Extended-spectrum and Metallo-beta lactamase enzymes mediated resistance in *Pseudomonas aeruginosa* in clinically isolated specimens

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Abstract

Pseudomonas aeruginosa is one of the leading opportunistic pathogens, frequently highlighted for the production of Extended-Spectrum Beta-Lactamase (ESBL) and Metallo-beta Lactamase (MBL) enzymes. This is believed to be the primary inhabitant of soil due to its adaptive nature and can survive in aquatic and even in a toxic environment. The current study aimed to screen ESBL, MBL producing, and Multiple Drug Resistant **(**MDR) strains of *P. aeruginosa*. Clinical specimens collected from patients were screened for the presence of *P. aeruginosa*. After identification, all the isolates were tested for the sensitivity pattern following the Kirby-Bauer disc diffusion method. The presence of ESBL and MBL enzymes were detected following DDST and IMP-EDTA detection tests, respectively. The sample size used in the study was 1369, and colonies for *P. aeruginosa* were obtained from 126 (9.20%) samples of culture media. 54.76% (69/126) of the positive cases were detected in the female population, whereas 45.24% (57/126) in the male population. High-frequency rate (n=43/126) was detected in the age group ≥31 year, followed by 21-30 (n=35/126), and 11-20 (n=34/126) age group, the minimal frequency (n=14/126) was detected in age group 0-10. The sensitivity pattern showed that the majority of the isolates were resistant, but class carbapenem (imipenem, meropenem), aminoglycosides (amikacin, gentamicin, tobramycin), and other antibiotics like sulbactam-Cefoperazone, tazobactam-piperacillin, tigecycline, and Fosfomycin exhibited the best efficacy against *P. aeruginosa*. It is concluded that the emergence of resistance due to ESBL and MBL enzymes in *P. aeruginosa* is directly linked with a public health concern because these strains are almost resistant to a wide range of antibiotics, and only limited antibiotics are potent against these organisms.

Keywords: Antimicrobial resistance; DDST; ESBL; imipenem-EDTA test; MBL; *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is worldwide known as an opportunistic pathogen, frequently isolated from clinical specimens and is associated with infections in immunocompromised patients as well as involved in hospital-acquired infections (Nseir *et al.*, 2006). Also, this bacterium possesses the ability to acquire resistant elements from other pathogenic bacteria as well as from the environment to compete multiple classes of antibiotics (Freitas & Barth, 2002). The emergence of antibiotic resistance in pathogenic bacteria predominantly depends /based on the misuse and inappropriate use of antibiotics in the community set (Tiersma, 2013), due to which Multi Drugs Resistant (MDR) is increasing day by day in pathogenic gram-negative bacteria (Bush, 2010).

In the strategy of resistance of *P. aeruginosa* to β-lactam antibiotics, several mechanisms are involved. One of the most medically important resistance mechanisms among them in pathogenic gram-negative *P. aeruginosa* is the production of β-lactamase enzymes (Livermore, 2002). In

this mechanism, the β-lactamase enzymes target and hydrolyze the amide bond of β-lactam ring of β-lactam antibiotics, e.g., carbapenems, monobactam, cephalosporin, and penicillin (Noyal *et al.*, 2009; Peshattiwar & Peerapur, 2011), and resulting in an inactive by-product of β-lactam antibiotics (Simoens *et al.*, 2006). The incidence and prevalence rate of Extended-Spectrum Beta-Lactamase (ESBL) producing bacterial infections increase with the passage of time (Thokar *et al.*, 2010). More than two hundred types of ESBL enzymes have been identified globally, most of which belong to family *Enterobacteriaceae* (Yazdi *et al.*, 2012). On the other hand, exceeding 80 types of Metallo-beta Lactamase (MBL) enzymes have been identified worldwide in different pathogenic bacterial strains (Bush & Jacoby, 2010). It is believed that early detection of these β-lactamase producing bacteria is medically important to prevent their intra and interhospital dissemination with an appropriate antibiotic therapy (Pandya *et al.*, 2011). The current study aimed to screen the presence of ESBL and MBL producing and multidrug resistant strains of *P. aeruginosa* to design treatment strategy that should be more targeted and effective against this pathogen.

