenem (MRP)	22	16
Nalidixic acid (NAL)	11	5
Nitrofurantoin (NIT)	10	6
Cefuroxime (CXM)	27	11
Ceftazidime (CAZ)	28	10
Colistin (CLS)	1	9
Gentamicin (GEN)	26	12
Contrimoxazole (COT)	22	16

ESBL detection by double disc synergy test (DDST)

A lawn culture of the bacterial isolate is prepared on Mueller-Hinton agar, ceftazidime (CAZ 30) and ceftazidime-clavulanic acid (CAC 30/10) discs, are placed 20–25 mm apart. The plate is incubated at 37°C for 18–24 hours. An enhanced zone of inhibition around the clavulanic acid-containing discs compared to the cephalosporin discs alone indicates ESBL production (Kumaran *et al.*, 2022).

CTX-M gene detection

Bacterial cells were harvested by centrifugation at 8000-10000 rpm for 3 minutes. The cell pellet was washed once with 1 mL of PBS and centrifuged again. Cells were lysed in 500 µL of lysis buffer (BGC) at 56°C for 20 minutes, followed by the addition of 5 µL RNase A. After incubation, 700 µL of binding buffer (BB) and magnetic beads (ZMB) were added, and the mixture was incubated at room temperature for 10 minutes. The beads were then separated using a magnetic stand, and the supernatant was discarded. The beads were washed twice with 500 µL of wash buffer, followed by air-drying for 5 minutes. Finally, DNA was eluted from the beads with 100 µL of elution buffer at 56°C for 5 minutes. All the procedures were followed as per the manufacturer's instruction. Magnetic-bead based gene extraction kit of 'Zymag' were used for the gene extraction. The eluted DNA was collected and stored at -20°C for further analysis. The Pcr cycles were carried out as per the procedure of Moghaddam *et al.*, 2014, PCR amplifications were performed to detect the presence of ESBL gene- CTX-M Group 1, using specific primers (listed in Table 1).

Table 2: Primer for *blaCTX-M*

CTXM-1	Forward Primer: 5′- TTAGGAARTGTGCCGCTGYA-3′ Reverse Primer: 5′-CGATATCGTTGGTGGTRCCCAT-3′

Table 3: PCR conditions for amplifying *blaCTX-M* and *blaPER* genes by polymerase chain reaction

Stage	Temperature (°C)	Time	No. of Cycles

Hot start	94	5 min	1
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	30 sec	35
Final extension	72	3 min	1

Results

Isolation and Identification of Klebsiella pneumoniae

Total 190 clinical specimens were processed and 98 were shown culture positive, and among the positive isolates, 38 (45%) isolates were identified as *Klebsiella pneumoniae*.

