

Original Article

Correlation between antibiotic resistance and biofilm formation in *Klebsiella* spp.

Dipan Barai¹, Mohibur Rahman Rafe¹, Najmun Nahar¹, Shahin Ara Begum², Humaira Akther¹, Anowara Begum^{1*}.

¹Department of Microbiology, University of Dhaka, Dhaka-1000, ²Laboratory Medicine, Green life Hospital Ltd, Dhaka

Antibiotic resistance among various microbial species is becoming a global threat to human health. MDR, or multidrug resistance, is an organism's ability to tolerate the effects of many antimicrobial treatments. *Klebsiella* spp. is the major bacteria that causes a variety of illnesses, including urinary tract infections. The primary goal of this study is to look into the relationship between antibiotic resistance and *Klebsiella* spp. biofilm production. This study included 26 clinical isolates of *Klebsiella* spp. (n=26) collected from a tertiary hospital in Bangladesh. Isolates were obtained from blood, wound swabs, urine, and other sources. Following isolation and identification, ampicillin resistance was found in all MDR *Klebsiella* spp. isolates, followed by Cefotaxime (84.61%), Polymyxin B (84.61%), Amoxicillin (80.76%), Trimethoprim (69.23%), Doripenem (57.69%), Ciprofloxacin (57.69%), Imipenem (50.0%), Meropenem (38.46%), and Oxytetracycline (34.61%). Out of 26 clinical isolates, about 26.92% (n=7) were positive for the *bla*-NDM-1 gene. However, 15.8% of *Klebsiella* spp. (n = 4) isolates tested positive for the *bla*OXA-1 gene. There were no *bla*-KPC gene positives. Plasmid profile analysis revealed that 24 of 26 *Klebsiella* spp. isolates included numerous plasmids ranging in size from less than 2kb to more than 10kb. Biofilm formation found that 31% of samples were extremely positive for biofilm formation, 69% were medium biofilm formation, and (n=4) isolates were positive for biofilm resistance gene out of a total of 26 isolates. This study provides an early report on the widespread presence of carbapenem-resistant *Klebsiella* spp., demonstrating the need for intensive surveillance systems and research initiatives in Bangladesh to reflect the influence of multidrug resistance features in clinical isolates and their risks.

Keywords: *Klebsiella* spp, MDR, PCR, Antibiotic, Biofilm, Plasmid.

Introduction:

Klebsiella species are Gram-negative, non-motile, encapsulated, lactose-fermenting and facultative anaerobic bacteria that cause several diseases, especially in countries with a poor health system¹. *K. pneumoniae*, its subspecies (*K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis*) and *K. oxytoca* are the frequently isolated and medically important members of this genus². Despite being a member of the normal flora of the human gastrointestinal tract, *K. spp.* is the most common pathogenic agent of nosocomial infections³. Pneumonia, urinary tract infection (UTI), blood stream and surgical wound infections, peritonitis, septicemia, meningitis⁴ and pyogenic liver abscess⁵ are the major diseases caused by *K. pneumoniae* in humans. *Salmonella* spp., *Escherichia coli*, and *K. pneumoniae* have the highest rates of antimicrobial resistance among *Enterobacteriaceae*^{5,7}. These infections have evolved resistance to widely used antimicrobial drugs, including carbapenems, aminoglycosides, fluoroquinolones, and extended-spectrum beta-lactams^{4,5}. Because of this, there are fewer therapeutic options, more expensive treatment options, longer hospital stays, and mortality^{6,7}. Numerous virulence factors, including the

capsule, lipopolysaccharide (LPS), fimbria and non-fimbria, adhesions, siderophores, and the capacity to produce biofilm, are responsible for the pathogenicity of *Klebsiella* spp. The bacterial aggregation known as biofilms are securely embedded in the extracellular matrix of polysaccharides, proteins, enzymes, and nucleic acid that permit irreversible attachment to any surface⁸. *K. pneumoniae* infections are becoming harder to treat because of their propensity to develop biofilms, necessitating the need for creative anti-biofilm strategies⁹. Biofilm production by KPC-producing *K. spp.* strains has been linked to high levels of antibiotic resistance¹⁰. Increased morbidity and mortality as well as persistent infections that are challenging to treat may arise from this¹¹.

