Prevalence of Extended-Spectrum Beta-Lactamase, AmpC Beta-Lactamase, and Metallo-Beta-Lactamase among Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract: *Objectives*: to study prevalence of ESBLs, AmpC beta-lactamase and metallo-beta-lactamase among clinical isolates of *P. aeruginosa*.

Materials and Methods: *P. aeruginosa* were isolated and identified by the traditional microbiological procedure, ESBLs were detected by combined disk diffusion method according CLSI recommendations, AmpC beta-lactamase was detected by iodometric methods, MBL was detected by disc potentiation test and Agar dilution method for antibiotic susceptibility testing.

Results: Out of 330 samples of different types of infections, 58 were positive for *P. aeruginosa*. All isolates showed high resistance to most of the tested antimicrobials but showed low resistance to amikacin. Forty-two (72.4%) isolates were detected as β-lactamase producers, 16 (27.5%) isolates were positive for ESBLs while 31(53.4%) were MBL producing strains. All ESBL and MBL producers were highly resistant to the tested antimicrobials. Plasmid profile showed that 9 isolates were plasmid mediated.

Conclusion: The study emphasizes a high prevalence of multidrug-resistant *P. aeruginosa* producing beta-lactamase enzymes of diverse mechanisms especially in burn units. Proper antibiotic policy and measures should be taken to minimize the emergence of this multiple β-lactamase producing pathogens and also the danger of their dissemination to other bacteria by plasmids which may carry resistance genes for other antimicrobials.

Keywords: *Pseudomonas aeruginosa*, AmpC β-lactamase, ESBLs, MBL, plasmid.

INTRODUCTION

Pseudomonas aeruginosa is a leading cause of nosocomial infections, including pneumonia, urinary tract infections, and bacteremia. The infections can be particularly severe in patients with impaired immune systems, such neutropenic or cancer patients [1]. *Pseudomonas aeruginosa* is intrinsically resistant to a wide range of drugs and pursue an extraordinary resistant mechanisms against other antimicrobials.

β-lactamases are important constituent of the antimicrobial resistance arsenal of *P. aeruginosa*. β-lactamases are identified in this pathogen as well as their biochemical properties [2]. β-Lactamases inactivate penicillins and cephalosporins by hydrolyzing the amide bond of the β-lactam ring. Molecular class C or AmpC primarily hydrolyses cephems (cephalosporins and cephamycins) but also hydrolyze penicillins and aztreonam. These enzymes are resistant to the currently available β-lactamase inhibitors such as clavulanate, tazobactam and sulbactam [3]. With rare exceptions, the hydrolysis of cephamycins, such as cefotetan and cefoxitin, is a property that can help to distinguish AmpCs from ESBLs. Genes encoding inducible chromosomal AmpC β-lactamases are part of the genomes of many Gram negative bacteria specially *P. aeruginosa*. High level production of AmpC may cause resistance to the first, second and third-generation cephalosporins, cephamycins, penicillins and β-lactamase inhibitor combination. Higher level AmpC production may occur as a consequence of mutation or when the organism is exposed to an inducing agent. Cephamycins (e.g. cefoxitin and cefotetan), ampicillin, and carbapenem are good inducer [4].

Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum cephalosporins (ESCs), such as cefotaxime (CTX), ceftriaxone, and ceftazidime (CAZ), and the monobactam aztreonam (ATM) [5]. Such enzymes are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and have been recently detected in *Pseudomonas aeruginosa* at low frequency [6-8]. These variants differ from their parent enzymes as they can hydrolyze broad spectrum b-lactam antibiotics such as penicillins and cephalosporins, including oxyimino β-lactams (cefotaxime, ceftazidime, and aztreonam). However, they do not hydrolyze cephamycins (cefoxitin) or carbapenems (imipenem or meropenem) [9]. Their activities are inhibited by

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clavulanic acid [10]. Plasmid mediated extended-spectrum enzymes were first reported in *Klebsiella pneumoniae* and later in almost all other *Enterobacteriaceae* and *P. aeruginosa.*

Emergence of MBL-mediated resistance is of serious concern. Carbapenems are effective therapeutic agents against highly resistant pathogens such as *P. aeruginosa* and Acinetobacter species. Spread of this resistance among these pathogens and transfer to other gram-negative bacteria would seriously restrict therapeutic options [11].