Chart 1. Prevalence of *Klebsiella pneumoniae*

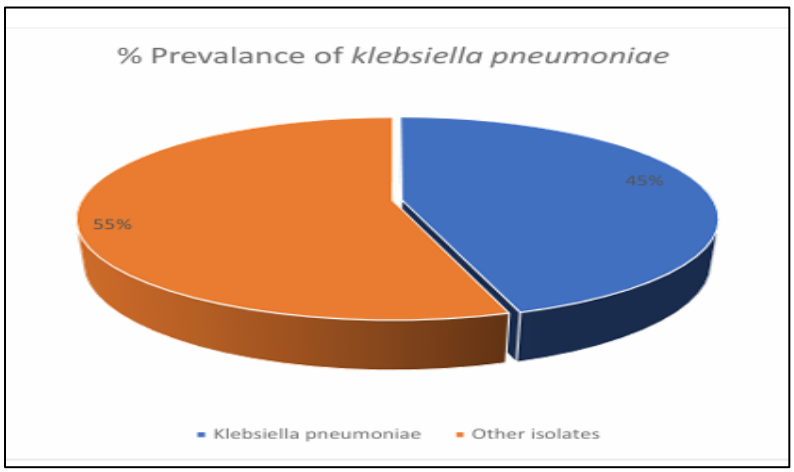
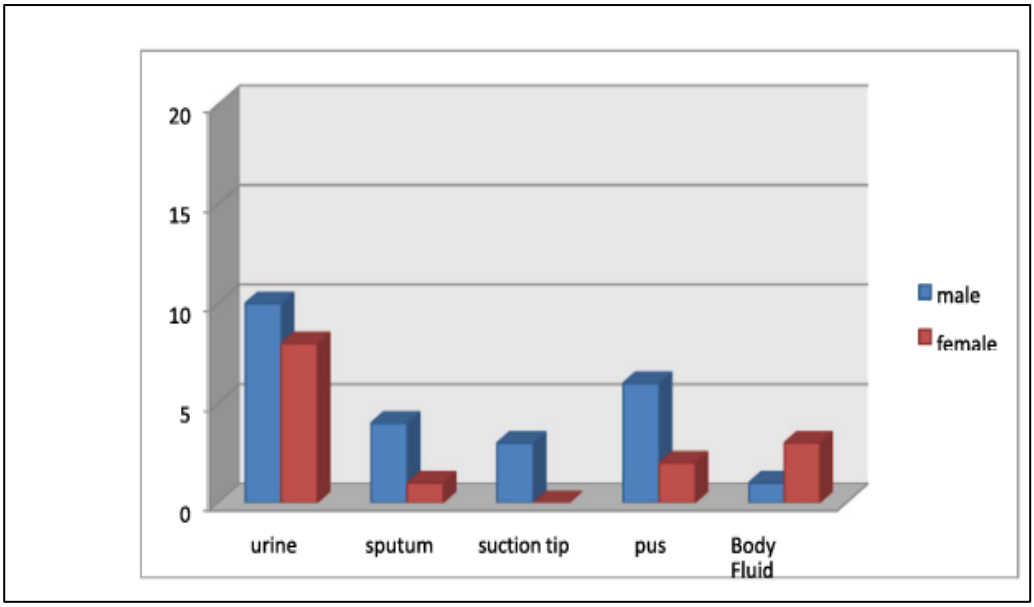


Table 4: Gender-Based Distribution of *Klebsiella pneumoniae* Isolates

SAMPLE	NUMBER OF *K.pneumoniae* ISOLATED FROM EACH SAMPLE	
	MALE	FEMALE
Urine	10	8
Respiratory samples	4	1

Suction tip	3	0
swab(wound)	6	2
Fluid	1	3

Chart 2: distribution of *K.pneumoniae* isolated from each samples



The majority of Klebsiella pneumoniae isolates were obtained from urine samples, indicating a significant prevalence in urinary tract infections. Additionally, the occurrence of K. pneumoniae was higher in male patients compared to female patients, suggesting a potential gender-based variation in susceptibility or infection rates.

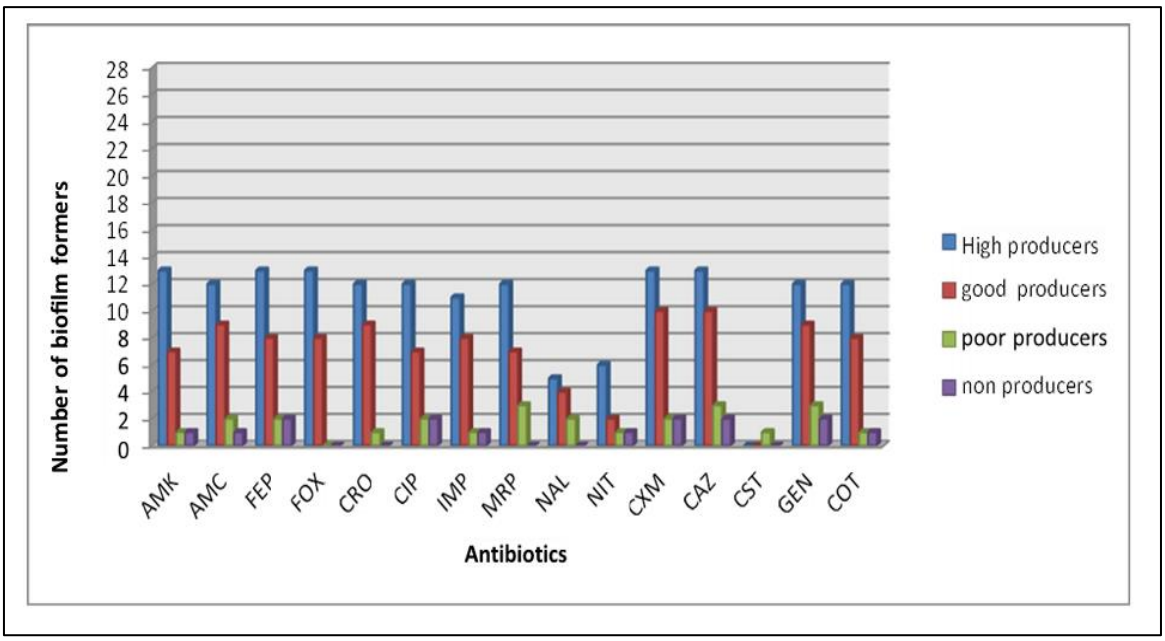
Figure 1: Hypermucoviscous colonies of *klebsiella pneumoniae*

