






## Article

# Biofilm Formation and Phenotypic Detection of ESBL, MBL, KPC and AmpC Enzymes and Their Coexistence in *Klebsiella* spp. Isolated at the National Reference Laboratory, Kathmandu, Nepal

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**Abstract:** *Klebsiella* spp. are associated with several nosocomial and opportunistic infections. Increasing antimicrobial resistance of *Klebsiella* species is aggravated by a number of intrinsic and extrinsic factors. The main aim of this study is to determine antimicrobial resistance due to production of  $\beta$ -lactamase enzymes, extended spectrum beta-lactamase (ESBL), metallo-beta-lactamase (MBL) and AmpC and *Klebsiella pneumoniae* carbapenemase (KPC) and biofilm formation in *Klebsiella* isolates. A total of 2197 non-duplicate specimens of urine, sputum and pus were obtained from the National Public Health Laboratory (NPHL), Kathmandu, Nepal, between February and August 2019. *Klebsiella* species were isolated, identified and screened for antimicrobial susceptibility testing with the disk diffusion method. Phenotypic detection of ESBL, MBL, KPC and AmpC production was observed and biofilm production was detected by the microtiter plate method. Out of a total of 2197 clinical specimens, bacterial growth was detected in 8% (175/2197) of the specimens. Of the total isolates, 86.3% (151/175) were Gram-negative bacteria and 37.7% (57/151) were *Klebsiella* spp. Of the total *Klebsiella* spp., 56% (32/57) were multi drug resistant (MDR), 16% (9/57) were ESBL, 26% (15/57) were MBL, 4% (2/57) were KPC (class A carbapenemase), 16% (9/57) were AmpC producers and 95% (54/57) were biofilm producers. Gentamicin was the most effective antibiotic, followed by cotrimoxazole, as 68% (39/57) and 47% (27/57) of the *Klebsiella* isolates were susceptible towards these drugs, respectively. The study results show evidence of  $\beta$ -lactamase production, high prevalence of MDR and biofilm producing *Klebsiella* species. Integrating the test parameters for phenotypic confirmation of ESBL, MBL, AmpC  $\beta$  lactamase and KPC in routine diagnostic procedures can help in the early detection and management of these resistant strains.

**Keywords:** antimicrobial resistance; multi drug resistant (MDR); extended-spectrum  $\beta$ -lactamase (ESBL); AmpC  $\beta$ -lactamase (ABL); carbapenemase; biofilm

## 1. 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 system [1]. *K. pneumoniae*, its subspecies (*K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis*) and *K. oxytoca* are the frequently isolated and medically important members of this genus [2]. Despite being normal flora organisms of the human gastrointestinal tract, *K. pneumoniae* is the most common pathogenic agent of nosocomial infections among *Klebsiella* spp. [3]. Pneumonia, urinary tract infection (UTI), blood stream and surgical wound infections, peritonitis, septicemia, meningitis [4] and pyogenic liver abscess [5]

are the major diseases caused by *K. pneumoniae* in humans. Antimicrobial resistance of Enterobacteriaceae (mainly *Escherichia coli*, *K. pneumoniae* and *Salmonella* spp.) has increased dramatically in recent years [4,5]. These pathogens have developed resistance against commonly used antimicrobial agents, such as the extended-spectrum beta-lactams, aminoglycosides, fluoroquinolones and carbapenems [4,5]. Therefore, drug-resistance problems are responsible for narrowed therapeutic options, increased cost of treatment, prolonged hospital stays and mortality [6,7].

The pathogenicity of *Klebsiella* species is attributable to a number of virulence factors, such as capsule, lipopolysaccharide (LPS), fimbrial and non-fimbrial adhesins, siderophores [8] and their ability to form biofilm [9]. Biofilms are the bacterial aggregates firmly lodged in the extracellular matrices of polysaccharides, proteins, enzymes and nucleic acid which facilitate anchoring to any surface irreversibly [10]. Because of their ability to form biofilms, infections caused by *K. pneumoniae* are becoming more challenging and warrant the need for innovative anti-biofilm approaches [11]. An examination utilizing signature labeled mutagenesis and surfaces covered with human extracellular framework (HECM) distinguished a protein associated with capsule biosynthesis essential for the biofilm production by *K. pneumoniae* [12]. A recent report indicated that capsule genes *wza* and *ORF14* are essential for *K. pneumoniae* to develop biofilm [13].

$\beta$ -lactamase enzymes, such as extended-spectrum  $\beta$ -lactamase (ESBL), AmpC  $\beta$ -lactamases and carbapenemase, are responsible for resistance to  $\beta$ -lactam antibiotics such as penicillins, cephamycin and carbapenem [14,15]. Carbapenems are used as the last resort drug for the life-threatening infections caused by multi drug resistant (MDR) pathogens and ESBL-producing Enterobacteriaceae [16–18]. However, over the past few years, due to the selective pressure of treating the ESBL and AmpC infections with carbapenems, resistance has emerged and *K. pneumoniae* is the most common carbapenem resistant Enterobacteriaceae (CRE) [19]. Prevalence of *Klebsiella pneumoniae* carbapenemases (KPC) and metallo- $\beta$ -lactamase producing strains among *K. pneumoniae* have been explored in different countries [19]. Carbapenem resistance due to metallo-beta-lactamase (MBL) production has been increasingly reported among clinical isolates from all around the world [20]. The rapidly increasing rate of MBL production among the members of Enterobacteriaceae, mainly *E. coli* and *K. pneumoniae*, has become one of the most common causes of hospital infections with limited therapeutic options [21]. A reliable estimate of the extent of drug resistant isolates of *K. pneumoniae* is thus essential to inform the control and management of hygiene and infection and treatment options for drug resistant strains.

