Antibiogram of ESBL-Producing *Klebsiella* Species and *Pseudomonas aeruginosa* from Clinical Specimens

Ashma Shrestha[1*], Kalyan Subedi[1], Sonali Kafle[2]
[1]Department of Microbiology, Kathmandu College of Science and Technology, Kathmandu, Nepal
[2]Alka Hospital Private Limited, Lalitpur, Nepal

***Corresponding author**: Ashma Shrestha, Department of Microbiology, Kathmandu College of Science and Technology (KCST), Kathmandu, Nepal; E-mail: asmas968@gmail.com

ABSTRACT

Objectives: The study aims to determine the antibiotic susceptibility pattern of extended spectrum beta-lactamase (ESBL)-producing isolates of *Klebsiella* and *Pseudomonas* from clinical specimens.

Methods: A total of 230 samples from different bodily fluids (urine, sputum, drain, pus, BAL, CSF, blood, and ascitic fluid) of patients were collected from various wards of Alka Hospital Private Limited, Lalitpur, Nepal. The samples were analyzed in the microbiological laboratory as per the CLSI guidelines. The Kirby-Bauer disc diffusion technique.was used to perform antibiotic susceptibility testing.

Results: Thirty-four (14.78%) samples showed culture positivity. Urine had the highest culture positivity; the patients of the age group 90-100 years were 66.67% culture positive. 100% of the samples from the SH/C ward were culture-positive. Of 34 isolates, 88.24% were ESBL producers, including 61.76% *K. pneumoniae*, 11.76% *K. oxytoca*, and 26.47% *P. aeruginosa*. MDR was observed in 96.3% of ESBL producers. Amikacin, Gentamicin, and Piperacillin-tazobactam were the most effective against ESBL-producing *P. aeruginosa*. Colistin and Polymyxin-B were the most effective antibiotics against *Klebsiella* species.

Conclusion: The study reports ESBL production significantly affected MDR development in selected bacterial specimens.

Keywords: ESBL, MDR, antibiotic resistance, *Klebsiella* spp., *Pseudomonas aeruginosa*.

INTRODUCTION

Gram-negative bacteria are responsible for a wide range of infections in humans. Some Gram-negative bacteria commonly isolated from clinical specimens are *Escherichia coli*, *Klebsiella* species, *Pseudomonas aeruginosa*, *Acinetobacter*, etc. The diseases caused by Gram-negative bacteria range from mild to severe. Gram-negative bacterial infections include pneumonia, sepsis, meningitis, urinary tract infections, peritonitis, gastrointestinal problems like cholera, and gastritis. The severity rate is high in the case of gram-negative bacterial infections due to their ability to develop resistance against different classes of antibiotics (Parija, 2012). The development of resistance to different antibiotic classes occurs in various ways. The most common form of developing antibiotic resistance is the presence of pumps (efflux pumps) in the cell walls of some Gram-negative bacteria that remove antibiotics. Some can change target regions for the antibiotics or produce proteins and enzymes like ESBL (extended-spectrum beta-lactamases) or carbapenemases, causing a breakdown in the antibiotics (Chessbrough, 2006).

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ESBL are the enzymes bacteria produce that can break down the beta-lactam ring in antibiotics like penicillins, monobactams, and cephalosporins. The breakdown makes these antibiotics ineffective against the bacteria. The bacteria producing ESBL are often resistant to different antibiotics, making infection caused by them impossible to treat (Rawat & Nair, 2010).

ESBL is produced mainly by Gram-negative bacteria, often associated with the spread of antibiotic-resistant bacterial infection in hospitals and healthcare settings. Person-to-person transmission of the disease from the ESBL-producing bacteria through contaminated surfaces and medical devices and acquired by individuals after long and haphazard exposure to antibiotics (Rawat & Nair, 2010). Among the Gram-negative bacteria capable of producing ESBL, species of the genus *Klebsiella* and *Pseudomonas aeruginosa* have been a significant concern in recent years. *Klebsiella* species belongs to the family Enterobacteriaceae, whereas *P. aeruginosa* belongs to the family Pseudomonadaceae (Parija, 2012).