2. Methodology

2.1. Study design and duration:

The current clinical study was carried out in the Microbiology laboratory of Khyber Teaching Hospital (KTH), Peshawar, during one year from January to December 2018. A total of 1369 different clinical specimens, including; wounds, swabs, High vaginal swabs (HVS), sputum, pus, tissue, and urine, were collected from indoor-patients (IPD) and outdoor-patients (OPD) of various wards (burn unit, ENT, etc.) of KTH.

2.2. Sample processing:

All the clinical specimens were collected in the presence of a medical officer from the mentioned wards, labelled properly, and were inoculated on suitable culture media, i.e., MacConkey agar (CM0007-OXOID), nutrient agar (CM0003-OXOID), CLED agar (CM0301-OXOID), blood agar (CM0055-OXOID). After inoculation, the culture media were overnight incubated at 37°C. After, the culture media were examined for the presence of *P. aeruginosa*. Furthermore, the growth of *P. aeruginosa* was confirmed on a selective medium (Cetrimide agar CM569-OXOID). Colonies grown on the selective medium were confirmed based on colony morphology, various biochemical tests, growth pigmentation, and staining characteristics per standard lab confirmation methods (Cheesbrough, 2006; Cowan & Steel, 2004).

2.3. Susceptibility pattern:

Prior to inoculation for susceptibility, Tryptic soy broth (CM129-OXOID) was prepared, autoclaved, and was filled to capped tubes, almost 5ml in each. Each bacterial isolate was overnight incubated in Tryptic soy broth at 35°C. Each tube's turbidity was adjusted to 0.5 McFarland index as per Clinical and Laboratory Standard Institute (CLSI) instructions. Routinely used different commercially available antibiotics (OXOID) (mentioned in Table 3) were subjected to Mueller Hinton agar (MHA) (CM0337-OXOID) following the Kirby-Bauer disc diffusion method to determine the susceptibility pattern of *P. aeruginosa* as prescribed by a study (Cavallo *et al.*, 2007). The minimum inhibitory concentration of each subjected antibiotic to bacterial isolates was determined by the disc diffusion method. The standard breakpoints were standardized as prescribed by CLSI instructions (Cockerill, 2011).

2.4. Detection of ESBL production:

Each strain of *P. aeruginosa* spp. isolated from different clinical specimens was screened according to CLSI for ESBL enzyme production via the synergy disc diffusion method. Prior to the ESBL detection test, the *P. aeruginosa* isolates stored at -20°C were refreshed on nutrient agar (CM0003-OXOID) for a better result.

A double disc-diffusion synergy test (DDST) was practiced to detect the ESBL production in *P. aeruginosa* strains. In this, commercially available antibiotic discs of a third-generation cephalosporin, cefotaxime, ceftazidime, and ceftriaxone were subjected on MHA medium at a distance of 25mm center to center from the amoxi-clav antibiotic disc (amoxicillin/clavulanic acid 20/10µg) as prescribed by a previous study (Hawser *et al.*, 2012). Enhancement in the inhibition zone of any disc of cephalosporin (third generation) after overnight incubation at 35±2°C toward amoxi-clav indicated the production of ESBL enzyme in the medium.

2.5. Detection of MBL production:

MBL production was detected in *P. aeruginosa* strains via the Imipenem-EDTA disc method (a disc diffusion method), in which 0.5M Ethylenediamine tetra acetic acid (EDTA) and two imipenem discs were used. Prior to inoculation, 0.5M EDTA was prepared with a ratio of 186.1g of EDTA (disodium EDTA.$2H_2O$) was dissolved in 1000ml distilled water, and pH was adjusted to 8.0.