While there have been a number of studies exploring production of beta-lactamase enzymes among *Klebsiella* species worldwide [22–24], there is a paucity of such studies in Nepal. The studies on biofilm mediated resistance and the resistance due to co-expression of beta-lactamase enzymes are sparse [25–27]. A few previous studies in Nepal have reported the varying prevalence of *Klebsiella* spp. For instance, the prevalence of nosocomial infections by *Klebsiella* spp. was 8.1% in a tertiary health care center in Bhairahawa [28], 15.6% in the Alka Hospital, Lalitpur [29] and 21.6% in the Tribhuvan University Teaching Hospital, Kathmandu [30]. The main objective of this study is to explore the burden of ESBL, AmpC, MBL, KPC and biofilm producing *Klebsiella* species, in addition to assessing co-existence of these factors and their role in development of drug resistance. This is a cross-sectional study carried out at the National Public Health Laboratory (NPHL), Kathmandu, between February and August 2019.

## 2. Materials and Methods

### 2.1. Study Design and Study Sample

A total of 2197 samples were processed and analyzed at NPHL. Specimens (total,  $n = 2197$ ) in this study constituted of urine ( $n = 2032$ ), sputum ( $n = 150$ ) and pus ( $n = 15$ ). These specimens were obtained in a sterile, clean, well-labeled and leak-proof container with no visible signs of contamination and were transported to the laboratory [31].

## 2.2. Sample Collection and Transportation

About 10–20 mL of early morning mid-stream urine (MSU) was collected in well-labeled, sterile, dry, wide-mouthed and leak-proof plastic container. Urine specimens that showed polymicrobial growth were not included in our study. Sputum samples were requested to be collected at early morning without a mix of saliva or nasal discharge and were collected in a wide-mouth leak-proof, disposable plastic container. In case of pus, a sterile cotton swab or aspirated syringes were used to collect the deepest portion of lesion or exudates rather than superficial debris. All the collected specimens were transported to the laboratory [32].

## 2.3. Macroscopic and Microscopic Examinations

Samples were examined for color, consistency, turbidity, presence of blood and pus based on the standard guidelines. Gram-stained smears of suitable specimens such as pus and sputum were prepared on a clean, grease-free glass slide and observed through a microscope for the presence of bacteria and pus cells [32,33].

## 2.4. Culture of Specimens and Identification of the Isolates

Sputum and pus samples were inoculated on blood agar and MacConkey agar, while urine was inoculated on Cysteine Lactose Electrolyte Deficient (CLED) agar using a standard calibrated loop (4 mm). All the inoculated media were incubated at 37 °C in aerobic conditions for 18–24 h. Thus, the obtained colony growths having Gram-negative rods suspected of *Klebsiella* spp. were further sub-cultured aerobically on nutrient agar (NA) overnight. Identification and confirmation of isolates were performed using microbiological tools and techniques such as colony morphology, staining and biochemical tests [32,33].

## 2.5. Antibiotic Susceptibility Testing

All identified bacterial isolates of *Klebsiella* spp. were tested for susceptibility against amoxiclav (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), cotrimoxazole (25 µg), norfloxacin (10 µg), nitrofurantoin (30 µg), cefotaxime (30 µg), gentamicin (10 µg), piperacillin-tazobactam (100/10 µg), amikacin (30 µg), tetracycline (30 µg), levofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg) and ceftazidime (30 µg) (HiMedia India Pvt. Ltd., Bengaluru, India), following the Kirby–Bauer method on Mueller-Hinton Agar (HiMedia India Pvt. Ltd.). Interpretation of results was based on the Clinical and Laboratory Standard Institute (CLSI) [34]. Isolates showing non-susceptibility (either a resistant or intermediate) to at least one antimicrobial agent in three or more of the categories were considered as multi-drug resistant [35].

## 2.6. Screening and Confirmation of ESBL Producers

The bacterial isolates were screened for possible ESBL production using ceftazidime (30 µg), cefotaxime (30 µg) and ceftriaxone (30 µg). Isolates showing reduced susceptibility to one or all of these drugs with zone of inhibition diameter for ceftazidime  $\leq 22$  mm, cefotaxime  $\leq 27$  mm, ceftriaxone  $\leq 25$  mm and aztreonam  $\leq 27$  mm were considered as possible ESBL producing isolates [29]. A phenotypic confirmation of ESBL was based on the combination disc method. A difference of  $\geq 5$  mm zone diameter of either cephalosporin discs or their respective cephalosporin-clavulanate disc was taken as phenotypic confirmatory test [34].

## 2.7. Screening and Confirmation of MBL Producers

Isolates resistant to carbapenems, such as imipenem (10 µg) or meropenem (10 µg), by disk diffusion test were suspected for possible MBL production. Phenotypic confirmation of MBL producers was performed by the imipenem-EDTA disk diffusion method. For this method, two imipenem disks (10 µg) were placed 20 mm apart and 5 µL of 0.5 M EDTA solution was added to one of the disks. The inhibition zones of the imipenem and imipenem-EDTA disc were compared after 16–18 h of incubation at 37 °C aerobically. The

increase in zone of inhibition of the imipenem and EDTA disk  $\geq 7$  mm than the imipenem disk alone was confirmed as MBL positive [36].

#### 2.8. Screening and Phenotypic Confirmation of KPC Producers

Isolates confirmed as carbapenem resistant in previous disc diffusion tests were screened for possible phenotypic KPC production using the meropenem-phenyl boronic acid (PBA) disk diffusion method. Two meropenem discs (10  $\mu\text{g}$ ) were placed on the plate at a distance of 20 mm and 20  $\mu\text{L}$  (containing 400  $\mu\text{g}$  of PBA) solution of concentration 20 mg/mL was added to one of the disc. The inhibition zones of the meropenem and meropenem disk with PBA were compared after 16–18 h of incubation at 37 °C aerobically. The increase in zone of inhibition with the meropenem disk with PBA  $\geq 5$  mm than the meropenem disk alone was confirmed as a KPC producer [37].