Klebsiella species are rod-shaped, capsulated, non-sporulating, non-motile, and facultative anaerobic bacteria commonly present in the environment, including soil and water. These bacteria can ferment lactose. These cause various human infections, from urinary tract infections (UTIs) to sepsis. Mostly, the conditions are nosocomial. The two most commonly isolated species are *K. pneumoniae* and *K. oxytoca*. Both species can cause UTIs, pneumonia, and sepsis in nosocomial settings. Besides these, *K. oxytoca* can also cause infections in the gastrointestinal tract, like diarrhea and wound infections (CDC, 2024a). Besides ESBL production, the capsule, fimbriae, and biofilm production ability assist *Klebsiella* species in developing resistance to different antibiotics. The increased rate of resistance to various types of antibiotics makes the infection caused by *Klebsiella* species challenging to control and treat (CDC, 2024a).

Pseudomonas aeruginosa is rod-shaped strict aerobic bacteria that are motile with the help of polar flagella and pili, non-sporulating, and non-lactose fermenters. It is commonly predominant in soil and water (Parija, 2012). However, it is also normal flora in the skin, respiratory, urinary, and gastrointestinal tracts. In healthy individuals, it is not harmful. However, people with suppressed immune systems and who are hospitalized can cause severe infections. Infections by *P. aeruginosa* are more common in patients with burns, severe skin trauma, and cystic fibrosis. It is also a primary causative agent in case of hospital-acquired infections. It can cause a wide range of diseases, including pneumonia, UTIs, respiratory distress, wound infections, and sepsis (CDC, 2024b).

The bacteria has an intrinsic mechanism of developing antibiotic resistance. The three significant mechanisms commonly implied by *P. aeruginosa* are the production of beta-lactamase enzyme, loss of upregulation of efflux pumps, and outer membrane proteins. Among the three methods, the production of ESBL by chromosomal and plasmid DNA is the most significant. Due to this property of some strains of bacteria, resistance to beta-lactam has become a considerable problem. Also, other mechanisms applied by the bacteria increase the potential of developing resistance to multiple drugs. (CDC, 2024b)

MDR (multi-drug resistance) bacteria means the bacteria developing insensitivity towards three or more classes of antibiotics. MDR is a significant problem in treating bacterial infections because it limits the effectiveness of the commonly used or available drugs. The limitation can lead to treatment failure, which increases the health care cost and accelerates the risk of prolonged infection. Hence, it leads to an increasing risk of death. MDR can be easily transferred from one bacteria to another, increasing the risk of spreading resistance to non-resistant bacteria and contributing to the antimicrobial resistance problem (Nikaido, 2009). ESBL-producing bacteria are often associated with a high chance of developing resistance to multiple drugs and beta-lactams, therefore considered MDR. In most hospital cases, ESBL, ESBL-producing bacteria are prone to MDR, which makes treating already complicated infections by these bacteria almost impossible to eradicate. The disease is also easily transferred between people, especially those with compromised immune systems (Government of Newfoundland and Labrador, 2011).

Antibiotic susceptibility testing (AST) is a laboratory technique that helps to determine the effectiveness of antibiotics against bacterial and fungal infections. It is crucial to select the correct antimicrobial treatment for particular diseases. The most common method of performing AST is the Kirby-Bauer disc diffusion method (Jorgensen et al., 2015). The Kirby-Bauer disc diffusion method involves the addition of antibiotic-impregnated paper discs onto the agar plate inoculated with the test organism. The antibiotics diffuse into the agar and create a concentration gradient. If the organism is susceptible to a particular antibiotic, the drug inhibits the growth, forming a clear zone of inhibition around the disc. The exact size of the zone of inhibition is measured and compared to interpretive criteria provided by standardized guidelines such as those from CLSI, 2020. This method is straightforward, easy to interpret, and cost-effective, with preliminary results within 18-24 hours (Bauer et al., 1966).

METHODS

Study design, study site, and sample size

A hospital-based cross-sectional study was conducted in Alka Hospital Pvt Ltd., Lalitpur, Nepal, for six months, from November 2021 to April 2022. Two hundred thirty bodily fluids (urine, drain, sputum, ascitic fluid, blood, pus, swabs, BAL, and CSF) were collected as samples from the patient visiting Alka Hospital. The basis for sample collection was signs and symptoms displayed by the patients; age, sex, and ward were other factors considered during sample selection. Samples leaked, contaminated, or from patients already under antibiotic treatment were discarded to study the exact susceptibility pattern of the isolated species. Samples from referral hospitals or clinics were also not considered.