In the imipenem-EDTA disc method, imipenem-resistant *P. aeruginosa* culture was inoculated on MHA medium. Two imipenem discs were placed, and an appropriate amount of prepared EDTA was loaded to one of the imipenem discs to obtain the desired EDTA concentration. The growth of *P. aeruginosa* was examined for the synergic effect of chelator agent (EDTA) and imipenem after overnight incubation at 35±2°C, which confirmed the production of MBL enzyme as prescribed by a study (Yong *et al.*, 2002).

2.6. Statistical analysis:

The data were statistically analyzed by applying the X^2 test (Chi-square test) using SPSS software version 20.0. P-value less than 0.05 (≥0.05) was considered as significant.

3. Results

Totally, 1369 clinical specimens were collected from indoor and outdoor patients having various infections and were screened for *P. aeruginosa*. Among the processed specimens, 126 (9.20%) exhibited colonies, whereas 1243 (90.8%) clinical specimens did not show the colonies of *P. aeruginosa* on culture media. Female population encompassed high frequency rate 69 (54.76%) in contrast to male population 57 (45.24%). The high-frequency rate was detected in the age group more than 31 years (≥31 years), followed by 21-30, and 11-20 age groups with frequencies 43, 35, and 34 respectively, whereas the age group 0-10 showed minimum frequency (n=14), among all age groups. Maximum frequency (n=25) of ESBL producing *P. aeruginosa* was confirmed in the age group 21-30, while minimum (n=2) in the age group 0-10 years. The relationship of two variables, age groups, and gender, was statistically cross-tabulated using the Chi-square test (X^2 test), which was highly significant (p-value=0.029≤0.05) Table 1.

Table 1. Gender-wise distribution of *P. aeruginosa*, ESBL, and MBL producing *P. aeruginosa* among different age groups and statistical relationship between gender and age group of patients.

Age group (year)	Total frequency			Male frequency			Female			*P-value*
	Total Positive	ESBL (n)	MBL (n)	Total Positive	ESBL (n)	MBL (n)	Total Positive	ESBL (n)	MBL (n)	
0-10	14	2	0	11	0	0	3	2	0	0.029
11-20	34	11	4	15	4	2	19	7	2	
21-30	35	25	5	11	9	1	24	16	4	
≥ 31	43	10	5	20	5	2	23	5	3	
Total	**126**	**48**	**14**	**57**	**18**	**5**	**69**	**30**	**9**	

The 126 positive samples were isolated from different clinical specimens. Out of these 126 positive isolates, pus contributed the highest frequency rate (n=70/126) among all clinical specimens, followed by urine (n=38/126), ear swab (n=9/126), tissue (n=4/126), wound (n=2/126), sputum (n=2/126), while high vaginal swab (n=1/126) contributed with lowest frequency rate. Among 126 positive isolates of *P. aeruginosa*, 38.10% (n=48/126) isolates showed the colonies as ESBL producing *P. aeruginosa*, 11% (n=14/126) showed the colonies as MBL producing *P. aeruginosa,* whereas 50.80% (n=64/126) showed no enzyme production. Among ESBL producing *P. aeruginosa*, 56.25% (n=27/48) were isolated from pus, 37.5% (n=18/48) from urine, and 6.25% (n=3/48) from ear swab, whereas no ESBL producing *P. aeruginosa* was isolated from the wound, tissue, high vaginal swab, and sputum. Among MBL producing *P. aeruginosa*, 50% (n=7/14) were detected in pus, 35.71% (n=5/14) in urine, 7.14% (n=1/14) in wound and tissue whereas, no MBL producing *P. aeruginosa* was detected in ear swab, sputum, and high vaginal swab Table 2.

Table 2. Frequency distribution of *P. aeruginosa* strains, ESBL producing, and MBL producing *P. aeruginosa* isolated from various clinical specimens.