#### 2.9. Screening and Confirmation of AmpC Producers

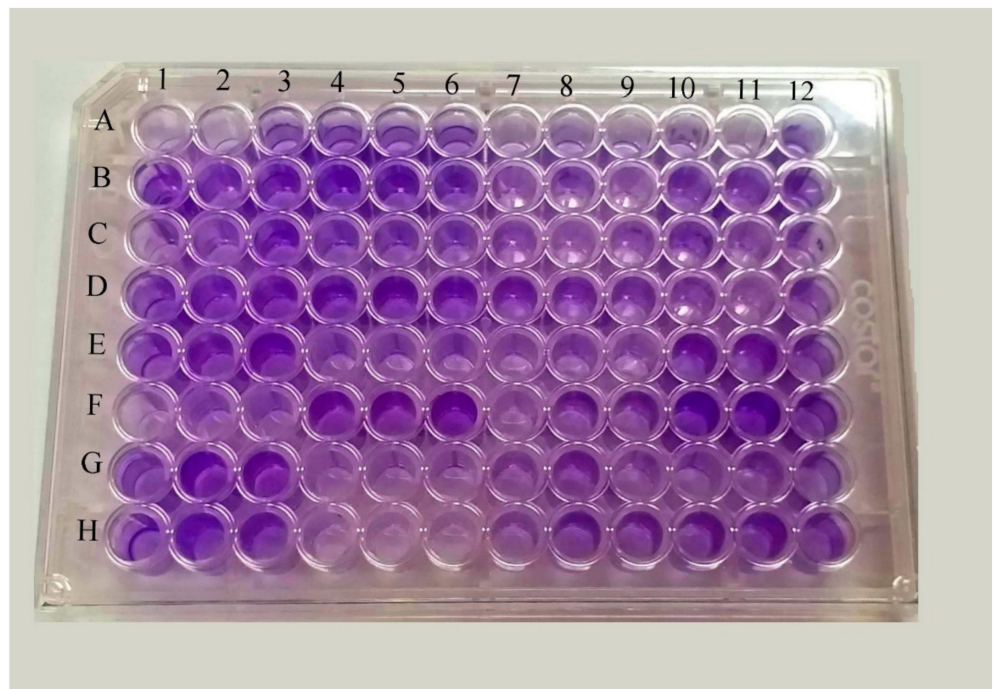
Bacterial isolates showing resistant or intermediate ( $\leq 18$  mm) onto a cefoxitin (CX) were screened as AmpC producers, which was further confirmed by an inhibition-based method. For this method, a PBA solution was prepared by dissolving 120 mg of PBA in 3 mL of DMSO (Dimethyl sulfoxide) into which 3 mL of sterile distilled water was added. A volume of 20  $\mu\text{L}$  of the stock solution of PBA solution was dispensed onto a cefoxitin (30  $\mu\text{g}$ ) disc. By using the standard disk diffusion method, the cefoxitin disc (30  $\mu\text{g}$ ) and cefoxitin (30  $\mu\text{g}$ ) disc with PBA (400  $\mu\text{g}$ ) were placed on an MHA plate already inoculated with the test strain. The inoculated plates were then incubated overnight at 37 °C. An organism showing an increase of  $\geq 5$  mm of zone diameter around the cefoxitin disk alone was considered as an AmpC producer [38].

#### 2.10. Detection of Biofilm Formation by Micro-Titer Plate Method

Biofilm production by the isolates was detected by a micro-titer plate method. For this method, a fresh loopful of test organism was inoculated into 10 mL of trypticase soya broth (TSB) with 1% glucose and incubated at 37 °C for 24 h. After incubation, the solution was diluted (1:100) with fresh TSB. Then, 200  $\mu\text{L}$  of diluted culture broth were added to each well of a microtiter plate having 96 wells. TSB with 1% glucose was used as the negative control. The plate was then incubated at 37 °C for 24 h. After incubation, the content of each well was removed by gentle tapping and wells were washed with 200  $\mu\text{L}$  of PBS 3–4 times to remove the free-floating bacteria. Biofilm formed by the bacteria adherent to the wells was fixed by 2% sodium acetate and then stained by 0.1% crystal violet. Excess stain was removed by washing the plate with de-ionized water and the plate was dried. Finally, all wells were filled with 200  $\mu\text{L}$  ethanol (95%) to release the dye from the cells. Optical density (OD) of stained adherent biofilm was then recorded with the help of a microtiter reader at the wavelength of 630 nm and results were interpreted as weak, moderate and strong biofilm producers. The optical density cut-off value (ODc) was determined by arithmetically averaging the OD of the wells containing sterile TSB broth and adding a standard deviation of +2. Samples with an OD higher than the cut-off value were considered positive, whereas those with the lower optical density than the cut-off value were considered as negative. Strains were classified using the following criteria:  $\leq \text{ODc}$  or  $\text{ODc} < - \geq 2 \times \text{ODc}$ , non-biofilm producer;  $2 \times \text{ODc} < - \leq 4 \times \text{ODc}$ , moderate biofilm producer;  $> 4 \times \text{ODc}$ , strong biofilm producer [39,40] (Figure 1).

#### 2.11. Quality Control

Stringent measures were utilized to ensure the quality standard and aseptic conditions. Each batch of media and the reagents, including antibiotic disks, were assured according to the CLSI guidelines. Purity plates were used to check the pure culture of the isolates and aseptic standards during biochemical tests. Control strains of *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used during the antibiotic susceptibility test (AST).



