Phenotypic detection of AmpC β-lactamase in *Pseudomonas aerogenosa* isolated from Burns and Wounds in Al-Rumetha Hospitals

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Abstract

Ampicillin hydrolyzing class C β-lactamase (AmpC) producing Pseudomonas aeruginosa has been recognized to be a serious opportunistic infection and due to the wide ability to create β-lactamases made this bacteria resistance to many antibiotics.The results of the current study showed that 36(27.7%) isolates of P. aeruginosa from 130 isolates examined for cefoxitin susceptibility by disk diffusion method. Out of thirty six P. aeruginosa isolates , 29(80.6%) were cefoxitin resistant . The modified three dimension test and AmpC disck test were used confirm the production AmpC β- lactamase in all cefoxitin resistant isolates , only 23(79.3%) isolates were phenotypically confirmed to produce AmpC beta-lactamase. The result of this study showed that 12/36 (33.3%) isolates were positive with blaAmpC gene by conventional PCR technique .

Keywords: *Pseudomonas aeruginosa, AmpC, MTDT, Cefoxitin*

Introduction

Pseudomonas aeruginosa is a responsible for opportunistic diseases which commonly causes infections in burned patients and is mostly resistant to varied antimicrobial agents, also this microorganism is leading to nosocomial infection among immunocompromised patients suffering from burn , cystic fibrosis, AIDS and cancer [1,2] . Commonly *P. aerugionosa* infections have been treated by fluoroquinolones, carbapenems, aminoglycosides and cephalosporins antibiotics . Nevertheless, resistance rate of *P. aeruginosa* to beta lactams , aminoglycosides , quinolones and carbapenems has been recorded from several countries [3] . *Pseudomonas aeruginosa* producing of AmpC enzyme pose a threat to public health and demands the need to detect by phenotypic methods, the occurrence of AmpC β-lactamases from various clinical and environmental isolates due to the fact that antibiotic resistance is an growing problem in hospitals in a worldwide . cephalosporinases such as AmpC β-lactamases are clinically important encoded on the chromosomes of the *Enterobacteriace*ae and a limited other organisms such as *Pseudomonas aeruginosa* where they responsible of resistance to penicillins, cefazolin, cephalothin, cefoxitin, and β-lactamase inhibitor-β-lactam combinations [4]. Resistance to multiple drugs in *Pseudomonas aeruginosa* is commonly the result of mixture of many mechanisms such as metallo β-lactamases (MBLs), AmpC β -lactamases, extended spectrum β -lactamases (ESBLs) , excessive expression of efflux pump and modifications of active site or outer membrane [5] . Hydrolytic enzymes such as AmpC β –lactamases cleave the β-lactam ring and confer bacterial resistance to to beta –lactam antibiotics [6]. MBLs and AmpC beta lactamases classified by Ambler as class B and class C respectively [7]. Biotyping and serotyping are phenotypic method while plasmid profile analysis and PCR are molecular methods used for diagnostic purpose [8]. Genes responsible for the beta-lactamase production in *P. aeruginosa* are typically resides either on plasmids or on the chromosome with the possible to exchange between bacterial populations [9].

Materials and Method

Sample Collection

A total of 130 clinical samples of *P. aeruginosa* from Al-Rumetha hospitals in Al-Muthanna province during the period of five months. Out of these , 60 wound and 70 burn swabs . Burn swabs were taken from the center of burn and wound lesion , depending on Collee *et al.* [11] a dry swab must first be soaked with a little amount of Brain-Heart Infusion broth , then organisms

were diagnosed using standard biochemical tests such as ability to produce oxidase , pigment formation, Gram staining , oxidation of glucose , growth at 42°C , gas and acid production on triple sugar iron agar as well as the samples were directly inoculated in CHROM agar™ Pseudomonas , blood agar , MacConkey agar , Nutrient agar and incubated for 24hr and at 37^0C under aerobic conditions .