Isolation and Identification

A loopful of the sample (urine, blood, sputum), a drop of the sample (ascitic fluid, BAL, and CSF), or the swab of the sample (pus swab and swab) was inoculated in MacConkey and Blood agar. The agar plates were incubated at 37°C for 24 hours. After 24 hours, an examination of colony morphology was performed. Gram staining was performed for each positive sample. Gram-negative bacilli or coco-bacilli shaped were further processed, and Gram-positive samples were appropriately discarded. The selected Gram-negative colonies were sub-cultured in a Nutrient agar plate for 24 hours at 37°C to obtain a pure culture of Gram-negative bacteria. Catalase, oxidase, oxidative fermentative (o/f), indole, Methyl red (MR), Vogas Proskauer (VP), urease, TSI (triple sugar iron), and citrate utilization tests were performed on the selected Gram-negative samples.

Identification of ESBL

Isolates producing ESBL were identified in two steps. The first step was to perform a screening test followed by a confirmatory test.

Screening test for ESBL: The disc diffusion test combines two or more cephalosporins. Here, CTX and CAZ were used to screen ESBL-producing isolates. Confirmatory test for ESBL: DDST confirmed that the suspected isolates were ESBL producers.

Antibiotic susceptibility testing

The isolated species were inoculated in NB. Kirby Bauer's AST disc diffusion method was used to study the susceptibility pattern of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*.

Identification of MDR isolates

The MDR mechanism of the isolates was studied by observing the susceptibility pattern of the isolates. The isolated Klebsiella species were said to MDR if these were resistant to any two of the following classes of drugs: cephalosporin, βlactams combination, aminoglycosides, fluoroquinolones, carbapenem, macrolide, and glycylcycline. For isolated Pseudomonas aeruginosa to be MDR, the isolates must be resistant to any two of the following classes of antibiotics: aminoglycosides, fluoroquinolones, carbapenem, and β-lactams combination (Weinstein, 2020).

Data analysis

The data from the study was analyzed using Microsoft Excel and SPSS statistics version 20.

RESULTS

Bacterial growth based on sample types

Of the 230 samples collected, 34 were culture-positive for the *Klebsiella* species and *P. aeruginosa*. The primary samples used for the testing were urine, pus, sputum, ascitic fluid, blood, CSF, swab, and BAL. The highest number of samples collected was urine (n=161); the fewest were CSF and ascitic fluid (n=1) (Figure 1).

Distribution of sample by gender and age

The relation between bacterial growth and gender was insignificant, with a p-value < 0.05 (Figure 2).

Distribution of samples by ward

Out of the three wards, samples from OPD were the highest (207), followed by IPD (18), and finally, SH/C ward (5). The relation between culture positivity and the ward is statistically significant with a p-value <0.05 (Table 1).

Distribution of isolates among clinical samples (Table 2 and 3)

ESBL in culture-positive cases

Of 34 positive samples, 30 (88.24%) were ESBL-producing, and 4 (11.76%) were non-ESBL producers (Table 4).

MDR among bacterial isolates (Table 5)

ESBL isolates based on MDR

The relation between ESBL production and MDR was statistically significant, with a p-value <0.05 (Table 6).

ESBL and MDR among identified bacteria

The relation between ESBL-producing isolates of *Klebsiella* and developing MDR was statistically significant with a p-value <0.05 (Table 7).

Association of ESBL production and MDR

The relation between ESBL-producing isolates of *P. aeruginosa* and developing MDR was statistically significant with p-value <0.05 (Table 8).

Antibiotic resistance pattern of *P. aeruginosa*

A single isolate of non-ESBL-producing *P. aeruginosa* was identified, and the antibiotic susceptibility pattern suggested the isolate was resistant to AK and MRP. It was susceptible to LEV, PIT, and GEN but showed an intermediary reaction to CAZ (Table 9).

Antibiotic resistance pattern of *K. oxytoca*

A single non-ESBL isolate of *K oxytoca* was identified, and it was resistant to CAZ and AZM antibiotics. The isolate was susceptible to MRP, PB, CL, TGC, and NX. The intermediary reaction was seen against PIT (Table 10).