**Figure 1.** Biofilm production test by the microtiter plate method: negative control (A1, A2, A3), strong producer (F10, F11, F12), moderate producer (B10, B11, B12), weak producer (H7, H8, H9) and non-producer (H4, H5, H6). NB: The higher the ODc, the higher the concentration of the purple color in the presented microtiter well. Concentration of color represents the strength of producing the biofilms from higher to lower as the concentration decreases.

### 2.12. Data Analysis

Data were entered into an excel spreadsheet and analyzed using SPSS Version 24 (IBM; Chicago, IL, USA) accessed on March, 2019. Categorical data were analyzed using the Chi-squared test. A *p* value of <0.05 was considered as statistically significant.

## 3. Results

### 3.1. Distribution of Growth Pattern of Bacterial Isolates

A total of 2197 different clinical specimens were processed for microbial culture, of which 175 (8.3%) showed growth of bacteria. Out of the 175 isolates, 151 (86.3%) were Gram-negative bacteria and 24 (13.7%) were Gram-positive bacteria. Among 151 Gram-negative isolates, 121 (80%) were from urine, followed by 28 (19%) from sputum and 2 (1%) from pus specimens, whereas, among 24 Gram-positive isolates, 19 (79.2%) were from urine, followed by pus sample (5; 20.8). Among the Gram-negative bacteria, *E. coli* (41.1%; 62/151) was the predominant organism, followed by *K. pneumoniae* (26.5%; 40/151), *K. oxytoca* (11.3%; 17/151) and *Pseudomonas aeruginosa* (10.6%; 16/151). Among Gram-positive bacteria, *Staphylococcus aureus* (62.5%; 15/24) was the most predominant organism, followed by *Enterococcus* spp. (33.3%; 8/24) and *Staphylococcus saprophyticus* (4.2%; 1/24) (Table 1).

*Klebsiella* spp. was mostly isolated from urine samples (66.7%; 38/57), followed by sputum (29.8%; 17/57) and pus samples (3.5%; 2/57). Males (57.5%; 23/57) were more prone to *Klebsiella* spp. infections than females (42.5%; 17/57). Patients of the age group above 60 years were more prone to *Klebsiella* spp. infections (Table 2).

**Table 1.** Distribution of growth pattern of bacterial isolates ( $n = 175$ ).

Specimens/Character	Culture Positive	
	Number	Percentage
Clinical specimens		
Urine ( $n = 2032$ )	140	80
Sputum ( $n = 150$ )	28	16
Pus ( $n = 15$ )	7	4
Gram type		
Gram-negative bacteria	151	86.3
Gram-positive bacteria	24	13.7
<b>Different type of Gram-positive bacteria (<math>n = 24</math>)</b>		
<i>S. aureus</i>	15	62.5
<i>Enterococcus</i> spp.	8	33.3
<i>S. saprophyticus</i>	1	4.2
<b>Different type of Gram-negative bacteria (<math>n = 151</math>)</b>		
<i>E. coli</i>	62	41.1
<i>K. pneumoniae</i>	40	26.5
<i>K. oxytoca</i>	17	11.3
<i>P. aeruginosa</i>	16	10.5
<i>Enterobacter aerogenes</i>	5	3.3
<i>Citrobacter freundii</i>	4	2.6
<i>Morganella morganii</i>	2	1.3
<i>Providencia</i> spp.	2	1.3
<i>Proteus</i> spp.	1	0.7
<i>Haemophilus</i> spp.	1	0.7
<i>Acinetobacter baumannii</i>	1	0.7
<b>Gender</b>		
Male	92	52.5
Female	83	47.5
<b>Age groups (Years)</b>		
0–15	10	5.7
16–45	80	45.7
46–60	45	25.7
>60	40	22.9

**Table 2.** Distribution of *Klebsiella* spp. among patients visiting NPHL ( $n = 57$ ).

Character	<i>K. pneumoniae</i> ( $n = 40$ )	<i>K. oxytoca</i> ( $n = 17$ )	Total
	N (%)	N (%)	N (%)
Clinical specimens			
Urine	25 (62.5)	12 (70.6)	37 (64.9)
Sputum	13 (32.5)	5 (29.4)	18 (31.6)
Pus	2 (5.0)	0	2 (3.5)
Gender			
Male	23 (57.5)	9 (52.9)	32 (56.1)
Female	17 (42.5)	8 (47.1)	25 (43.9)
Age groups (in years)			
0–15	1 (2.5)	0	1 (1.8)
15–45	13 (32.5)	7 (41.2)	20 (35.1)
45–60	10 (25)	5 (29.4)	15 (26.3)
>60	16 (40)	5 (29.4)	21 (36.8)