Antibiogram:

Antibiotic susceptibility of the all isolates were determined by Kirby-Bauer disk diffusion method according to CLSI recommendations [12]. The antibiotics used comprise disks (Himedia, India): Ceftazidime (CEF, 30 ug), Imipenem (IMP, 10 ug), Meropenem (MEM, 10 ug), Cefotaxime (CTX, 30 ug), Amoxicillin/ Clavulanic acid (AMC, 20/10 ug),Amikacin (AK, 10 ug), Erythromycin (E, 10 ug), Piperacillin (PI, 10 ug), Chloramphenicol (CHL, 30 ug), Cefoxitin (30 µg), Aztreonam (ATM, 10 ug), Rifampicin (R, 5 ug), Ciprofloxacin (5 µg), Tetracycline (30 µg). Moreover, different class of antimicrobials used by VITEK2 automatic system for confirmatory susceptibility tests of isolates [13].

Detection of AmpC β -lactamases

The ability of *Pseudomonas aeruginosa* isolates to produce AmpC enzyme was screened by testing cefoxitin susceptibility . The isolates that exposed resistance to the antimicrobial activity of cefoxitin were supposed initially to produce AmpC enzyme when their inhibition zone diameter ≤ 18 mm; and this acceptable phenotypic confirmation [14].

Modified three dimensional test (MTDT) :

According to Manchanda and Singh [15] and Parveen *et al.* [16] This test was accomplished .

AmpC Disk Test:

This test also carried out as recommended by Parveen *et al.* [16] Basak *et al.* [17] .

Molecular detection of AmpC –lactamase :

DNA extraction

The method of extraction Plasmid DNA was done by using the genomic DNA Mini kit, based on the manufacturer's instructions (Geneaid, korea).

Determination of DNA product and purity :

The use of nanodrop spectrophotometer for purpose of genomic DNA checking by measurement of the concentration and purity of extracted genomic DNA via evaluation the absorbance at (260 /280 nm) [18].

Preparing the Primers suspension :

The stock solution is prepared according to the manufacturer's instructions in leaflet through dissolving lyophilized primers after Spinning down with TE buffer (Promega, USA) , Whereas the working solution of the primer was also prepared according to the manufacturer's instructions, these primers were synthesized and supplied by (Biocorp, Canada).

Amplification :

The whole volume of the PCR mixture set up in 20 µl includes 5 µl of lyophilized AccuPower® PCR PreMix (Bioneer, korea), 10 pico/ µl of each specific primer for target gene and 5µl of P. aeruginosa DNA template , so as according to presented in (table1)the amplification conditions started with thermocycler program .

Table 1. The amplification conditions of PCR thermocycler for blaAmpC gene .

Steps	Temperature oC	Time	Cycle number
Initial Denaturation	94	30sec	35
Denaturation	94	30 sec	
Annealing	60	1min	
Extension	72	1 min	
Final extension	72	10min	

Results

This study showed that out of the One hundred and thirty samples were taken from Al-Rumetha hospitals , 36 (27.7%) P. aeruginosa isolates were diagnosed , The frequency of P. aeruginosa isolates from the wound and burn swab samples presented in (Fig.1). The highest P. aeruginosa percentage occurred in the burn 23(32.9%) followed by wound infections 13 (21.7%)($p \leq 0.05$) .