Resistance to β -lactam antibiotics including penicillins, cephamycin, and carbapenem is caused by β -lactamase enzymes like extended-spectrum β -lactamase (ESBL), Amp C β -lactamases, and carbapenemase^{12,13}. For life-threatening infections brought on by multidrug resistant (MDR) pathogens including *Enterobacteriaceae* that produce ESBL, carbapenems are administered as a last option^{14,15}. *K. spp.* is the most prevalent carbapenem resistant *Enterobacteriaceae* (CRE), however over the past few years, due to

*Corresponding author:

Prof. Dr. Anowara Begum, Department of Microbiology, University of Dhaka, Dhaka-1000, E-mail: anowara@du.ac.bd

the selection pressure of treating the ESBL and AmpC infections with carbapenems, resistance has arisen¹⁶.

The transmission of antibiotic resistance genes from environmental bacteria to clinically significant bacteria is another key function of *K. spp*¹⁷. Antimicrobial resistance has a variety of ways that will harm the effectiveness of treatment. Antibiotic resistance has been identified by the World Health Organization (WHO) as one of the top three global health issues¹⁸.

There have been numerous research looking into the synthesis of beta-lactamase enzymes among *Klebsiella* species all over the world^{19, 20}, but there haven't been many of these in Bangladesh. Studies on resistance caused by co-expression of beta-lactamase enzymes and resistance caused by biofilms are rare^{21, 22}. The variable incidence of *Klebsiella* spp. has been documented in a few earlier investigations conducted in Bangladesh. Investigating the prevalence of *Klebsiella* species that produce biofilms, ESBL, and KPC as well as assessing their coexistence and role in the development of drug resistance were the main objectives of this study.

Materials and Methods

Sample Collection, Isolation, identification and antibiotic susceptibility tests of Klebsiella spp.

Samples for this study was collected from tertiary hospital in Bangladesh. Identification of *Klebsiella* spp. isolate was conducted by culturing on MaConkey agar, gram staining and biochemical testing. Kirby Bauer method was used to perform antibiotic susceptibility tests. The Clinical and Laboratory Standards Institute 2020 was used to classify as sensitive, intermediate or resistant bacteria (CLSI 2020)²³. *Klebsiella* spp. isolates that showed resistance to three or more different classes of antimicrobials were classified as multidrug resistant (MDR) *Klebsiella* spp.²⁴. Total 26 isolates were tested for antimicrobial susceptibility following Kirby Bauer method²⁵ with 10 different groups of antibiotics using the standard agar disk diffusion method on Muller-Hinton agar (Oxoid Ltd., Basingstoke, UK).

Bacterial isolates were initially cultivated for 1–1.5 hours in Muller–Hinton broth (MHB) (Oxoid, UK) to a turbidity corresponding to 0.5 MacFarland standard. On MHA plates, a bacterial lawn was produced, and an antibiotic disc was added. The plates were incubated for 16-18 hours at 37°C. The zones of inhibition were measured, and the susceptibility was scored according to the standards by the clinical Laboratory Standards institute (CLSI, 2020).

Biofilm formation assay

Biofilm formation assay was performed in accordance with the protocol developed by George A.O'Toole²⁶ with some minor modification.

Growing a biofilm

Biofilm formation was estimated in 96-well polystyrene plates. Initially, all the samples were grown in Luria broth (LB) medium for 24 h at 37°C with shaking. After overnight incubation, a 1:100 dilution was prepared by adding 10 µl of culture into micro-centrifuge tube containing 990 µl of fresh LB broth. Each well received 250 µl of diluted culture (3 duplicates for each isolate). Then, the microtiter plate was incubated at 37°C for 24 hours.

Staining the biofilm

After overnight incubation, the bacterial cells were dumped out by turning the plate over and shaking out the liquid. Unattached cells and media components were removed by washing gently 2-3 times by submerging in a small tub of sterile water for lowering the background staining and the plates were then air dried for 5-10 minutes. Then 125 µl of 0.1 % solution of crystal violet was added to each well of the microtiter plate. The microtiter plate was incubated at room temperature for 10-15 minutes. The plates were rinsed 3-4 times with water by submerging them in a tub of water, shaking them out, then vigorously blotting them on a stack of paper towels to remove all extra cells and dye. The microtiter plate was turned upside down for a few hours or overnight.