### 3.2. Antibiotic Susceptibility Pattern of the Isolates

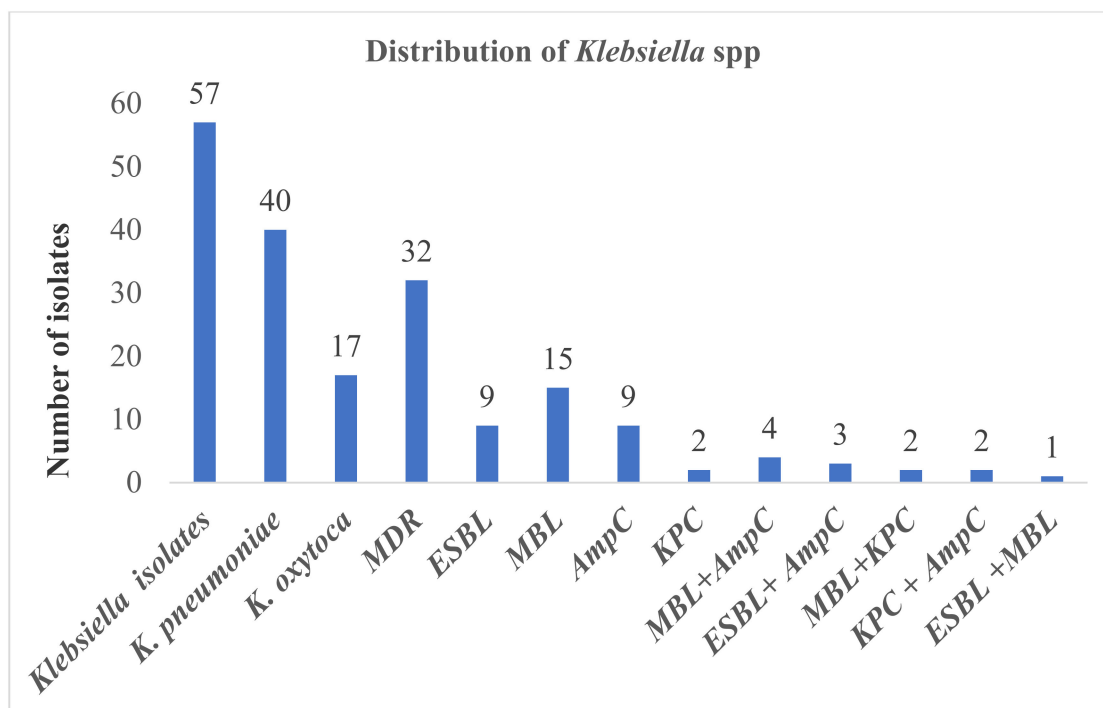
Fifteen antibiotics of different classes (on recommendation of CLSI, 2019 guidelines) were tested against *Klebsiella* spp. Among the antibiotics used, gentamicin was the most

effective drug (68.4%), followed by cotrimoxazole (47.4%), ceftriaxone (45.6%), tetracycline (45.6%), ciprofloxacin (42.1%) and amoxiclav. (33.3%) (Table 3).

**Table 3.** Antibiotic susceptibility profile of *Klebsiella* spp. (n = 57).

Mode of Action	Antimicrobial Category	Antibiotics	<i>K. pneumoniae</i> (n = 57)		
			Sensitive	Intermediate	Resistant
			N (%)	N (%)	N (%)
Cell wall synthesis inhibitors	Aminopenicillin	Amoxiclav (30 µg)	19 (33.3)	4 (7.1)	34 (59.6)
		Ceftriaxone (30 µg)	26 (45.6)	2 (3.5)	29 (50.9)
	Extended spectrum cephalosporins	Cefoxitin (30 µg)	21 (36.8)	4 (7.0)	32 (56.2)
		Cefotaxime (30 µg)	26(45.6)	1(1.8)	30 (52.6)
	Carbapenems	Imipenem (10 µg)	20 (35.0)	5 (8.8)	32 (56.2)
		Meropenem (10 µg)	20 (35.0)	5 (8.8)	32 (56.2)
Penicillins and beta-lactamase inhibitors	Piperacillin-tazobactam (100/10 µg)		20 (35.0)	6 (10.5)	31 (54.5)
Protein synthesis inhibitors	Aminoglycosides	Amikacin (30 µg)	25 (43.9)	3 (5.3)	29 (50.8)
		Gentamicin (10 µg)	39 (68.4)	1 (1.8)	17 (29.8)
	Tetracyclines	Tetracycline (30 µg)	26 (45.6)	5 (8.8)	26 (45.6)
Nucleic acid synthesis inhibitors	Fluoroquinolones	Ciprofloxacin (5 µg)	24 (42.1)	3 (5.3)	30 (52.6)
		Norfloxacin (10 µg)	20 (35.1)	2 (3.5)	35 (61.4)
		Levofloxacin (5 µg)	21 (36.8)	4 (7.0)	32 (56.2)
	Nitrofurans	Nitrofurantoin (300 µg)	26 (45.6)	6 (10.5)	25 (43.8)
Folate pathway inhibitors	Trimethoprim and Sulfamethoxazole	Cotrimoxazole (25 µg)	27 (47.4)	1 (1.8)	29 (50.9)

Among the total 57 *Klebsiella* isolates, 32 (56%) were MDR, including 26 (81.3%) *K. pneumoniae* and 6 (18.8%) *K. oxytoca* isolates (Figure 2).



**Figure 2.** Distribution of ESBL, MBL, AmpC and KPC producing *Klebsiella* spp.

### 3.3. ESBL, AmpC, MBL, KPC Production among *Klebsiella* spp.

Among the total 57 *Klebsiella* spp. only 9 (16%; 9/57) isolates were confirmed as ESBL producers. The highest percentage of ESBL production was found among *K. pneumoniae* (7/40; 17.5%), followed by *K. oxytoca* (2/17; 11.8%). In the AmpC detection test, 9 (16%) isolates were confirmed as AmpC producers. The highest percentage of AmpC production was found among *K. pneumoniae* (8/40; 20%), followed by *K. oxytoca* (1/17; 5.9%). In the MBL test, 15 (26%) were confirmed as MBL producers. The highest percentage of MBL production was found among *K. pneumoniae* (13/40; 32.5%), followed by *K. oxytoca* (2/17; 11.8%). In the KPC detection test, only two isolates (2/40; 5%) were confirmed as KPC producing *K. pneumoniae* (Figure 3).