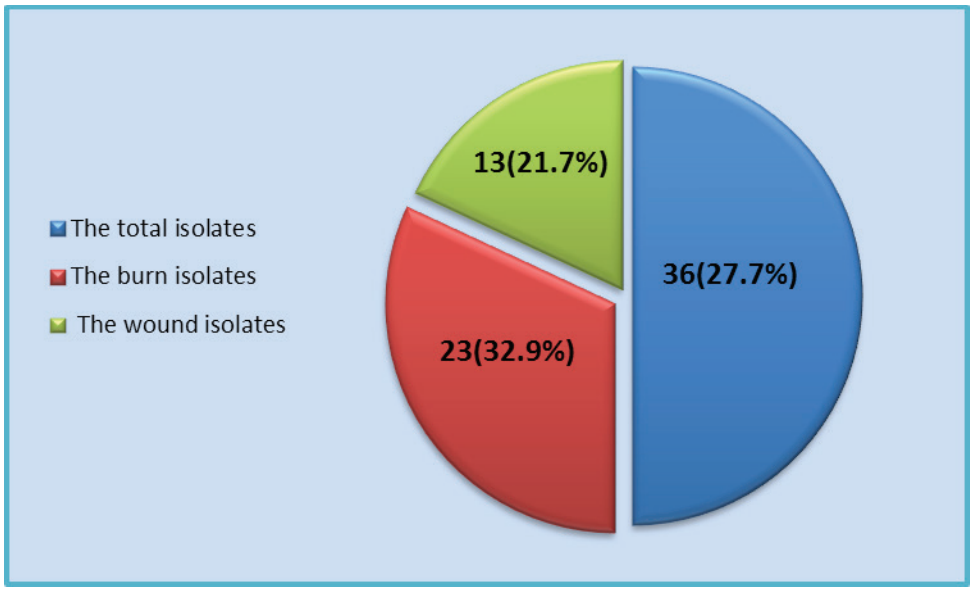


Fig (1): Frequency of P. aeruginosa according to infection sites on chrome agar.

All the P.aeruginosa isolates were tested for various antibacterial agents to determine the resistant rate which include (Ceftazidime , Imipenem , Meropenem , Cefotaxime , Amoxicillin/ Clavulanic acid ,Amikacin , Erythromycin , Piperacillin , Chloramphenicol , Cefoxitin , Aztreonam , Rifampicin , Ciprofloxacin and Tetracycline) . Fig. (2) show that the isolates were diverse in susceptibility to antimicrobial agents (Chloramphenicol 100%, Piperacillin 100%, Rifampicin 94.4% Erythromycin 94.4% ,Ceftazidime 94.4% , Cefotaxime 94.4% , Cefoxitin 80.6% , Amoxicillin/ Clavulanic acid 75% , Aztreonam 61.1% Ciprofloxacin 55.6% , Amikacin 47.2% , Tetracycline 41.7 % Meropenem 27.8%, Imipenem 19.4%) .The isolates that resist three classes of antibiotics or more are considered multidrug resistant .

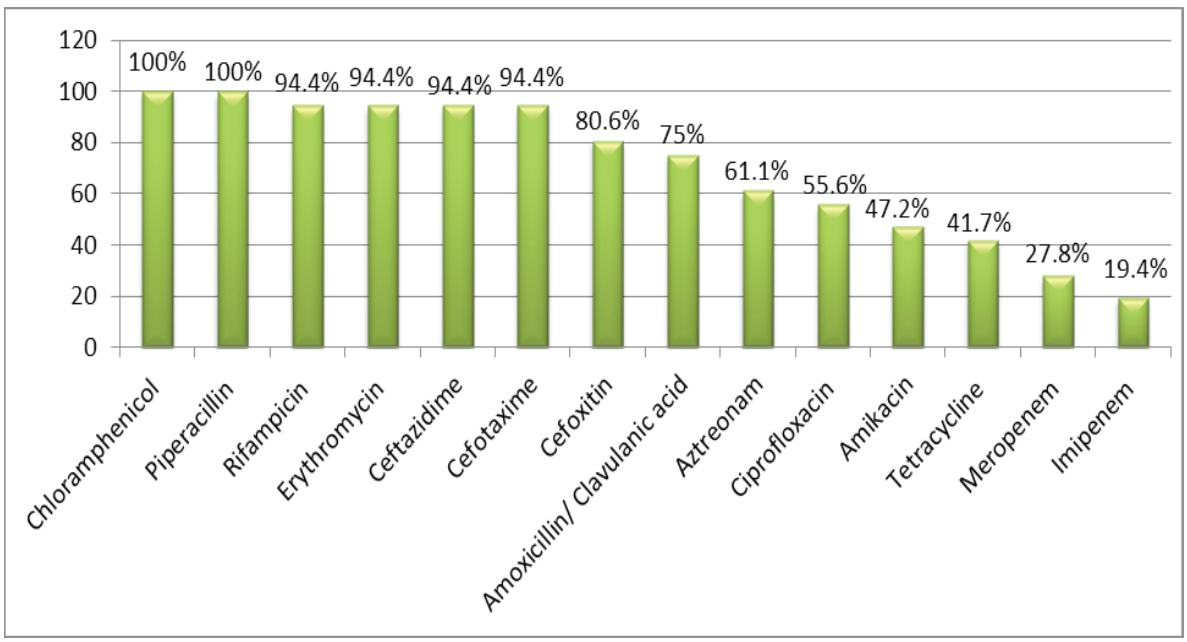


Fig. (2) : Antibiotics susceptibility profile for P. aeruginosa isolates (number=36).