Quantifying the Biofilm

In order to dissolve the crystal violet that was adhered to the cells, 125 µl of 33% acetic acid was applied to each well of the microtiter plate and was incubated at room temperature for 10-15 minutes. Finally, the absorbance was measured at 560nm in a plate reader using a blank of 33% acetic acid in water.

Extraction of Chromosomal DNA

The Boiled DNA method²⁷ was followed for extracting chromosomal DNA. After extraction of DNA, DNA Concentration was measured and purity checked by Colibri Microvolume Spectrometer (Titertek- Berthold, Berthold Detection Systems GmbH, Bleichstrasse, Pforzheim, Germany).

PCR amplification

Amplification was performed in 14.25 µl reaction volumes for each specimen containing 2.5 µl of template DNA. The reaction volume was prepared by mixing the components in eppendorf tubes and after well mixing by pipetting a volume of 11.75µl was transferred to each PCR tube. Then templates were added to each PCR tubes and transferred to thermal cycler for amplification and run was 35 cycles. After PCR, the PCR product was visualized by agarose gel electrophoresis (Bio Rad, USA). After PCR, the PCR product was visualized by gel electrophoresis (Bio Rad, USA)

Table 1: Primer list for Antibiotic resistance genes

Target genes	Primer name	Primer sequence (5'-3')	Amplicon Size (ref.)	Annealing Temp (°C)
<i>bla</i> NDM-1	NDM-F	GGTTTGGCGATCTGGTTTTC	465 (Talukdar Rahman <i>et al.</i> , 2013)	58
	NDM-R	CGGAATGGCTCATCACGATC		
<i>bla</i> OXA-1	OXA-1F	ACACAATACATATCAACTTCGC	814 (Talukdar Rahman <i>et al.</i> , 2013)	54
	OXA-1R	AGTGTGTTTAGAATGGTGATC		
<i>bla</i> KPC	KPC-F	CATTCAAGGGCTTCTTGCTGC	498 (Dallenne <i>et al.</i> , 2010)	52
	KPC-R	ACGACGGCATAGTCATTTGC		

Table 2: PCR conditions for gene amplification

Segment	Process	Temperature(°C)	Time
Segment 1	Denaturation	95	45 seconds
Segment 2	Annealing	Primer specific AT	45 seconds
Segment 3	Extension	72	45 seconds

Table 3: Primer list for Biofilm gene

Target gene	Primer name	Primer sequence (5'-3')	Amplicon Size (bp)	Annealing T(°C)
<i>fim</i> -H	fimH-F	TGCAGAACGGATAAGCCGTGG	508	63
	fimH-R	GCAGTCACCTGCCCTCCGGTA		

Table 4: PCR conditions for biofilm gene amplification

Segment	Process	Temperature(°C)	Time
Segment 1	Denaturation	95	45 seconds
Segment 2	Annealing	63	45 seconds
Segment 3	Extension	72	45 seconds

Isolation of plasmid DNA

FAVORGREN (FavorPrep Cat.No: FAPDE100) plasmid Miniprep Kit was used for plasmid extraction. Whole process was done according to manufacturer's instruction.

Results

Antibiotic resistance pattern of *Klebsiella* spp.

The Kirby-Bauer disk diffusion assay was used to determine the antibiotic resistance of all *Klebsiella* spp. isolates to a total of 10 antibiotics from 7 different classes. The tested isolates showed a varying extent of resistance patterns against these antibiotics. Among the MDR *Klebsiella* spp. isolates, all isolates were resistant to Ampicillin and followed by Cefotaxime (84.61%), Polymyxin B (84.61%) Amoxicillin (80.76%), Trimethoprim (69.23%), Doripenem (57.69%), Ciprofloxacin (57.69%), Imipenem (50.0%), Meropenem (38.46%), Oxytetracycline (34.61%), respectively.