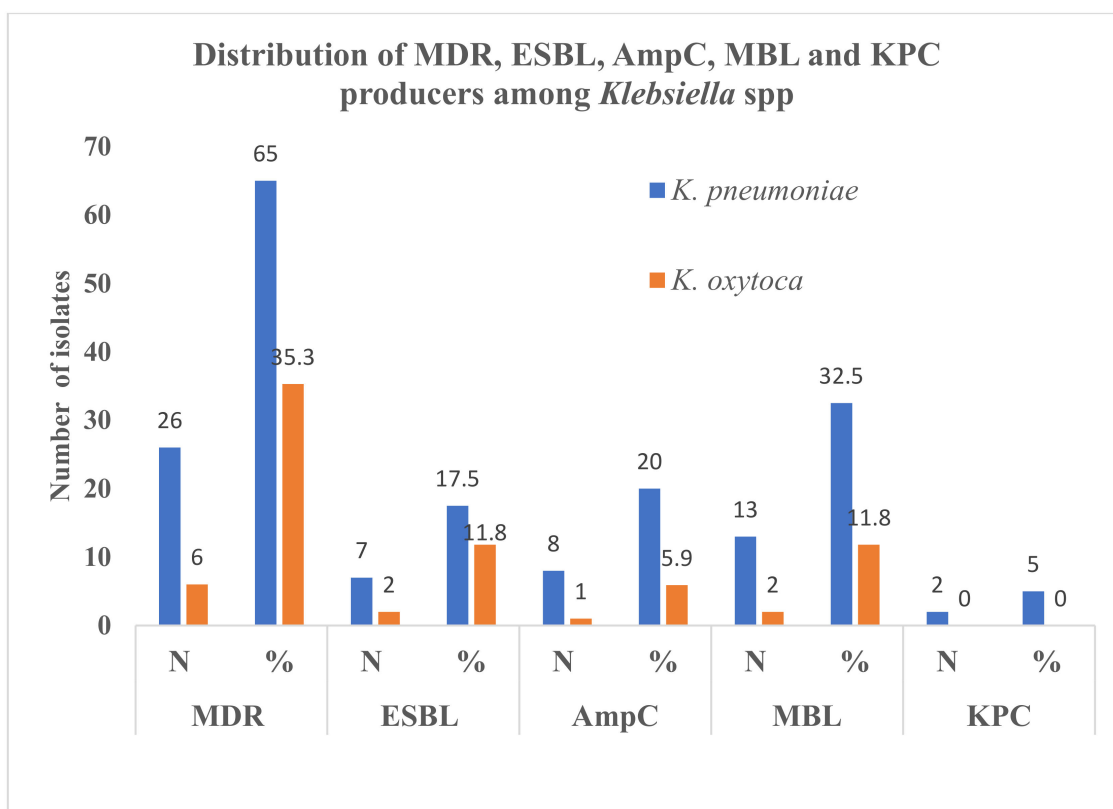


Figure 3. Distribution of MDR, ESBL, AmpC, MBL and KPC producers among *Klebsiella* isolates ( $n = 57$ ).

### 3.4. Distribution of ESBL, AmpC, MBL and KPC Producers in Differential Specimens and Age Groups

ESBL, AmpC, MBL and KPC were detected in larger proportions in urine samples and among the age group of 16–60 years. Urine samples constituted 78% of ESBL, 67% of AmpC, 60% of MBL and 50% of KPC producers. Sputum and pus samples contributed in decreasing order. The age group of 16–60 years alone constituted the 66.7% (6/9) of ESBL, 88.9% (8/9) of AmpC, all (2/2) KPC and 60% (9/15) of MBL producing isolates. Similarly, 62% (20/32) of MBL and 50% (1/2) of KPC were detected in urine samples (Table 4).

### 3.5. Detection of Biofilm

Out of 57 *Klebsiella* isolates, 54 (94.8%) isolates were confirmed as biofilm producers, of which 4 isolates (7%) were strong producers, 34 (59.7%) were moderate and 16 (28.1%) were weak biofilm producers (Table 5).



**Table 4.** Demographic and clinical character of MDR, ESBL, AmpC, MBL and KPC producing *Klebsiella* spp. according to clinical specimens, gender and age.

Character	MDR	ESBL	AmpC	MBL	KPC
	N (%)	N (%)	N (%)	N (%)	N (%)
Clinical specimens					
Urine	20 (62.5)	7 (77.8)	5 (55.6)	9 (60)	1 (50)
Sputum	10 (31.3)	0 (0)	3 (33.3)	6 (40)	1 (50)
Pus	2 (6.3)	2 (22.2)	1 (11.1)	0 (0)	0 (0)
<i>p</i> value	0.44	0.07	0.74	0.28	0.8
Gender					
Male	21 (65.6)	8 (88.9)	4 (44.4)	9 (60)	0
Female	11 (34.4)	1 (11.1)	5 (55.6)	6 (40)	2 (100)
<i>p</i> value	0.11	0.7	0.11	0.52	0.04
Age groups (in years)					
0–15	1 (3.1)	0 (0)	0 (0)	1 (6.7)	0 (0)
16–45	12 (37.5)	4 (44.4)	5 (55.5)	6 (40)	1 (50)
46–60	8 (25)	2 (22.2)	3 (33.4)	3 (20)	1 (50)
>60	11 (34.4)	3 (33.3)	1 (11.1)	5 (33.3)	0 (0)
<i>p</i> value	0.63	0.83	0.15	0.55	0.52

**Table 5.** Biofilm production among *Klebsiella pneumoniae* and *K. oxytoca* isolates in relation to species, clinical specimens, gender and age.