Antibiotic resistance pattern of *pneumoniae*

Two isolates of non-ESBL-producing *K. pneumoniae* were identified. The same antibiotics were used to observe their antibiotic resistance patterns. Both samples were resistant to CAZ. One isolate was resistant, and the other was susceptible to NX and CL. One isolate was sensitive, and one showed an intermediary reaction to MRP, AZM, and TGC. Both isolates were susceptible to PIT and PB (Table 11).

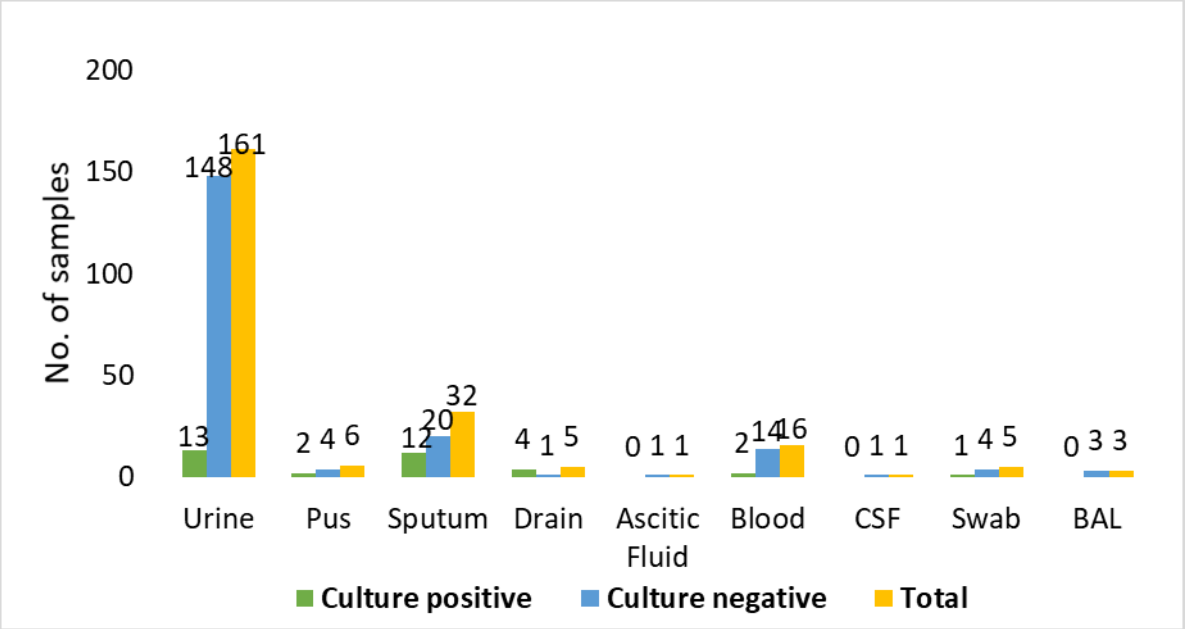

Figure 1: Bacterial growth pattern by clinical specimen types

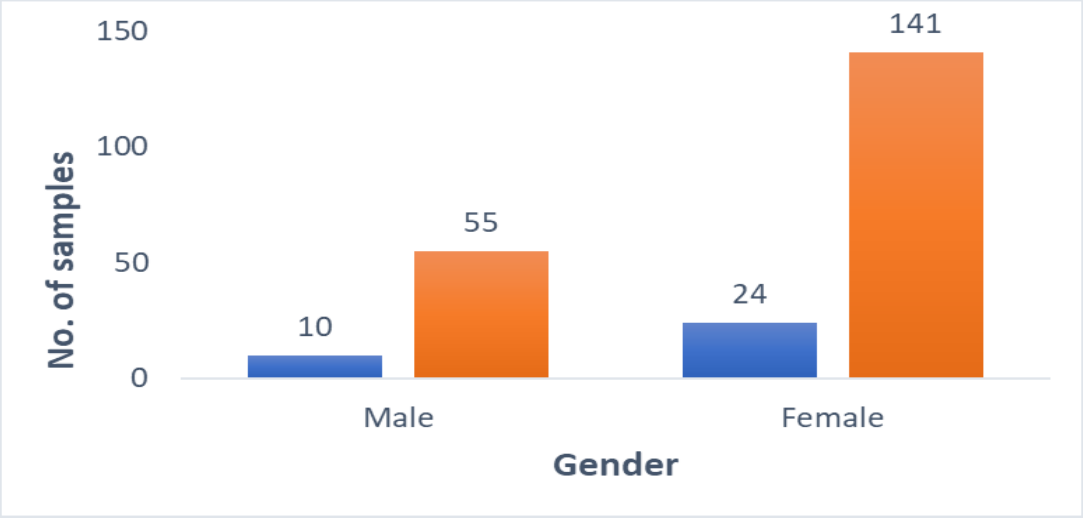

Figure 2: Gender-wise distribution of samples

Table 1: Ward-wise distribution of bacterial isolates

Ward	*K. pneumoniae*	*K oxytoca*	*P. aeruginosa*	Growth positive	Growth negative	Total
OPD (207)	16	4	6	26	181	3.6×10^{-7}
SH/C (5)	4	-	1	5	0	
IPD (18)	1	-	2	3	15	
Total	**21**	**4**	**9**		**196**	

Table 2: Sample distribution by age

Age	*K. pneumoniae*	*K oxytoca*	*P. aeruginosa*	Growth positive	Growth negative	Total
0-10	0	0	0	0 (0)	6 (3.06%)	6 (2.61)
10-20	0	0	0	0 (0)	11 (5.61%)	11 (4.78%)
20-30	1 (50%)	1 (50%)	0	2 (5.41%)	35 (94.59%)	37 (16.09%)
30-40	2 (40%)	2 (40%)	1 (20%)	5 (9.26%)	49 (90.74%)	54 (23.48%)
40-50	1 (33.33%)	1 (33.3%)	1 (33.33%)	3 (9.09%)	30 (90.9%)	33 (14.35%)
50-60	4 (66.67%)	0	2 (33.33%)	6 (18.75%)	26 (81.25%)	32 (13.91%)