Detection of antibiotic resistance genes

Out of 26 isolates, about 7 of them were positive for *bla*NDM-1 gene, the prevalence was 26.92%. Out of 26 isolates, about 4 of them were positive for *bla*OXA-1 gene, the prevalence was 15.8%. There was no positive isolates for *bla*-KPC-1 gene.

Biofilm formation

From the 26 isolates, 8 isolates (31%) were very strong biofilm forming and 18 isolates (69%) were medium biofilm forming.

Out of 26 isolates, 4 isolates (15.38%) were found PCR positive for biofilm resistance genes.

Plasmid profile in *Klebsiella* spp.

Analysis of plasmid profile of the *Klebsiella* spp. isolates (n=26) was performed followed by visualization of the extracted plasmid on Agarose gel. Plasmid profile analysis revealed that under experimental conditions, 24 out of 26 *Klebsiella* spp. isolates harbored multiple plasmids with varying sizes ranging from less than 2 kb to more than 10 kb.

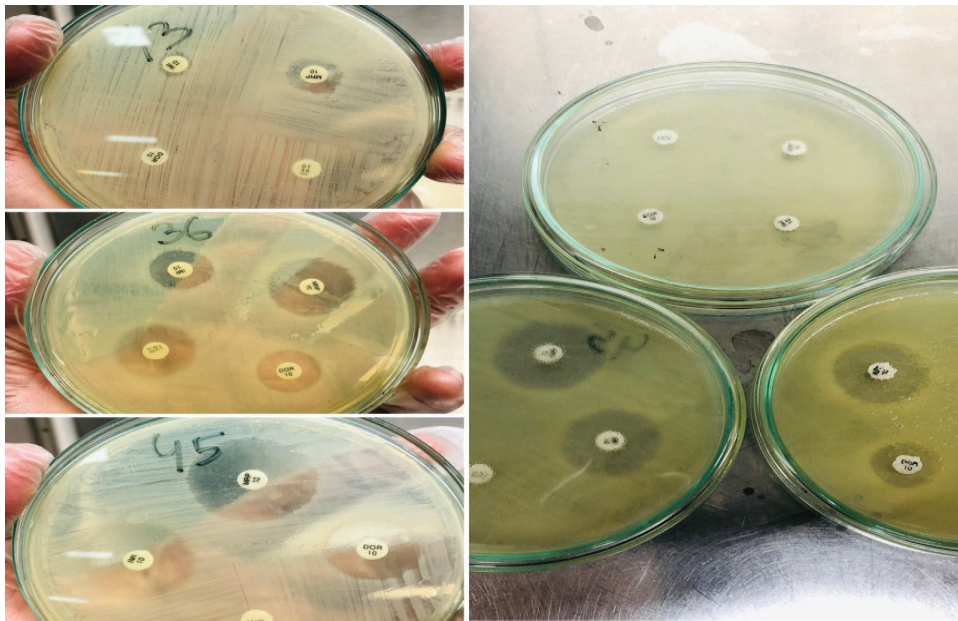


Fig. 1: Representative image of antibiotic susceptibility testing in *Klebsiella* spp. isolates by Kirby-Bauer method.