Character	Biofilm Producers			Biofilm Non-Producers	
	Strong	Moderate	Weak	Total Producer	Non-Producer
	N (%)	N (%)	N (%)	N (%)	N (%)
<i>Klebsiella</i> spp. ( <i>K. pneumoniae</i> and <i>K. oxytoca</i> )	4 (7%)	34 (59.7%)	16 (28.1%)	54 (94.8%)	3 (5.2%)
<i>K. pneumoniae</i>	2 (50)	24 (70.6)	13 (81.3)	39 (72.2)	1 (33.3)
<i>K. oxytoca</i>	2 (50)	10 (29.4)	3 (18.8)	17 (27.8)	2 (66.7)
Clinical specimens					
Urine	4 (100)	19 (55.9)	12 (75)	35 (64.8)	2 (66.7)
Sputum	0 (0)	13 (38.2)	4 (24)	17 (31.5)	1 (33.3)
Pus	0 (0)	2 (5.9)	0 (0)	2 (3.7)	0 (0)
Gender					
Male	1 (25)	20 (58.8)	9 (56.3)	30 (55.6)	2 (66.7)
Female	3 (75)	14 (41.2)	7 (43.8)	24 (44.4)	1 (33.3)
Age group (in years)					
0–15	0 (0)	0 (0)	1 (6.3)	1 (1.9)	0 (0)
16–45	2 (50)	12 (35.3)	5 (31.3)	19 (35.2)	1 (33.3)
46–60	1 (25)	10 (29.4)	4 (25)	15 (27.7)	0 (0)
>60	1 (25)	12 (35.3)	6 (37.5)	19 (35.2)	2 (66.7)
Relation of biofilm production according to MDR, ESBL, MBL, AmpC and KPC production					
MDR producers	1 (3.1)	21 (65.6)	8 (25)	30 (93.7)	2 (6.3)
ESBL producers	1 (11.1)	5 (55.6)	3 (33.3)	9 (100)	0 (0)
MBL producers	1 (6.7)	8 (53.3)	5 (33.3)	14 (93.3)	1 (6.7)
AmpC producers	0 (0)	7 (77.8)	2 (22.2)	9 (100)	0 (0)
KPC producers	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)

### 3.6. Biofilm Production among ESBL, AmpC, MBL and KPC Producing *Klebsiella* spp.

Among total (9) ESBL producers, 1 (11%) was strong, 5 (56%) were moderate and 3 (33%) were weak biofilm producers. Similarly, of the total (9) AmpC producing *Klebsiella* spp., 7 (78%) were moderate and the remaining 2 (22%) were weak producers. Among total (15) MBL producers, 1 (6.7%) was a strong, 8 (53.3%) were moderate and 5 (33.3%) were weak producers. In AmpC producing *Klebsiella* spp., all (2/2; 100%) were moderate biofilm producers (Table 5).

### 3.7. Co-Production of ESBL and AmpC, ESBL and Carbapenemase (MBL and KPC), MBL and KPC, MBL and AmpC and KPC and AmpC

Among total MDR *Klebsiella* spp., 3 (33.3%) isolates were found to be both ESBL and AmpC producers. Among the total MDR *Klebsiella* spp., only one (6.7%) isolate produced both ESBL and MBL. In the study, none of the isolates produced both ESBL and KPC.

All of the imipenem and meropenem-resistant isolates (2/2) were also positive in the MBL and KPC production tests. Among MDR *Klebsiella* species, 4 (44.4%) produced both MBL and AmpC. Among total MDR *Klebsiella* species, 2 (22.2%) isolates produced both KPC and AmpC.

## 4. Discussion

This study was carried out to determine the beta-lactamase enzymes (ESBL, MBL, KPC and AmpC) and biofilm production among *Klebsiella* spp. from the clinical samples (urine, pus and sputum) obtained at NPHL, Kathmandu. Because of their ability to produce beta-lactamase enzymes and biofilms, *Klebsiella* spp. are resistant to a wide range of antibiotics [41,42]. As most of the beta-lactamase genes are on plasmid mediated mobile genetic elements, the resistant strains are now disseminating rapidly, leading to increases in mortality, morbidity and healthcare costs [26]. Co-expression of several beta-lactamase genes in an organism may complicate the problem of drug-resistance, thereby limiting treatment options [14]. Detection of these factors and their existing association with drug resistance is crucial in diagnostic laboratories.

In this study, one-third of the isolated Gram-negative bacteria was constituted by *Klebsiella* spp. This finding was consistent with previous studies [25,26,41–45]. Various factors, such as study location, type of sample, seasonal variation and environmental conditions, including temperature, pH and humidity of the study site, may be responsible for the difference in the rate of prevalence in other studies [26]. *Klebsiella* spp. are opportunistic pathogens which cause various infections, such as UTI, pneumonia, blood stream infections and wound infections. In this study, most of the organisms (66.7%) were isolated from urine samples, followed by sputum (29.8%) and pus (3.5%). This may be due to the presence of a large number of urine samples compared with other clinical specimens. Several studies in the past have shown the prominence of urine samples, suggesting the crucial role of *Enterobacteriaceae* in UTIs in Nepal [25,26,46] and in Spain [47].

In the antibiotic susceptibility test, fifteen antibiotics of different classes were tested against *Klebsiella* spp. Nitrofurantoin is regarded as having better pharmacokinetics for the treatment of UTIs rather than other infections [48]. Among the antibiotics used, gentamicin was found to be most effective (68.4%), followed by cotrimoxazole (47%), ceftriaxone (45.6%), ciprofloxacin (40.7%) and amoxiclav (33.3%) against isolates of *Klebsiella* spp. However, in a study conducted in Iran, the highest resistance was associated with gentamicin and cefepime, whereas the lowest rate was associated with imipenem, meropenem and ceftazidime [42]. Over the years, the prevalence of antimicrobial resistance and MDR has increased to a high level at an alarming rate in Nepal [49]. Our study also reported more than half of the total isolated *Klebsiella* spp. as MDR. This study is in harmony with previous studies from Indonesia [50] and Brazil [51].