60-70	5 (62.5%)	0	3 (37.5%)	8 (38.1%)	13 (61.90%)	21 (9.13)
70-80	3 (100%)	0	0	3 (15.79%)	16 (84.21%)	19 (8.26%)
80-90	4 (80%)	0	1 (20%)	5 (35.71%)	9 (64.29%)	14 (6.09%)
90-100	1 (50%)	0	1 (50%)	2 (66.67%)	1 (33.33%)	3 (1.3%)
Total	**21 (9.13)**	**4 (1.73%)**	**9 (3.91%)**	**34 (14.78%)**	**196 (85.22%)**	**230 (100%)**

Table 3: Distribution of isolates among sample type

Isolates	Urine	Drain	Sputum	Blood	Pus	Swabs	Total
K. oxytoca	3 (75%)	-	1 (25%)	-	-	-	4 (11.76%)
K. pneumoniae	8 (38.1%)	4 (19.05%)	6 (28.57%)	1 (4.8%)	1 (4.76%)	1 (4.76%)	21 (61.8%)
P. aeruginosa	2 (22.22%)	-	5 (55.56%)	1 (11.11%)	1 (11.1%)		9 (26.47%)
Total	**13 (38.24%)**	**4 (11.76%)**	**12 (35.29%)**	**2 (5.88%)**	**2 (5.9%)**	**1 (2.9%)**	**34 (100%)**

Table 4: Distribution of ESBL in culture-positive cases

	K. oxytoca	*K. pneumoniae*	*P. aeruginosa*	Total
ESBL	3 (10%)	19 (63.33%)	8(26.67%)	30 (88.24%)
Non-ESBL	1 (25%)	2 (50%)	1 (25%)	4 (11.76%)
Total	**4**	**21**	**9**	**34**

Table 5: Distribution of MDR among the bacterial isolates

	K. oxytoca	*K. pneumoniae*	*P. aeruginosa*	Total
MDR	2 (50%)	17 (80.95%)	7 (77.78%)	26 (76.47%)
Non-MDR	2(50%)	4 (19.05%)	2 (22.22%)	8 (23.53%)
Total	**4**	**21**	**9**	**34**

Table 6: Distribution of ESBL isolates based on MDR

	ESBL	Non-ESBL	Total	P-value
MDR	26	1	27	0.0042
Non-MDR	4	3	7	
Total	**30**	**4**	**34**	