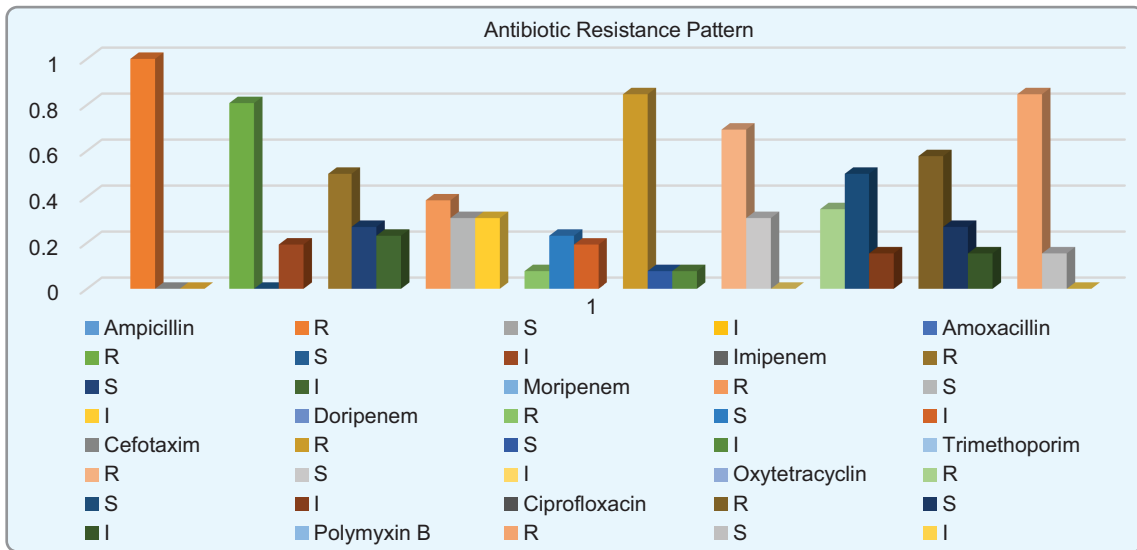


Fig. 2: Antibiotic resistance frequency of MDR *Klebsiella* spp. to different antibiotics