Specimen type	Total Positive N (%)	ESBL Positive N (%)	MBL Positive N (%)	Non-ESBL & MBL N (%)
Pus	70 (55.56)	27 (56.25)	7 (50)	36 (56.25)
Wound	2 (1.59)	0 (0)	1 (7.14)	1 (1.56)
Ear Swab	9 (7.14)	3 (6.25)	0 (0)	6 (9.38)
Sputum	2 (1.59)	0 (0)	0 (0)	2 (3.13)
High vaginal swab	1 (0.79)	0 (0)	0 (0)	1 (1.56)
Urine	38 (30.16)	18 (37.5)	5 (35.71)	15 (23.44)
Tissue	4 (3.17)	0 (0)	1 (7.14)	3 (4.69)
Total	**126 (9.20)**	**48 (38.10)**	**14 (11)**	**64 (50.80)**

Commercially available 24 antibiotics (OXOID) were subjected to find out the susceptibility pattern of gram-negative *P. aeruginosa*. The results of the susceptibility pattern exhibited that the bacteria vary in susceptibility pattern to all tested

antibiotics. The majority of the isolates were resistant to these antibiotics, while some antibiotics showed good results against the tested isolates. Imipenem (84.92%) and meropenem (86.51%) (class carbapenem) exhibited the best result against *P. aeruginosa* among all antibiotics, followed by amikacin (83.33%), gentamicin (60.32%), tobramycin (43.65%) from class aminoglycosides, sulbactam-cefoperazone (83.33%), tazobactam-piperacillin (80.95%), tigecycline (75.4%) and fosfomycin (67.46%). On the other hand, bacterial isolates were highly resistant to sulfamethoxazole-trimethoprim (87.30%), followed by ampicillin (85.71%) (penicillin), nitrofurantoin (79.37%) (nitrofurans), amoxi-clav (76.19%) (Augmentin), chloramphenicol (73.81%) (cell wall inhibitor), nalidixic acid (86.51%), norfloxacin (82.54%), levofloxacin (62.70%), ciprofloxacin (57.14%) (class quinolones), cefotaxime (72.22%), ceftazidime (70%), and cefoxitin (61.9%) (class cephalosporin), and aztreonam (57.14%) (class monobactam) Figure 1.