Samples were screened for ESBL, MBL, KPC and AmpC  $\beta$  lactamase and more than half of the *Klebsiella* spp. isolates were found positive, but the percentage was lower during confirmatory tests. However, the detected load of ESBL is still enough to challenge a number of treatment regimens. The more clinically important species, *K. pneumoniae* was the dominant (17.5%) producer of ESBL over *K. oxytoca* (12%). This finding of higher prevalence of ESBL producers is similar to previous studies reported from Kathmandu [25], Birgunj [52], Model Hospital, Kathmandu [53] and Taiwan [54]. The factors leading to the high prevalence of ESBL producers could be indwelling catheters, invasive procedures and excessive use of cephalosporins and severity of the illness [55]. The AmpC production test was conducted using the inhibitor-based method using phenyl boronic acid (PBA) [38]. A

significant number of isolates of both species (*K. pneumoniae* and *K. oxytoca*) were screened and tested positive, which is consistent with the previous studies from Nepal [27,56,57].

Although carbapenems are the last choice for the treatment of isolates producing ESBL and AmpC, the proportion of carbapenem resistant *Klebsiella* spp. is on the rise [58]. Our study reported 56% imipenem and meropenem resistant *Klebsiella* spp., 26% of which tested positive for MBL and 3.5% of which were KPC producers. The findings of our study are in line with the studies reported from India [59–61], but contrast with other studies reported from Nepal [26,62]. This might be due to variation in study sites, nature of illnesses and the clinical specimens.

The occurrence of multiple  $\beta$ -lactamases among bacteria was also observed. In this study, 3 (33.3%) isolates were found to be ESBL and AmpC producers having similarity with a study in Taiwan [54]. Similarly, in our study, 6.7% isolates were co-producers of ESBL and MBL; 22% were KPC and AmpC producers and, of 2 KPC isolates, both isolates were co-producers with MBL and AmpC  $\beta$  lactamase. The relationship of co-occurrence among the pairs ESBL and MBL and MBL and KPC was statistically significant. The maximum percentages of ESBL, AmpC, MBL and KPC producers were found in urine samples. The significant association among these parameters was also explained in some other studies [14]. This may be due to involvement of more study subjects with complaints of UTIs, thus inclusion of urine as the largest sample size in our study.

Biofilm production in this study was found in 95% *Klebsiella* spp. with more than half of the total biofilm producers being MDR. In contrast to our findings, other studies have reported, comparably, more strong biofilm producers than ours does [63,64]. Biofilm producers were found to be highly resistant to cefotaxime, levofloxacin, piperacillin-tazobactam and imipenem (80%). Gentamicin was an effective drug against these recalcitrant strains. However, varying degrees of antibiotic resistance by biofilm producing isolates were observed in some other studies reported from Nepal [49,65].

#### *Strengths and Limitations*

This study should serve as a valuable reference for future studies determining the prevalence of ESBL, MBL, KPC, AmpC  $\beta$  lactamase and biofilm producers. This study compiled a set of enzymes, their prevalence and their relationships. The findings and conclusions may be a reference tool for policy makers and clinicians to design and implement treatment regimens.

There are several limitations to this study. As this study was conducted in a single laboratory, studies with large numbers of study samples, at multiple healthcare facilities and communities would be desirable to estimate the actual burden of antimicrobial resistance (AMR) in the country. As our study relied on phenotypic methods for detection of enzymes, further analysis with molecular detection of responsible genes and their characterization would be more sensitive and reliable. Although a large sample was screened ( $n = 2197$ ), the study ended up with only 57 *Klebsiella* species, which limited the statistical analyses and inferences.

#### **5. Conclusions**

The detection of a high prevalence of beta-lactamase enzymes and biofilm production among isolated *Klebsiella* species in our study showed the important roles of these factors in conferring resistance. The co-expression of any of these factors together potentially further reinforces the multi-drug resistance among bacterial isolates against the routinely used antibiotics, such as beta-lactams and carbapenems. Since there is no single diagnostic method (either phenotypic or genotypic when used alone) which has ideal (100 percent) sensitivity and specificity, an algorithm approach, or the integration of molecular/genotypic tests in addition to the routine diagnostic procedures can help in the early detection and management of infections caused by resistant isolates. In addition to antibiotic therapy, anti-biofilm approaches (chelating agents and lantibiotics) could be considered to inform the best treatment options.

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**Informed Consent Statement:** Written informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** All the data pertaining to this study are within the manuscript.

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## Abbreviations

AMR, antimicrobial resistance; AST, antibiotic susceptibility test; ATCC, American Type Culture Collection; BA, blood agar; CDC, Center for Disease Control; CLED, Cysteine lactose electrolyte deficient; CLSI, Clinical Laboratory Standard Institute; CTX-M, Cefotaxime, Munich; ECDC, European Centre for Disease Prevention and Control; EDTA, Ethylene Diamine Tetra acetic acid; ESBL, Extended Spectrum Beta Lactamase; GES-1, Guaiana Expanded Spectrum; GIM, German imipenemase; OXA, Oxacillin-hydrolyzing; KPC, *Klebsiella pneumoniae* Carbapenemase; MBL, Metallo-beta-lactamase; MDR, multi-drug resistance/resistant; MHA, Mueller-Hinton Agar; MIC, minimum inhibitory concentrations; NA, nutrient agar; NDM, New Delhi Metallo-beta-lactamase; NPHL, National Public Health laboratory; PBA, Phenyl Boronic Acid; PER, *Pseudomonas* extended resistance; SHV, Sulphydryl Variable; SIM, Seoul Imipenemase; SPM-1, Sao Paulo Metallo-beta lactamase; TEM, Temocillin-hydrolyzing; VEB, Vietnamase Extended Spectrum Beta lactamase; VIM, Verona integron encoded metallo-beta lactamase.

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