Two methods were used to check of production AmpC β- lactamase among cefoxitin resistance P. aeruginosa isolates , of the 36 P. aeruginosa isolates , 29(80.6%) were cefoxitin resistant by the disk diffusion method and initially regarded as AmpC β-lactamase producers Fig.3.

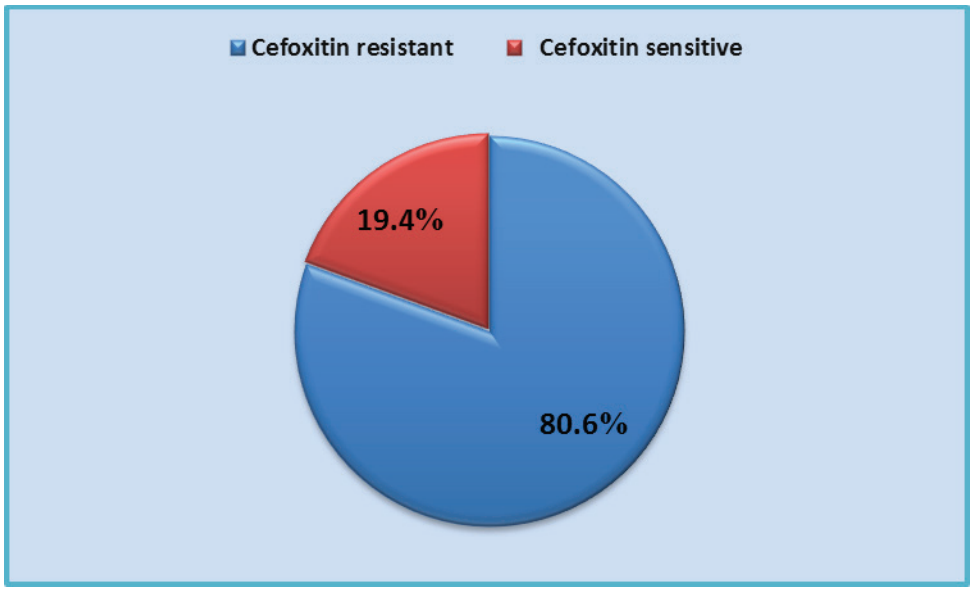


Fig (3) : Cefoxitin susceptibility of β-lactam resistant P. aeruginosa isolates (n=36).

The modified three dimension test was also used to confirm the production AmpC β- lactamase in all cefoxitin resistant isolates , only 23(79.3%) isolates were phenotypically confirmed to produce AmpC beta-lactamase. However, there is another method used to detect production AmpC β- lactamase among cefoxitin resistance P. aeruginosa isolates , this test is AmpC disk test and 23(79.3%) out of the 36 P. aeruginosa isolates were positive for AmpC beta-lactamase as demonstrated in(Table 2) .

Table (2): AmpC β-lactamase production among P. aeruginosa isolates .

Cefoxitin susceptibility of isolates	No. (%) of phenotypic AmpC β-lactamase producer isolates		No. (%) of AmpC- negative isolates
	MTDT	AmpC Disk Test	
Resistant(n=29)	23(79.3%)	23(79.3%)	6(20.7%)
Susceptible (n=7)	0(0%)	0(0%)	0(100%)
Total (n=36)	23(63.9%)	23(63.9%)	6(16.7%)
L.S.D. 0.05 of isolate susceptibility =2.987 methods= 1.547 Interaction=3.656			

Molecular detection of AmpC β- lactamase production among the P. aeruginosa isolates 36(27.7%) with blaAmpC gene by PCR technique . This result showed that 12/36 (33.3%) isolates were positive with blaAmpC gene as presented in Fig. 4 and 5.