Table 7: Distribution of ESBL and MDR among *Klebsiella* species

	ESBL	Non-ESBL	Total	P- value
MDR	19	1	20	0.031
Non-MDR	3	2	4	
Total	**22**	**3**	**24**	

Table 8: Distribution of MDR and ESBL among *P. aeruginosa*

	ESBL	Non-ESBL	Total	P-value
MDR	7	0	7	0.047
Non-MDR	1	1	2	
Total	**8**	**1**	**9**	

Table 9: Antibiotic susceptibility pattern of ESBL-producing *P. aeruginosa*

Antibiotic	Resistant	Susceptible	Intermediate	Total
AK	6 (75%)	2 (25%)	0	8
CAZ	6 (75%)	2 (25%)	0	8
LEV	3 (37.5%)	3 (37.5%)	2 (25%)	8
MRP	6 (75%)	2 (25%)	0	8
PIT	2 (5%)	5 (62.5%)	1 (12.5%)	8
GEN	2 (25%)	6 (75%)	0	8
Total	**25 (52%)**	**20 (41.67%)**	**3 (6.23%)**	**48**

Table 10: Antibiotic susceptibility pattern of ESBL-producing *K. oxytoca*

Antibiotic	Resistant	Susceptible	Intermediate	Total
CAZ	2 (75%)	1 (25%)	0	3
MRP	1 (25%)	2 (75%)	0	3
PIT	1 (25%)	2 (75%)	0	3
AZM	3 (100%)	0	0	3
PB	0	3 (100%)	0	3
CL	0	3 (100%)	0	3
TGC	0	3 (100%)	0	3
NX	2 (75%)	1 (25%)	0	3
Total	**9 (37.5%)**	**15 (62.5%)**	**0**	**24**

Table 11: Antibiotic susceptibility pattern of ESBL-producing *K. pneumoniae*

Antibiotic	Resistant	Susceptible	Intermediate	Total
CAZ	18 (94.7%)	0	1 (5.3%)	19
MRP	10 (52.68%)	5 (26.32%)	4 (21.1%)	19
PIT	12 (63.16%)	5 (26.32%)	2 (10.53%)	19
AZM	16 (84.21%)	0	3 (15.79%)	19
PB	3 (15.79%)	8 (42.11%)	8 (42.1%)	19
CL	2 (10.53%)	6 (31.58%)	11 (57.89%)	19
TGC	6 (31.52%)	2 (10.53%)	11 (57.89%)	19
NX	15 (78.9%)	4 (21.1%)	0	19
Total	**82 (53.95%)**	**30 (19.74%)**	**40 (26.3%)**	**152**

DISCUSSION

The study's main goal was to study the antibiotic susceptibility pattern among ESBL-producing strains of *Klebsiella* species and *Pseudomonas aeruginosa*. The Cronbach formula was used to determine this study's sample size, which estimated the total sample size between 214 and 235 based on two different studies. So, this study collected 230 samples. The two studies that were the references for sample size estimation were one conducted in Everest Hospital, where 16.7% *P. aeruginosa* were ESBL producers, and another study conducted in Sahid Gangalal Hospital found 18.75% ESBL-producing *Klebsiella* species (Guragain et al., 2019 and Shilpakar et al., 2021).

The studies isolated 30 ESBL isolates of *Klebsiella* species and *P. aeruginosa*. Out of the 30 ESBL producers, 26 were MDR. However, out of the non-ESBL producers, 4, only one was found to be an MDR strain. The MDR strains among ESBL producers are significant, with a P-value of 0.0042. This result suggests that producing ESBL enzyme is one of the reasons for developing MDR in bacteria.

Among the targeted organisms, *Klebsiella pneumoniae* had the highest number of isolates in all the samples. 19 (90.48%) of the 21 samples were ESBL producers out of isolated *K. pneumoniae*. 11.76% (4 out of 34 samples) of *K. oxytoca* was identified from the isolated samples. 3 (75%) of the 4 samples were ESBL producers. Among the ESBL-producing isolates of *Klebsiella* species isolated in this study, 86.36% were MDR strains. A single non-ESBL-producing *Klebsiella* species is MDR. There is a statistically significant relation between ESBL-producing enzymes and the development of MDR among isolated *Klebsiella* species P-value = 0.031.