The presence of ESBLs and AmpC beta-lactamases in a single isolate reduces the effective-ness of the beta lactam/beta-lactamase inhibitor combinations, while MBLs and AmpC beta-lactamases confer resistance to carbapenems. Often, these enzymes are co-expressed in the same isolate.

Most of the methods aimed to detect ESBLs in Klebsiella and other *Enterobacteriacea* with little or no chromosomal β-lactamase activity, e.g., *E. coli* and *Proteus mirabilis* [12, 13]. Detection of ESBLs in species with inducible chromosomal β- lactamases, such as *Enterobacter* spp., proved a difficult task. *P. aeruginosa* presents further difficulties, because it not only has an inducible AmpC enzyme but also has a much greater degree of impermeability than *Enterobacteriaceae*, as well as efflux-mediated resistance [4, 6, 14, 15]. Our work aims to study the prevalence of Extended Spectrum β-lactamases (ESBL), AmpC β lactamases and Metallo β lactamase (MBL) enzymes, producing *Pseudomonas aeruginosa* strains isolated from different clinical samples, the antibiotic susceptibility profile of Extended Spectrum β-lactamase (ESBL), Amp C β-lactamase and Metallo β-lactamase (MBL) producing *Pseudomonas aeruginosa* and their plasmid profile.

MATERIALS AND METHODS

Isolation and Identification

Fifty eight *P. aeruginosa* strains were isolated from 330 clinical samples collected from patients of different infections (urinary tract infections, wound infections, respiratory tract infections and infections associated with burns) and identified according to standard microbiological procedures [16, 17].

Antibiotic Susceptibility Testing and MIC Determination

Antimicrobial susceptibility testing was performed using agar dilution method according to the clinical and laboratory standard institute (CLSI) 2011 [18]. The antibiotics included were ampicillin, amoxicillin, ciprofloxacin, gentamicin, amikacin, cefotaxime, carbenicillin, piperacillin amoxicillin/clavulenic acid, ampicillin/sulbactam, ceftazidime, meropenem, azetreonam and cefipime (Hi-Media, Mumbai). Preparation of stock solutions of the tested antibiotics was performed according to manufacturer's instructions.

Detection of Amp C β-Lactamases by Iodometric Method

The tested *Pseudomonas aeruginosa* isolates were tooth-picked onto the surface of nutrient agar plates. After overnight inoculation at 37°C, the plates were overlaid with 1% molten agarose containing 0.2% soluble starch and 1% penicillin. The plates were incubated for 15 minutes at room temperature to solidify; iodine solution was poured onto the agar plates. After 10 seconds the residual iodine solution was damped out and the plates were incubated at room temperature until discoloration zone appeared around β-lactamase producing colonies [19].

Detection of Extended Spectrum β-Lactamases (ESBLs) by Double-Disc Synergy Test

DDSTs were performed by placing disks of ceftazidime, cefotaxime, aztreonam, and cefepime (30 µg each) at a distance of 30 or 20 mm (center to center) from a disk containing AMC (amoxicillin, 20µg, and clavulanic acid 10µg) [14]. ESBL production was inferred when the cephalosporin zone was expanded by the clavulanate. Enhancement of zone of inhibition is indicative of presence of an ESBL.

Kirby-Bauer Disc Approximation to Detect Inducible β-Lactamase

This disc approximation technique [20] was used to detect inducible β-lactamase enzyme production. We used cefoxitin (FOX) β-lactamase inducer, and cefotaxime (CTX) and ceftazidime (CAZ) as the third generation cephalosporin reporter agents [21].

A cefoxitin producing no zones of inhibition was placed on a seeded plate at a distance from CTX and CAZ equivalent to the radius of the zone produced by theses discs when tested alone. Incubate for 24h at 37°C, then (a) the radius of the zones of inhibition between FOX disc and the tested discs were measured. In addition, (b) the radii on the far side of the tested discs were measured. Isolates were

considered positive for inducible β- lactamases when the radius of (a) was smaller than that of (b) by 4 mm or more.