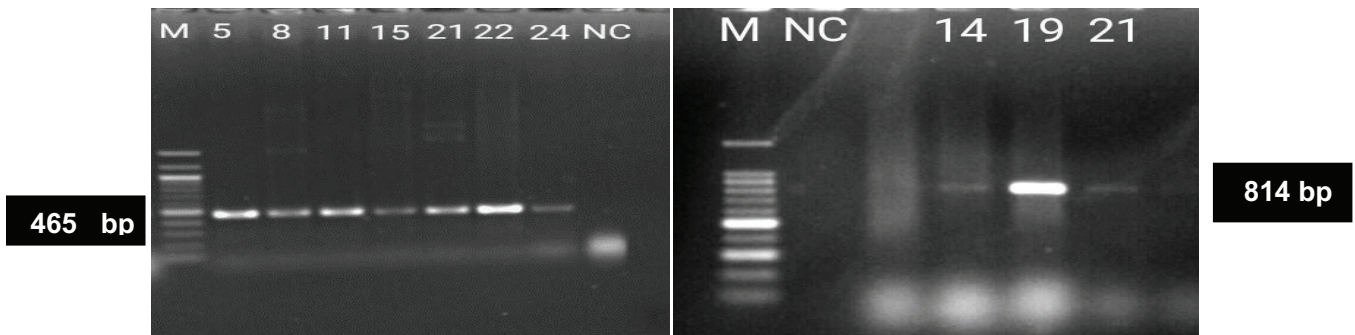


Fig. 3 & 4: Agarose Gel electrophoresis for *bla*NDM-1 and *bla*-OXA 1 protein

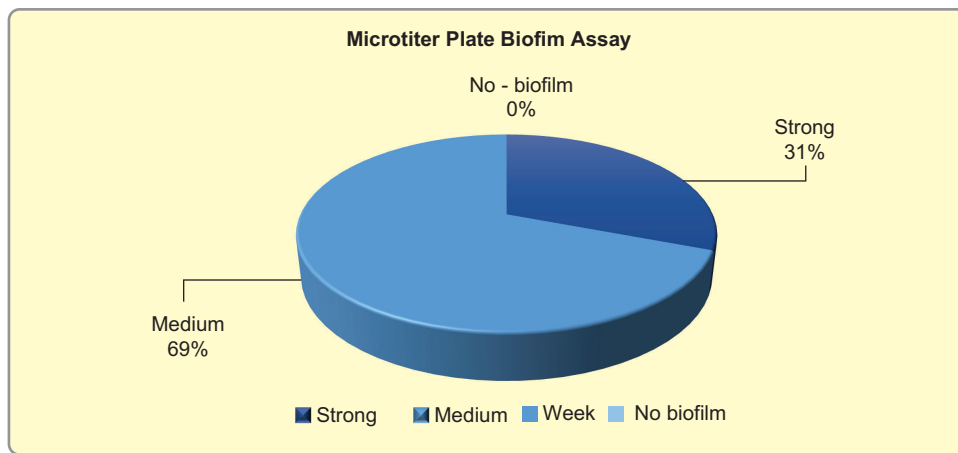


Fig. 5: Graphical representation of Biofilm Assay

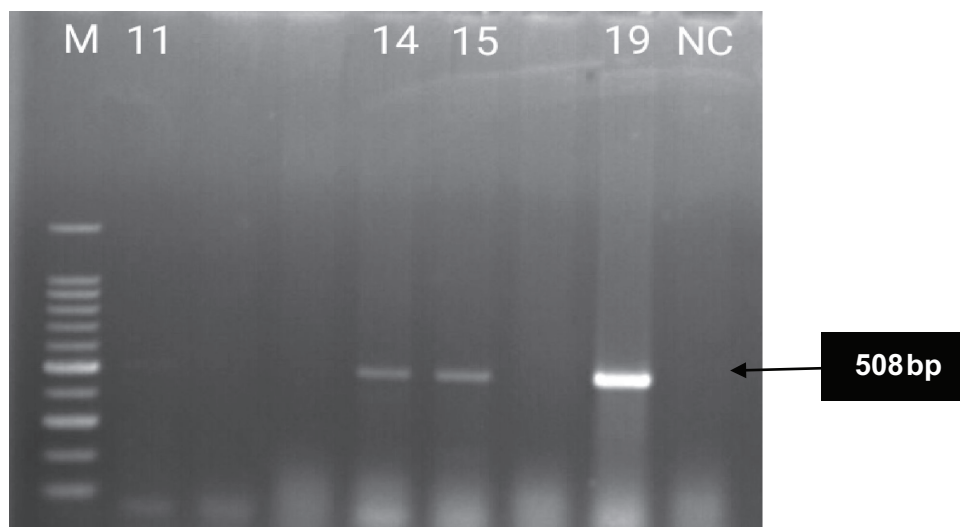


Fig. 6: Agarose gel electrophoresis for *fim-H* gene

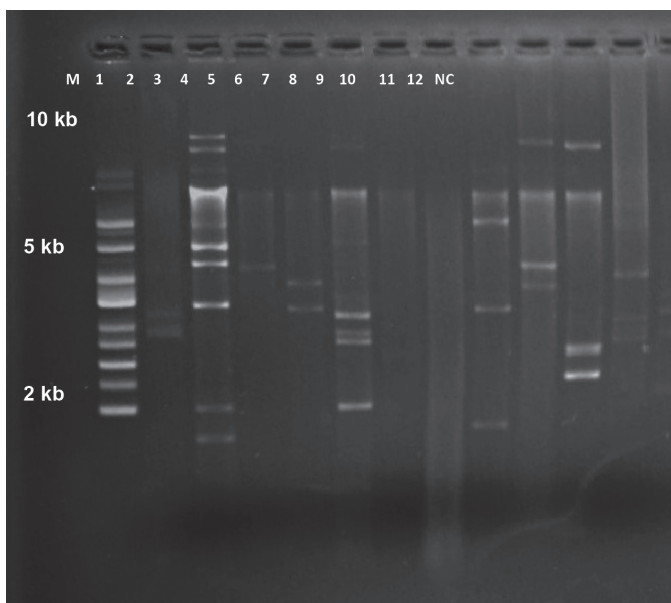


Fig. 7: Plasmid profile of *Klebsiella* spp.

Discussion

Antimicrobial resistance has quickly spread around the world, posing a severe threat to public health. Additionally, the high mortality and morbidity rates result from the widespread dissemination of multidrug-resistant bacteria, making it impossible to treat infections with current antibiotics²⁸. The genetic identification of antibiotic resistance determinants is required to understand and control the spread of multidrug-resistant (MDR) diseases. Critical relevance can be shown in the rise of MDR *Klebsiella* spp. gram-negative bacteria that cause nosocomial, urinary tract, and wound infections²⁹. *Klebsiella* species contain extended-spectrum beta-lactamases (ESBLs) and carbapenemases that can hydrolyze more recent carbapenems. Infections caused by extended-spectrum beta-lactamase (ESBL)-producing bacteria have traditionally been treated with carbapenems antibiotic³⁰. For the treatment of infections linked to healthcare that are life-threatening, these medicines are also regarded as a last resort³¹. Unfortunately, carbapenem resistance