Observed hypermucoviscous colonies for all the 38 isolates, and the string test were positive for them.

Biofilm Formation

Out of 38 *isolates*, 27 were biofilm producers. Among them 13 isolates were high producer, 10 isolates were good producers and 4 isolates were poor producers. Highest number of biofilm producers were isolated from the urine and most of them are high biofilm producers. All the 38 *klebsiella pnemoniae* were analysed for antibiotic sensitivity pattern and compared with the biofilm producers.

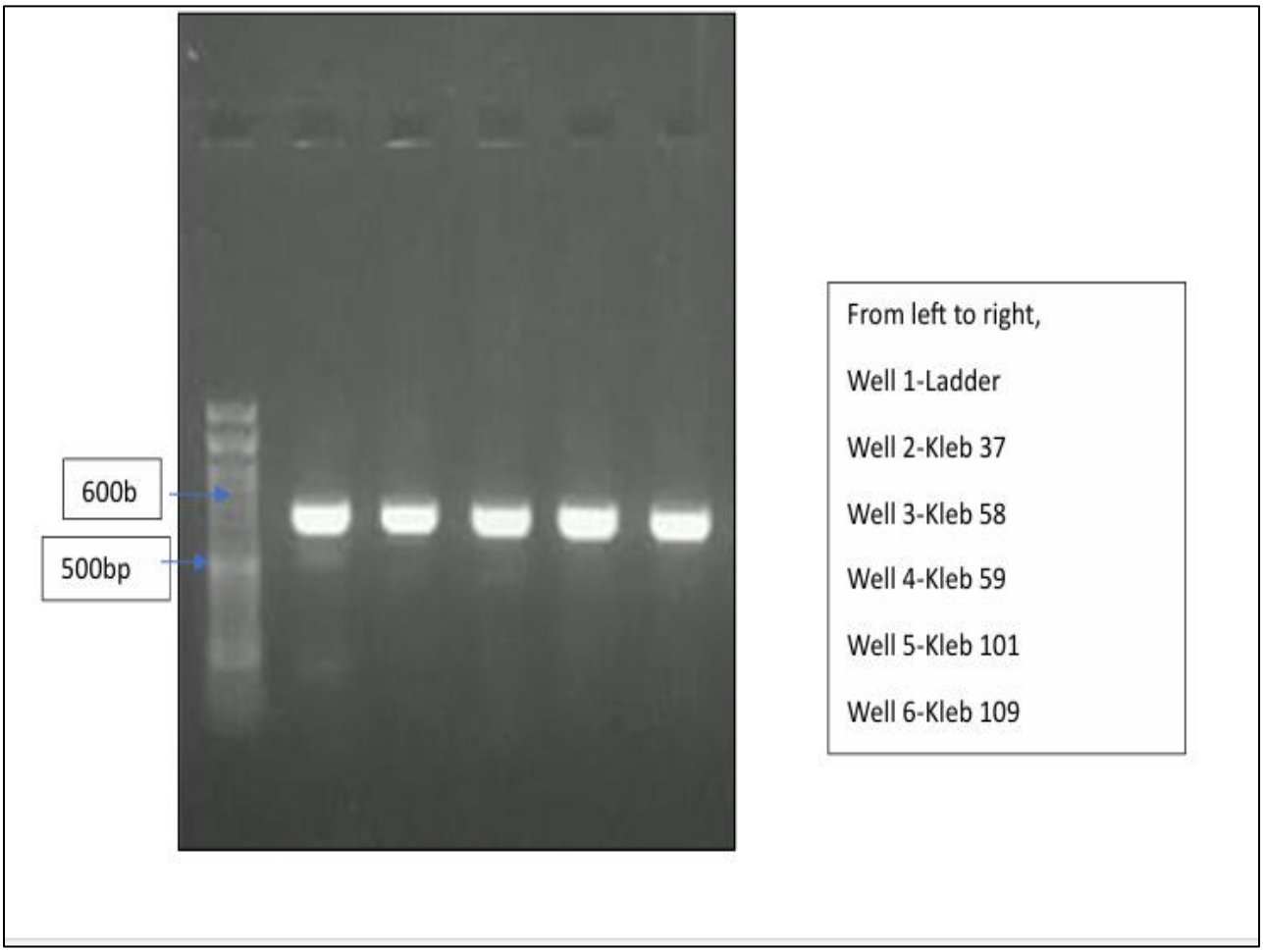
Chart 3: Comparitive analysis of antibiotic-resistant patterns among high, moderate (Good), poor and Non-producers