Detection of Metallo-Beta-Lactamases (MBL) by Disc Potentiation Test

MBL producing *P. aeruginosa* was suspected when the isolate was resistant to meropenem and imipenem [22]. All meropenem resistant strains were screened for metallobetalactamase (MBL) production by disc potentiation test with EDTA impregnated imipenem and meropenem discs.

A 0.5 M EDTA solution (pH 8) was sterilized by autoclaving. Test organisms were inoculated onto plates of Muller Hinton agar. Two 10 µg imipenem discs and two 10 µg meropenem discs were placed on inoculated plates and 5 µl of EDTA solution was added to one imipenem and one meropenem discs (each alone). The zone of inhibition around imipenem and meropenem discs alone and those with EDTA was recorded and compared after 16-18 h incubation at

37°C. An increase in zone size of at least 7mm around the imipenem-EDTA disc and meropenem-EDTA discs were recorded as positive result [23].

Isolation and Analysis of Plasmid DNA Product

Centrifuge 2 ml of a culture and wash pellet in 2 ml of the following TE: 0.05 M Tris, pH 8.0, 0.01 M EDTA. Resuspend in 40 µl of the same TE buffer. Fill 0.6 ml of freshly prepared lysis buffer (TE buffer used above with 4% SDS, pH adjusted to 12.45) into Eppendorf tube and add the cell suspension to the lysis buffer, mix gently. Complete lysis by incubating at 37°C for 20-30 min. Add 30 µl of 2 M Tris, pH 7 for neutralization, mix gently. Add 0.24 ml of 5 M NaCl for precipitation of chromosomal DNA and incubate on ice for 4 hrs. Centrifuge for 10 min and transfer supernatant into new tube for ethanol precipitation. Plasmid DNA was separated by electrophoresis in 0.8% agarose gel (Sigma) in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]). The gel was stained with ethidium bromide, and plasmid bands were visualized with UV light [24].

Table 1:   Prevalence of *P. aeruginosa* Isolated from Different Types of Infections

Type of infection	No. of samples	No. of *Pseudomonas aeruginosa* isolates (%*)
Urinary tract infection	50	11 (22)
Respiratory tact infections	150	10 (6.6)
Wound infections	100	15 (15)
Burns	30	22 (73)
Total	330	58 (17.5)

*percents of isolates were correlated to the number of each type of collected samples.
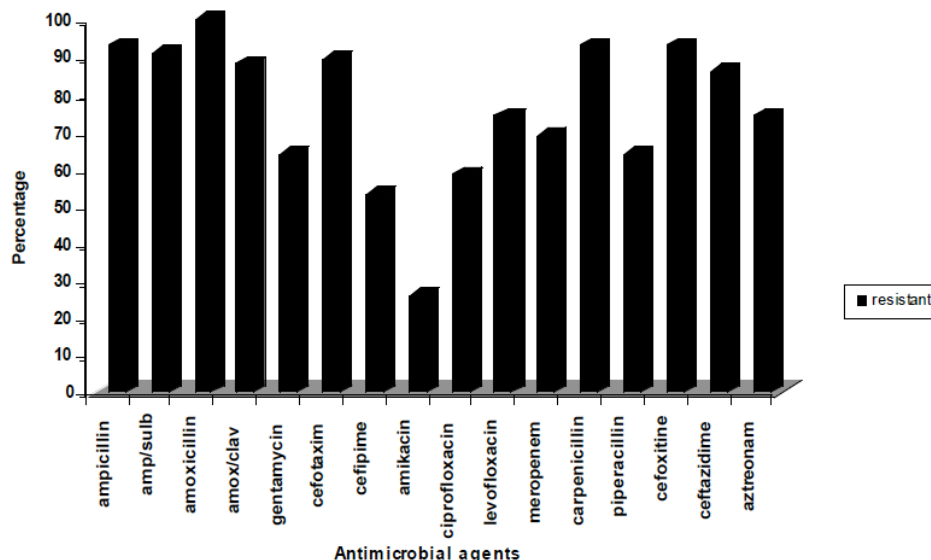

Figure 1: Resistance pattern of the isolated *P. aeruginosa* to different antimicrobial agents.