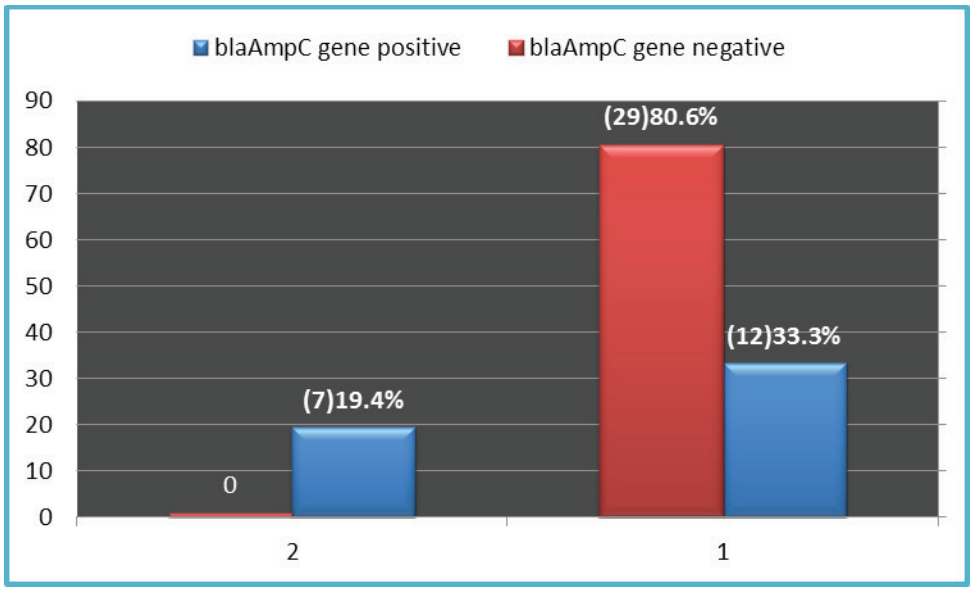
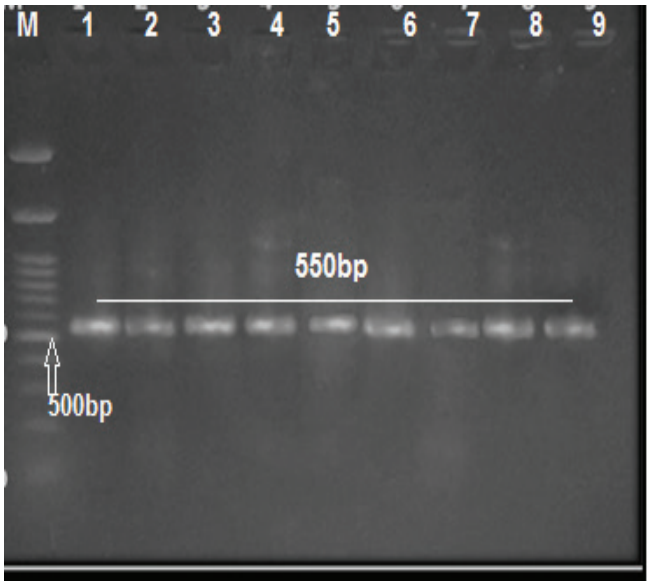


Fig (4): Occurrence of blaAmpC gene among β-lactam resistant P. aeruginosa isolates (n=36).



Y_2O_3 nanofill-erssrs

Fig (5): Agarose gel stained with ethidium bromide to investigate of AmpC β-lactamase gene among P. aerogenosa isolates by PCR technique, Lane (M), 1500 bp DNA marker , Lanes (1, 2,3,4,5,6,7,8 and 9) P. aeruginosa showing (550 bp) blaAmpC gene .