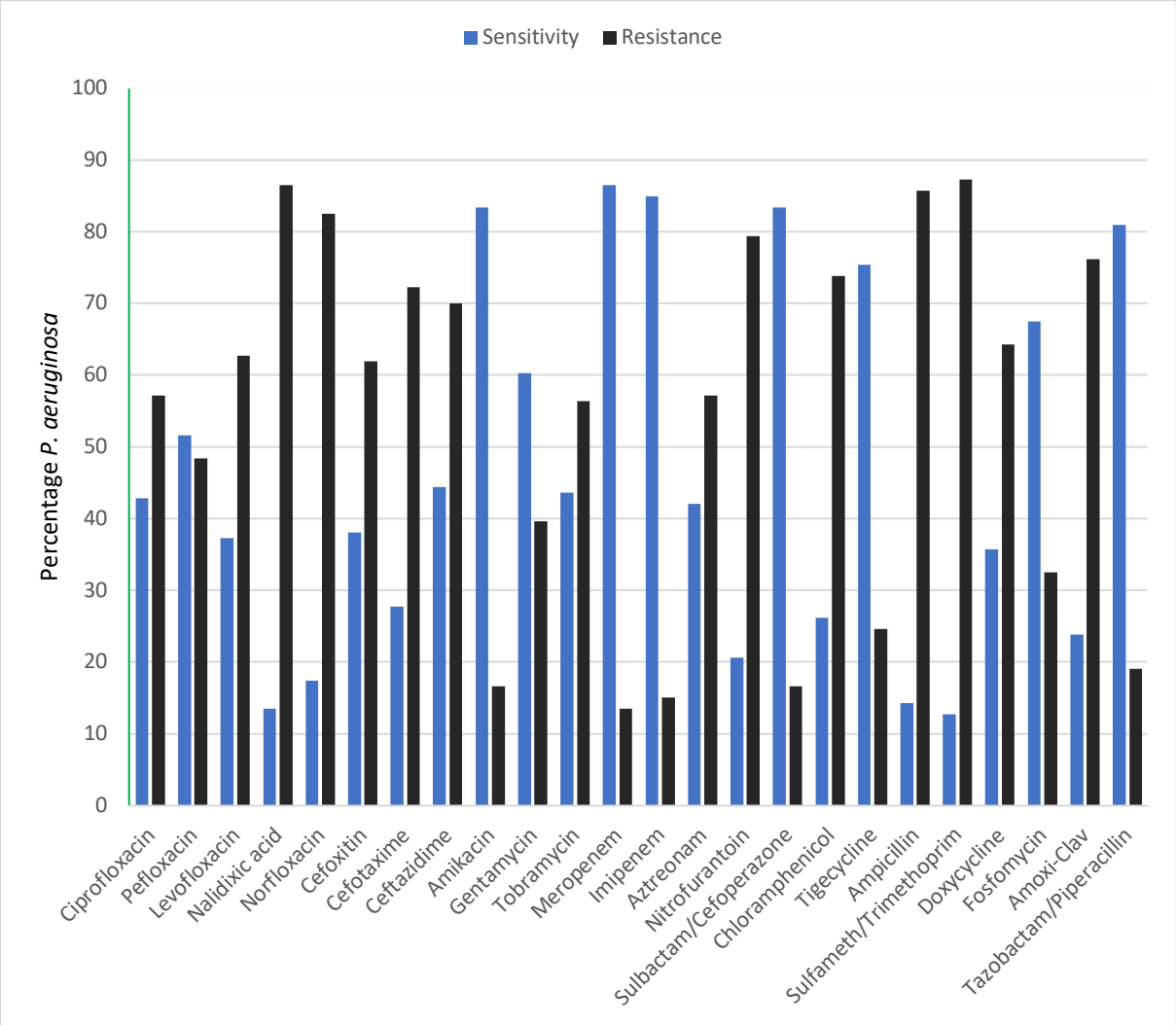

Fig. 1. Susceptibility pattern of *P. aeruginosa* species detected by disc diffusion method.

The susceptibility pattern showed that ESBL and MBL producing *P. aeruginosa* vary in their susceptibility pattern to all antibiotics. In the case of ESBL producing *P. aeruginosa*, most of the antibiotics, i.e., levofloxacin, nalidixic acid, norfloxacin, amikacin, gentamicin, cefoxitin, nitrofurantoin, chloramphenicol,

tigecycline, fosfomycin, and amoxi-clav, exhibited 100% efficacy to isolates of *P. aeruginosa*, followed by sulbactam-cefoperazone, doxycycline, tobramycin, ciprofloxacin, and sulfamethoxazole-trimethoprim, whereas other antibiotics, i.e., pefloxacin, meropenem, imipenem, cefoxitin, cefotaxime, ceftazidime, ampicillin, aztreonam, and tazobactam-piperacillin showed no sensitivity to ESBL producing *P. aeruginosa*.

On the other hand, MBL producing *P. aeruginosa* were highly resistant to most of the subjected antibiotics, i.e., meropenem, imipenem, ceftazidime, cefotaxime, aztreonam, and ampicillin (100% each), followed by nalidixic acid, nitrofurantoin (92.86% each), levofloxacin, norfloxacin, sulfamethoxazole-trimethoprim, doxycycline (85.71% each), pefloxacin, tobramycin, and chloramphenicol (78.57% each) whereas tazobactam-piperacillin and sulbactam-cefoperazone showed the best result to tested antibiotics followed by amikacin (71.43%), gentamicin and tigecycline (64.29% each) Table 3.

Table 3. Susceptibility pattern of ESBL and MBL producing *P. aeruginosa* isolated from clinical specimens.