Curing of ESBL Enzyme Production

Extended spectrum β-lactamase producing strains showing plasmid bands were grown overnight in 5 ml L-B containing 0.25-16 µg/ml ethidium bromide and acridines. Subsequently, the cultures were diluted and spread on nutrient agar plates to give 100 colonies/plate. ESBL deficient organisms were then detected by DDST [25].

RESULTS

A total of 58 *P. aeruginosa* isolates were obtained from 330 samples collected from different infections, *P. aeruginosa* was mostly isolated from burn units followed by urinary tract infections (Table **1**). Antibiotic resistance pattern revealed that all *Pseudomonas aeruginosa* showed resistance to most of the tested antimicrobials. All isolates were completely resistant to amoxicillin and showed high resistance to the tested β-lactams and cephalosporins. Low resistance was shown against amikacin (25.8%) (Figure **1**).

All *P. aeruginosa* isolates were tested for AmpC β-lactamase production by iodometric method. It was found that 72.4% of *P. aeruginosa* were AmpC β-lactamase producers (Figure **2**), *P. aeruginosa* isolates showed no inducible AmpC β-lactamase production. DDST revealed that ESBL production was found in 27.5% of *P. aeruginosa* isolates. All ESBL producing strains were AmpC β-lactamase positive. Thirty-one isolates were MBL producers while 25 (43.1%) of them were AmpC β-lactamase positive (Table **2**).

It was found that 75.8% of *P. aeruginosa* isolates were multi-drug resistant (Table **3**). Most of multi-drug resistant isolates were obtained from wound infections as all wound samples were multidrug resistant followed by burns (90.9% of burn samples). ESBLs and MBL production was mostly observed among strains isolated from burn units.

Antibiotic resistance pattern of ESBLs producers revealed that all tested ESBLs producing isolates were completely resistant to all tested antibiotics except gentamicin (68.7%) and amikacin (62.5%) (Table **4**).
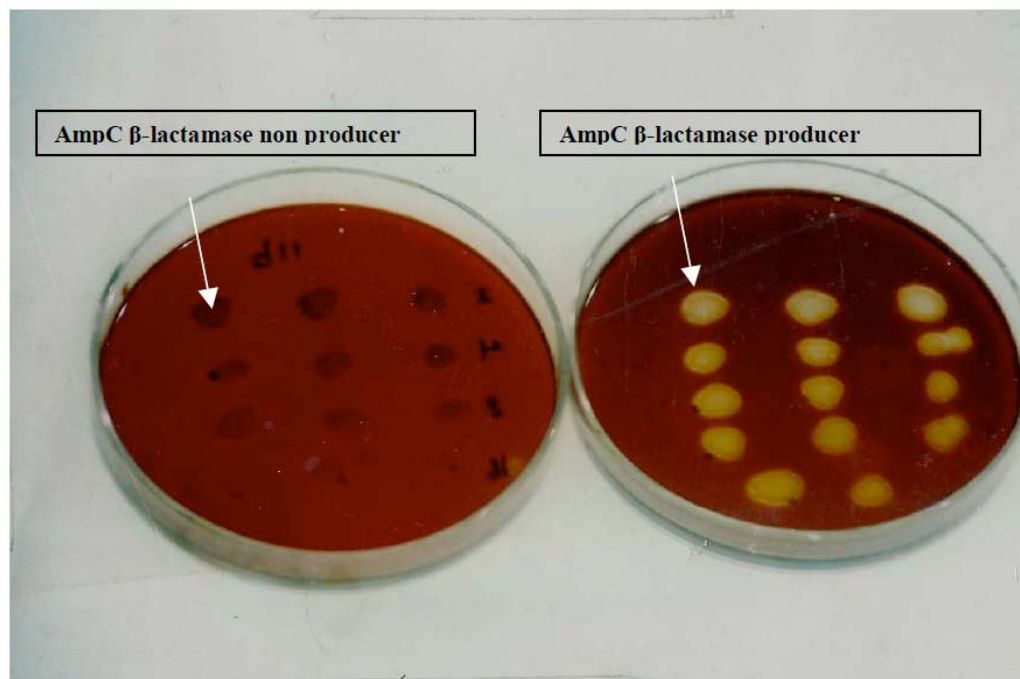

Figure 2: AmpC β-lactamase production by *P. aeruginosa* isolates.