The biofilm producers were showed more resistance to antibiotics in comparison to non-biofilm producers, that the non-biofilm producing strains were more sensitive to antibiotics. The *K.pneumoniae* strains were showed high resistance to majority of the antibiotics. In this high resistance towards the cefuroxime (77.1%), ceftazidime (74%) gentamicin (68.4%), Meropenem (58 %), and imipenem (55 %). Due to this high resistance pattern of klebsiella pneumoniae, we tried using colistin disc for 10 selective multi drug resistant strains, The colistin were identified as a sensitive drug for 9 out of 10 strains.

The betalactamase production were confirmed by using Double disc test with cac and caz. Among all the resistant strains especially the strains shown resistant towards beta lactam antibiotics, 5 strains were positive for betalactamase production and the all 5 strains were tested positive for the presence of *blaCTX-M gene.*

Figure 1. The pattern of PCR amplification products of *blaCTX-M* amplified products analysed on a 1% agarose gel.



The agarose gel electrophoresis (AGE) image shows the PCR amplification products of *Klebsiella pneumoniae* isolates targeting the *blaCTX-M* gene. The gel consists of multiple lanes, with the first lane containing a molecular weight (MW) marker for size estimation.

Distinct single bands are visible in all tested lanes, appearing around the 550–600 bp region, which corresponds to the expected size of the blaCTX-M-1 amplicon. The presence of clear, well-defined bands without significant smearing indicates successful amplification with minimal degradation or nonspecific amplification. These results confirm that the *blaCTX-M* gene is present in the tested *Klebsiella pneumoniae* isolates, indicating their potential for extended-spectrum beta lactamase (ESBL) production.

CONCLUSION

The research highlights a critical association between the high rate of biofilm formation among isolates, particularly those from urine samples, and increased antimicrobial resistance. Biofilm-producing strains demonstrated significantly greater resistance across multiple antibiotic classes, including cefuroxime, ceftazidime, gentamicin, meropenem, and imipenem. This correlation underscores the challenge in effectively treating *K. pneumoniae* infections associated with this virulent phenotype. The detection of the blaCTX-M gene in five multidrug-resistant (MDR) isolates confirms the presence of Extended-Spectrum Beta-Lactamase (ESBL) production, thereby emphasizing the urgent necessity for enhanced antimicrobial stewardship and molecular surveillance to control the spread of these resistant mechanisms. Despite the high overall resistance rates, colistin demonstrated encouraging effectiveness against most of the tested MDR strains, suggesting its continued potential as a valuable last-resort therapeutic agent. These findings collectively underscore the critical need for continuous monitoring of resistance patterns and biofilm-associated infections, alongside the implementation of alternative therapeutic strategies, to effectively combat the rising global threat posed by MDR *Klebsiella pneumoniae*.

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