Antibiotics (Code)	ESBL		MBL	
	Sensitive N (%)	Resistant N (%)	Sensitive N (%)	Resistant N (%)
Ciprofloxacin (CIP)	46 (95.83)	2 (4.17)	6 (42.86)	8 (57.14)
Pefloxacin (PEF)	0 (0)	48 (100)	3 (21.43)	11 (78.57)
Levofloxacin (LEV)	48 (100)	0 (0)	2 (14.29)	12 (85.71)
Nalidixic acid (NA)	48(100)	0 (0)	1 (7.14)	13 (92.86)
Norfloxacin (NOR)	48 (100)	0 (0)	2 (14.29)	12 (85.71)
Amikacin (AK)	48 (100)	0 (0)	10 (71.43)	4 (28.57)
Gentamicin (CN)	48 (100)	0 (0)	9 (64.29)	5 (35.71)
Tobramycin (TOB)	46 (95.83)	2 (4.17)	3 (21.43)	11 (78.57)
Meropenem (MEM)	0 (0)	48 (100)	0 (0)	14 (100)
Imipenem (IMP)	0 (0)	48 (100)	0 (0)	14 (100)
Cefoxitin (FOX)	48 (100)	0 (0)	4 (28.57)	10 (71.43)
Cefotaxime (CTX)	0 (0)	48 (100)	0 (0)	14 (100)
Ceftazidime (CAZ)	0 (0)	48 (100)	0 (0)	14 (100)
Aztreonam (ATM)	0 (0)	48 (100)	0 (0)	14 (100)
Nitrofurantoin (F)	48 (100)	0 (0)	1 (7.14)	13 (92.86)
Sulbactam/Cefoperazone (SCF)	47 (97.92)	1 (2.08)	12 (85.71)	2 (14.29)
Chloramphenicol (C)	48 (100)	0 (0)	3 (21.43)	11 (78.57)
Tigecycline (TGC)	48 (100)	0 (0)	9 (64.29)	5 (35.71)
Ampicillin (AMP)	0 (0)	48 (100)	0 (0)	14 (100)
Sulfamethoxazole/Trimethoprim (SXT)	44 (91.67)	4 (8.33)	2 (14.29)	12 (85.71)
Doxycycline (DO)	47 (97.92)	1 (2.08)	2 (14.29)	12 (85.71)
Fosfomycin (FOS)	48 (100)	0 (0)	6 (42.86)	8 (57.14)
Amoxi-clav (AMC)	48 (100)	0 (0)	4 (28.57)	10 (71.43)
Tazobactam/piperacillin (TZP)	0 (0)	48 (100)	12 (85.71)	2 (14.29)

4. Discussion

Pseudomonas aeruginosa is the leading cause of nosocomial infections worldwide, especially among. immunocompromised patients. The bacterium is majorly isolated with high resistance from different clinical specimens such as pus, sputum, urine,

blood, wound, ear swab, high vaginal swab, body fluid, tissues, and other clinical specimens (Ahmad *et al.*, 2016; Gupta *et al.*, 2016). The current study detected 9.20% positive of the total 1369 collected samples, in which the male contributed 45.24% less than the female contributed 54.76% of the positive samples. Similar findings were obtained in the previous study, in which a high frequency of disease was observed in the female than the male population. Our study agrees with the previous study, where it denoted that the male population was less infected than the female population (Ahmad *et al.*, 2016). High frequency rate (n=43) was detected in our study in the age group ≥31 year, followed by 21-30 (n=35), 11-20 (n=34) and 0-10 (n=14). A previous study also documented that the infection of *P. aeruginosa* was detected in patients having age between 21-50 years (Javiya *et al.*, 2008). The relationship of two variables in our study, age groups, and gender, was statistically cross-tabulated using the Chi-square test, which was highly significant (*p-value*=0.029) Table 1.