Table 2:   Distribution of AmpC β-Lactamase, ESBLs and MBL Production Among the Isolated *P. aeruginosa* Isolates

Total number of isolates	No. of isolates (%)				
	AmpC β-lactamase Producers	ESBLs producers	AmpC+ ESBLs	MBL producers	MBL+ AmpC
58	42 (72.4)	16 (27.5)	16 (27.5)	31 (53.4)	25 (43.1)

Table 3:   Distribution of Multidrug Resistant (MDR), ESBLs and MBL Producing Isolates in Clinical Specimens

Specimen	*P. aeruginosa*	No. of isolates (%)*		
		MDR isolates	**ESBL producing strains**	**MBL producing strains**
Urine	11	2 (18.8)	1 (9)	1 (27.27)
Sputum	10	7 (70)	0 (0)	4 (45.4)
Wound	15	15 (100)	5 (33.33)	10 (80)
Burn	22	20 (90.9)	10 (45.5)	16 (90.9)
Total	58	44/58 (75.8)	16/58 (27.4)	31/58 (53.4)

*percents of isolates were correlated to the number of each type of collected samples.

Table 4:   Antibiotic Resistance Pattern of ESBL and MBL Producing *Pseudomonas aeruginosa*

	No. of resistant isolates (%)									
	AMP/SULB	**AMC**	**GEN**	**AMK**	**CIP**	**LEV**	**MEM**	**CARB**	**PRL**	**AZT**
ESBL producers n= 16	16 (100)	16 (100)	11 (68.7)	10 (62.5)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	13 (81.25)
MBL producers n = 31	26 (83.8)	27 (87.09)	27 (87.09)	17 (54.8)	26 (83.8)	26 (83.8)	31 (100)	31 (100)	25 (80.6)	28 (90.3)

AMP/SULB: Ampicillin/sulbactam, AMC: Amoxicillin/clavulenic, GEN: gentamicin, AMK: Amikacin, CIP: Ciprofloxacin, LEV: Levofloxacin, MEM: Meropenem, CARB: Carbenicillin, PRL: Piperacillin, AZT: Aztreonam.
ESBL: Extended spectrum β-lactamase producing strains, MBL: metallo-β lactamase producing strains.

MBL producers showed high resistance to all tested antibiotics but moderate resistance to amikacin (54.8%).

On analysis of plasmid profile of ESBLs producing strains, It was found that out of 16 ESBLs producers, 9 strains harbored plasmid (Figure **3**). Furthermore, Plasmid mediated ESBL cured cells showed no enzyme activity which proved that ESBL enzymes were plasmid mediated in these strains. The Antibiogram of these 9 strains revealed that 3 of them were resistant to all antibiotics except Amikacin (2 strains from wound and one strain isolated from burn) while the other 6 strains were completely resistant to all tested antibiotics (Table **5**).
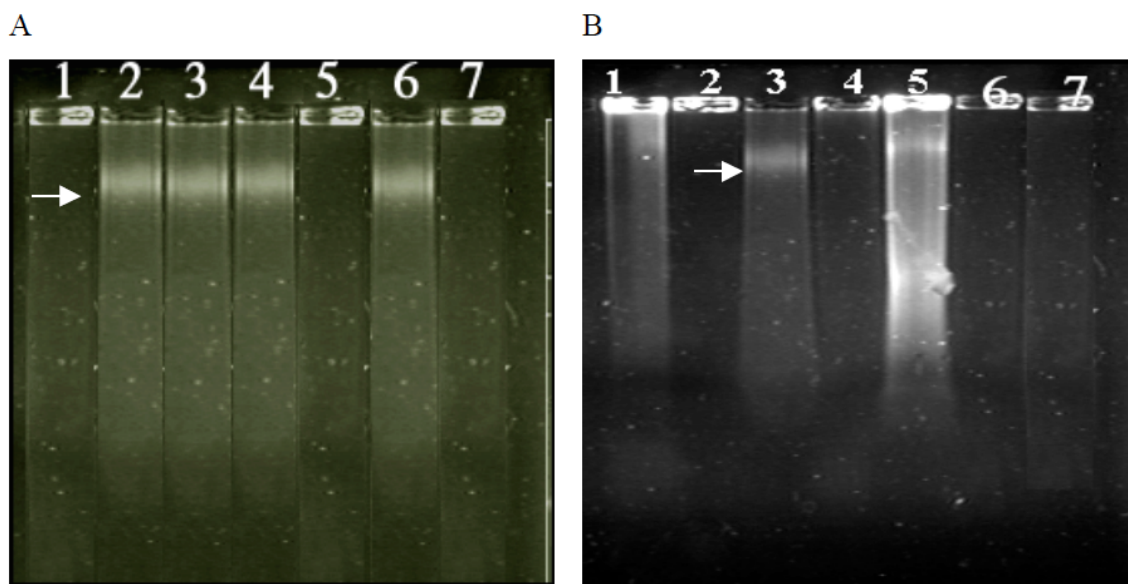