Discussion

The present study showed that the highest rate of *P. aeruginosa* occurred in the burn infections23(32.9%) followed by wound infections 13 (21.7%)($p \leq 0.05$) .In Al-Muthanna , AL-Aaajipi [19] found that the P. aeruginosa prevalence rate was (44.64%) from the burn which is higher than our study . The current results are agreement with Azeez and Bakr [20] who found the highest P. aeruginosa prevalence rate occurred in the burn(40%) followed by wound infections (17.5%) . As well as, Nasih et al. [2014] recorded (17%) P. aeruginosa from the patients. Another study showed that (38%) P. aeruginosa isolates among 100 burn samples in Baghdad hospitals [21]. P. aeruginosa is currently one of the major bacterial pathogens responsible for nosocomial infections and due to resistance to various antibiotics the treatment of these infections has become very difficult. The results described in (Fig.2) demonstrates that the isolates were (100%) resistant to Chloramphenicol and Piperacillin respectively , similar studies were performed by other researcher Azeez and Bakr [20] showed that among 50

isolates of P. aeruginosa, 100% resist to Penicillin, Lincomycin, Vancomycin, Piperacillin, Rifampicin, and Chloramphenicol,as well as Ali 2016 [23] illustrated that out of the 60 P. aeruginosa isolates , 30% resist to fourteen various antibacterial agents but all isolates were resistant to G, L, CEF, ATM, PI, and CTX . The reasons of wide range of resistance is dependent upon excessive use or misuse of antibiotics , hospital setting, transmission of resistance genes among bacterial species [24,25]. In this study, 94.4% of the isolates were resistant to Rifampicin , Erythromycin ,Ceftazidime and Cefotaxime ,also 80.6% to cefoxitin , this resistance is due to the presence of other mechanisms such as AmpC type β-lactamases or MexAB-OprM Efflux Pumps[26-27]. This resembles with the results of Chika et. al. [28] who reported P. aeruginosa isolates were resistant to Ceftazidime (92%) and cefoxitin (80%). Furthermore, the percentage of the susceptibility to Meropenem was 27.8% and Imipenem 19.4% therefore considered most effective antibiotics , this is agreed with Azeez and Bakr [20] who found the lowest resistance ratio was for Imipenem 4% and Meropenem 20% . Therefore due to the sensitivity of the bacterial isolates to imipenem and meropenem, making them the best therapeutic option. This sensitivity is due to the patients infected with bacterial isolates producing AmpC beta-lactamases used imipenem as last option [29, 30]. But this results mismatched with Salimi and Eftekhar,2013 [31] who showed P. aeruginosa was 93.20% resistant to Imipenem and 94.17% to Meropenem .The production of AmpC beta-lactamase is one among numerous resistance mechanisms discovered in P. aeruginosa from both the community and hospital environment is fast becoming a international risk and the appearance and development of these multidrug resistant organisms is a warning to existing drugs[32, 33]. when antibiotics are over prescribed so as when people lack of interest in personal hygiene this leads to resistance to antibiotics . The primary screening test of the molecular class C β- lactamases (AmpC) used in the laboratory is modified three-dimensional test , this results revealed 23(79.3%) isolates were phenotypically confirmed to produce AmpC beta-lactamase in this test , all the isolates were resistant to Cefoxitin while the remaining 6(20.7%) cefoxitin resistant isolates were negative for molecular class C β- lactamase due to the redection of antibiotic penetration across porins or these isolates probably have genes for plasmid-mediated and/ or chromosomal AmpC beta-lactamase production but silent genes or not encoded in these isolates [30] .

Conclusion

The best way to diagnose AmpC β- lactamase is PCR technique , The current study showed high rates of resistance to Phenicols, Penicillins, Ansamycins, Macrolides, Cephalosporin III and Cephamycin in P. aeruginosa isolated from patient in Al-Rumetha Hospitals, Iraq , which could help many doctors prescribe the best antibiotics for treatment of P. aerogenosa infections. Furthermore, the current study also showed most of these isolates had ampC genes and there was important correlation between resistant to β-lactam antibiotics and existence of ampC gene.

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Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under Biology Dep./ Education for Pure Science College/Al-Muthanna University and all experiments were carried out in accordance with approved guidelines.

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