Our study also reported that 38.10% (n=48/126) of the positive isolates were detected as ESBL producing *P. aeruginosa,* whereas 11% (n=14/126) detected as MBL producing *P. aeruginosa* Table 2. The frequency rate of ESBL producing *P. aeruginosa* is quite high compared to MBL producing *P. aeruginosa*, but most isolates were non-ESBL and MBL producers. The mean difference in the frequency distribution of ESBL and MBL producers in our study is supported by the previous report (Al-Agamy *et al.*, 2012). According to our study Table 2, the majority of the *P. aeruginosa* spp. were isolated from pus (n=70), followed by urine (n=38), ear swab (n=9), and tissue (n=4). The specimen collection procedure is defended by the previous study (Ahmad *et al.*, 2016).

The sensitivity results of our research study highlighted that all the bacterial isolates vary in their susceptibility pattern to all subjected antibiotics. The majority of them combated the selected antibiotics; however, some antibiotics showed the best results against the tested gram-negative bacteria. A previous study is in line with our study (Ahmad *et al.*, 2016). Some antibiotics such as meropenem (86.51%) and imipenem (84.92%) (class carbapenem) illustrated best activities to *P. aeruginosa*, followed by amikacin (83.33%), gentamicin (60.32%), tobramycin (43.65%) (class aminoglycosides), sulbactam-cefoperazone (83.33%), tazobactam-piperacillin (80.95%), tigecycline (75.4%) and fosfomycin (67.46%). The previous study also confirmed that these antibiotics illustrated the best potential and are commonly prescribed for the treatment against *P. aeruginosa* (Al-Agamy *et al.*, 2012).

The resistant pattern of bacterial isolates in the current study was; sulfamethoxazole-trimethoprim 87.30%, ampicillin 85.71% (penicillin), nitrofurantoin 79.37% (nitrofurans), amoxi-clav 76.19% (Augmentin), chloramphenicol 73.81% (cell wall inhibitor), nalidixic acid 86.51%, norfloxacin 82.54%, levofloxacin 62.70%, ciprofloxacin 57.14% (class quinolones), cefotaxime 72.22%, ceftazidime 70%, and cefoxitin 61.9% (class cephalosporin), and aztreonam 57.14% (class monobactam) Figure 1. The resistant pattern of *P. aeruginosa* was documented previously; according to them, *P. aeruginosa* is highly resistant to most of the antibiotics, i.e., quinolones, cephalosporin, penicillin, monobactam, etc. and is difficult to treat easily (Javiya *et al.*, 2008; Zafer *et al.*, 2014).

The result of our study revealed that some antibiotics such as pefloxacin, meropenem, imipenem, cefotaxime, ceftazidime, ampicillin, aztreonam, and tazobactam-piperacillin exhibited no potency against ESBL producing *P. aeruginosa*, whereas some classes of antibiotics such as class quinolones, aminoglycoside, and other antibiotics like chloramphenicol, nitrofurantoin, etc. were quite potent against ESBL producing *P. aeruginosa*. While most of the antibiotics,

such as class quinolones, aminoglycosides, carbapenem, cephalosporines, and other antibiotics like ampicillin, nitrofurantoin, nalidixic acid, etc. were completely resisted by MBL producing *P. aeruginosa* **(**Table 3). The previous studies also reported that most antibiotics showed no potency against ESBL and MBL producing *P. aeruginosa* (Ahmad *et al.*, 2016; Rafiee *et al.*, 2014).

5. Conclusions

Our study concluded that the emergence of resistance in *P. aeruginosa* goes high, which is directly linked with ESBL and MBL enzymes. These *P. aeruginosa* strains are almost resistant to a wide range of commercially available antibiotics, and only limited antibiotics are potent against these organisms. Third generation cephalosporine and carbapenem showed zero potency against ESBL and MBL producing *P. aeruginosa,* which is a major public health concern, because these antibiotics were the drug of choice. Furthermore, the current study highlights that ESBL is much more prevalent in the study area than MBL as a resistant element in *P. aeruginosa* to cause hospital-acquired infections and life-threatening diseases. So, our study highly recommends that DDST and IMP-EDTA should be used for detection of ESBL and MBL enzymes, respectively, and effective antibiotics should be prescribed in the study area to overcome dissemination of multidrug-resistant *P. aeruginosa* infections.

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