Figure 3: Plasmid profile for ESBLs producing *P. aeruginosa*. **A**: Lane 1, 5, 7: negative for plasmid. Lane 2, 3, 4 & 6 positive for plasmid bands. **B**: Lane 1, 2, 4, 6 & 7 show no plasmid. Lane 3 & 5: positive for plasmids.

Table 5: **Antibiogram of Plasmid Mediated ESBLs Producing Strains**

Strains	No. of isolates	specimen	Susceptible	Resistant
1	2	Wound	Amikacin	Ampicillin, ampicillin/sulbactam, cefotaxim, cefipime, amikacin, ciprofloxacin, levofloxacin, meropenem, carbenicillin, piperacillin, cefoxitin, ceftazidime, Gentamicin, cefipime, azetronam
2	1	Burn	Amikacin	Ampicillin, ampicillin/sulbactam, cefotaxim, cefipime, amikacin, ciprofloxacin, levofloxacin, meropenem, carbenicillin, piperacillin, cefoxitin, ceftazidime, Gentamicin, cefipime, azetronam
3	5	Burn	-	Ampicillin, ampicillin/sulbactam, cefotaxim, cefipime, amikacin, ciprofloxacin, levofloxacin, meropenem, carbenicillin, piperacillin, cefoxitin, ceftazidime, Gentamicin, cefipime, azetronam and amikacin
4	1	Wound	-	Ampicillin, ampicillin/sulbactam, cefotaxim, cefipime, amikacin, ciprofloxacin, levofloxacin, meropenem, carbenicillin, piperacillin, cefoxitin, ceftazidime, Gentamicin, cefipime, azetronam and amikacin

DISCUSSION

Pseudomonas aeruginosa is one of the most important microorganisms which causes problems clinically as a result of its high resistance to antimicrobial agents and is therefore a particularly dangerous bug. Despite the discovery of ESBL, Amp C β-lactamases and MBL at least a decade ago, there remains a low level of awareness of their importance and many clinical laboratories have problems in detecting ESBL & Amp C β-lactamases. Failure to detect these enzymes has contributed to their uncontrolled spread and commonly to therapeutic failures.

Problems arise especially with organisms that produce an inducible Amp C β- lactamases, as clavulanate can induce high level production of Amp C β- lactamases, which may obscure recognition of ESBLs [4]. According to Clinical & Laboratory Standards (CLSI) interpretive definitions, ESBLs do not always increase MICs to levels characterized as resistant [26]. Not only that ESBL producing organisms may give false sensitive zones in routine disk diffusion test. The number of infections caused by extended spectrum β-lactamases producing *P. aeruginosa* is on rise and poses a threat to patients due to therapeutic failure if they remain undetected [27]. Metallo-beta lactamase (MBL) producing *P. aeruginosa* is an emerging threat and a cause of concern for treating physicians as it can hydrolyze carbapenems which are given as a last resort to the patient having infection with ESBL and AmpC β-lactamase producing *P. aeruginosa*. The MBLs have become more notorious as therapeutically available inhibitors are not available and for their potential for rapid and generalized dissemination to different other Gram negative bacilli.