in bacteria has increased and is well known. The overuse of antibiotics, which constitutes antibiotic abuse, is a significant contributor to this issue. For the past 22 years, a sharp rise in beta-lactam-resistant *Klebsiella* species has been noted in Bangladesh³². The issue has worsened with the identification and spread of the new carbapenemase known as New Delhi Metallo β -lactamase (NDM). This enzyme allows bacteria to withstand several antibiotic types, transforming them into superbugs. A prevalent variant of NDM native to South Asian countries, including Pakistan, Nepal, and Bangladesh, is NDM-1. In Bangladesh, natural water tests, sewer samples, and even clinical samples have all revealed the presence of this³³.

The study presents the antibiotic susceptibility profile of multidrug-resistant isolates collected from the clinical origin, their carbapenemase production ability, biofilm formation and plasmid profile analysis. All the screened isolates of this study were tested by Kirby–Bauer disk diffusion method for antimicrobial susceptibility pattern. All isolates were resistant to Ampicillin followed by Cefotaxime (84.61%), Polymyxin B (84.61%), Amoxicillin (80.76%), Trimethoprim (69.23%), Doripenem (57.69%), Ciprofloxacin (57.69%), Imipenem (50.0%), Meropenem (38.46%), and Oxytetracycline (34.61%), respectively. There are older reports from Bangladesh about multidrug-resistant gram-negative bacteria, particularly regarding *Klebsiella* species³⁴. This finding was consistent with reports from Pakistan and India, respectively³⁵. According to most reports, the *Enterobacteriaceae* family members disseminate multidrug resistance properties through mobile genetic elements such as conjugative plasmids which indicates that the isolate were fecal in origin. Out of 26 clinical isolates of *Klebsiella* spp. (n=26) about 26.92% *Klebsiella* spp. (n=7) isolates were positive for *bla*NDM-1 gene. New Delhi Metallo-beta-lactamase 1 (*bla*NDM-1) is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics, including the carbapenem family of antibiotics that are a mainstay for the treatment of antibiotic-resistant bacterial infections³⁶. 15.38 % (n =4) of these isolates were positive for the *bla*OXA-1 gene. Hence these isolates were positive for extended-spectrum beta-lactamase (ESBL) genes. With the beginning of the twenty-first century, bacterial strains harboring the *bla*OXA-1 gene have emerged and disseminated worldwide. They are now an important cause of nosocomial and community-onset urinary tract and bloodstream infections in humans³⁷. Among all isolates of *Klebsiella* spp (n=26), there was no positive isolate for *bla*-KPC genes.

Approximately 31% (n = 8) of all *Klebsiella* species isolates (n = 26) had strong biofilm development, while 69% of the isolates (n = 18) had medium biofilm formation. Four isolates, or 15.38 % of the 26 total, tested positive for the biofilm resistance gene by PCR.

To examine the plasmid profile of the clinical isolates, plasmid DNA was extracted. In experimental settings, 24 out of 26 *Klebsiella* spp. isolates were found to harbor numerous plasmids, ranging in size from roughly less than 2 kb to more than 10 kb, according to plasmid profile analysis. MDR is frequently linked to outbreaks and resurgent infectious illnesses. The MDR determinants are frequently encoded on conjugative plasmids that can transmit from cell to cell by conjugation³⁸.

The interpretation of this study may be limited for some reason. The patient records did not include information about previous antibiotic therapy. Only available antibiotics were used in antibiotic susceptibility testing. As a result, the percentage of MDR isolates may have increased since the isolates may have been resistant to medications that were not employed in this investigation. Only a tiny number of antibiotic resistance genes were subjected to Polymerase Chain Reaction (PCR), and surprisingly, some of them revealed resistance genes like *bla*-NDM-1, *bla*-OXA-1 and *bla*-KPC.

However, for a thorough understanding, other genes associated with antibiotic resistance must be studied. Additionally, there were only about 26 isolated cells total (n=26). If the study used substantially bigger samples, the results would be more significant.

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