A study conducted by Ghimire et al., 2018 at Sahid Gangalal National Heart Centre, Kathmandu, Nepal, showed that out of the 80 isolated Gram-negative organisms, 18 (22.5%) isolates were *K. pneumoniae*, and 2 (2.5%) were *K. oxytoca*. In the same study, 8 of the 18 (44.44%) *K. pneumoniae* were confirmed ESBL producers, and both *K. oxytoca* were ESBL producers. 47.1% of the ESBL-producing *K. pneumoniae* were MDR, and 100% of the ESBL-producing K oxytoca were MDR. (Ghimire et al., 2018) The MDR and ESBL production had some significant relationships in the study by Ghimire and the team. This may be due to the ability of ESBL producers to develop MDR by resisting different classes of antibiotics through gene mutations and changes in the targets of various antibiotics.

9 (26.47%) *P. aeruginosa* was identified from the growth pool. Among the nine isolates of *P. aeruginosa*, 8 were ESBL producers (23.53%). 87.5% of ESBL-producing *P. aeruginosa* were MDR strains. The non-ESBL strain was not MDR. This result showed a statistically significant relation between ESBL production and the development of MDR among isolated *P. aeruginosa*. (P-value = 0.047) This study resembled a study conducted by Shrestha et al. (2018), which showed a 7.9% growth rate for *P. aeruginosa* in the pus sample, i.e., 25 out of 316 were positive for *P. aeruginosa*. Among the positive growth, 36% of samples were ESBL producers. Another study that resembled the result from this study showed that 16.25% positive growth of *P. aeruginosa* and 38.46% of the *P aeruginosa* were ESBL producers. Among the ESBL producers, 55.6% were MDR

species (Ghimire et al., 2018).

The antibiotics with the lowest resistivity against *P. aeruginosa* were GEN, 30%, MRP, 17%, and IPM, 15%. The most effective antibiotic was CL, with 0 isolates resistant to it. The antibiotic CAZ used in this study showed results similar to those of Kothari et al., 2020. However, the most effective drug against *P. aeruginosa* for this study (PIT, 62.5%) and Kothari et al., 2020 (CL, 100%) showed different results. They used piperacillin from the β-lactam antibiotic class with the results of 61% isolates resistant to it. The difference in the type of antibiotic susceptibility can be because of the difference in strains due to various factors, including genetic in bacteria used for the study.

PB, CL, and TGC were the most effective antibiotics against ESBL-producing *K. oxytoca*, with a 100% susceptibility rate. AZM was the most ineffective drug, with all three isolates resistant. MRP and PIT had 33.33% resistant isolates, whereas CAZ and NX had 66.67%.

CAZ was the most ineffective drug against ESBL-producing *Klebsiella* species and *P. aeruginosa*. PIT was among the antibiotics effective against *P. aeruginosa* and *K. oxytoca*. At the same time, CL was the most effective against ESBL-producing *K. pneumoniae*. A study conducted by (Paudel et al., 2021) in Sahid Gangalal Hospital showed that CL was the most sensitive antibiotic, whereas AMP was least effective with 100% isolates resistant to the drug, which supports this study (Paudel et al., 2021).

Conclusion

The study concludes PB was the most effective antibiotic against ESBL-producing *Klebsiella pneumoniae*. Whereas CAZ was the most ineffective drug against ESBL-producing *K. pneumoniae*. PB, CL, and TGC were the most effective antibiotics against ESBL-producing *K. oxytoca*. In contrast, AZM was the most ineffective drug against ESBL-producing *K. oxytoca*. PIT and GEN were the most effective antibiotics against ESBL-producing *P. aeruginosa*. While CAZ, AK, and MRP were the most ineffective drugs against ESBL-producing *P. aeruginosa*. *K. pneumoniae* was the most predominant bacterial isolate, followed by *P. aeruginosa* and *K. oxytoca*. Urine was the most collected sample with the highest culture positivity. The highest number of samples was from the patients in the age group 30-40 years. However, culture positivity was highest in the samples from patients of 90-100 years. Samples from OPD were the highest, and the same ward had the highest cultural positivity. The relationship between cultural positivity and the patient's gender does not correlate. In this study, ESBL was a significant cause of the development of multi-drug resistance among the selected and isolated strains of *Klebsiella* species and *P. aeruginosa*.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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