Hence, accurate identification of MBL producing strains are very urgently needed. Though PCR gives specific and accurate results, it's use is limited to few laboratories because of it's high cost and different types of ESBLs, AmpCβ-lactamases and metallo-betalactamases (MBLs) present worldwide [4].

Our study showed that *P. aeruginosa* was mostly isolated from burn units, followed by urinary tract infections that agree with results obtained by Mahmoud *et al.* [28]. It was found also that 72.4% of *P. aeruginosa* were positive for AmpC β-lactamase and 27.5% were positive for ESBLs which were close to results obtained by Okesola and Oni [29] and Aggarwal *et al.* [30] while higher rates was shown by Ali *et al.* [31] and Anjum and Mir, [32]. In this study there is no inducible ESBLs and all ESBLs producers were AmpC β-lactamase positive. Livermore and Woodford [33] and Upadhyay *et al.* [34] reported that AmpC β-lactamase production was shown by 59.4% of *P. aeruginosa* isolates and the co-existence of AmpC β-lactamase and ESBLs was reported in 3.3%. Also 7% of isolates were positive for inducible ESBLs that disagree with our results. A high burden of coexisting different beta-lactamase enzymes (i.e., AmpC -32.7%, AmpC + ESBL - 24.5% and AmpC + MBL - 48.5%) in clinical isolates of *P. aeruginosa*, were reported by Kumar *et al.*, [35] which represent a serious therapeutic challenge for clinicians caring for burn patients. Similar results were shown with our study (AmpC -72.4%, AmpC + ESBL – 27.5% and AmpC + MBL – 43.1%) that indicate the needs to apply strict infection control practices as physical isolation in a private room, use of gowns and gloves during patient contact and hand washing before and after each patient visit, appropriate empirical antimicrobial therapy and early detection of these β-lactamase-producing isolates could help to reduce the burden of infections. Our study revealed

that no single antibiotic showed 100% sensitivity to all *P. aeruginosa* strains but multi-drug resistance was shown by most of *P. aeruginosa* isolates that was documented by many researchers [28, 36, 37]. In various studies across the world, varying resistance (4-60%) has been seen towards meropenem and imipenem [38, 39]. We found 68.9% resistance to meropenem that higher than results obtained by Varaiya *et al.*, [22]. Our study showed low resistance against amikacin, similar to results obtained by Mahmoud *et al.* [28], moderate resistance was shown against cefipime, gentamicin and ciprofloxacin, high resistance was observed against antipseudomonal antibiotics and meropenem while lower degrees of carbapenem resistance was noted by Jaykumar [40] and Varaiya *et al.* [22]. Results showed that ESBLs producing organisms were completely resistant to all tested antimicrobials but low activity was shown by amikacin and gentamicin. This suggests a possible existence of co-resistance to quinolones and aminoglycosides on the gene responsible for ESBLs production [41]. In our study, 9 (56.25%) of the 16 ESBLs isolates harbor plasmids that was close to results obtained by Mahmoud *et al.* [28]. The remaining 7 isolates were plasmidless and β-lactamase production in these strains could be chromosomally mediated. The presence of plasmids in different types of strains indicated that horizontal gene spread might be responsible for the high frequency of ESBLs detected in *P. aeruginosa* in this study [42].

The present study emphasizes the high prevalence of multidrug resistant *Pseudomonas aeruginosa* producing β-lactamase enzymes of diverse mechanisms. To combat these problems, epidemiological studies should be undertaken in hospital settings to monitor the source of infection. Early detection of these β-lactamase producing isolates in a routine laboratory could help to avoid treatment failure, as often the isolates producing this enzyme show a susceptible phenotype in routine susceptibility testing. Furthermore, strict antibiotic policies and measures to limit the indiscriminative use of cephalosporins and carbapenems in the hospital environment should be undertaken to minimize the emergence of this multiple β-lactamase producing pathogen whose spread would leave no other option to treat Gram-negative nosocomial infections.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

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