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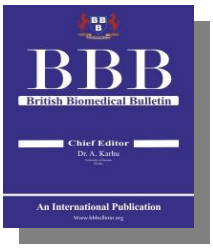

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Genotypic Detection of Cefepime Resistance in Iraqi Clinical Isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Study background and aims: *Pseudomonas aeruginosa* is opportunistic pathogen commonly implicated in serious nosocomial infections .It resisted many Antibiotics including β-lactams group. The aims of this study were to detect ESBLs production in clinically isolated *P. aeruginosa* and the prevalence of Cefepime resistance gene using PCR technique.

Material and method: Eighteen isolates of *P. aeruginosa* were isolated from patients suffering various infections. They diagnosed using Api 20E Kit followed by genotypic detection using a housekeeping gene (*rpsl*) by PCR. Rapid ESBLs detection kit was used to detect four types of β-lactamases including: preliminary screening for β-lactemase, ESβLs type, Metallo-β- lactamases (MβL) and AmpC enzyme. *BlaCTX-M and* bla_{CMY} genes responsible for 3rd and 4th generation cephalosprine (cefepime) resistance respectively were screened by PCR.

Results: Out of 18 isolates,15(83.3%) were positive in Preliminary screening of β-lactamases, ESβLs type were detected in 13 isolates (72.2%), the rate of MβL producers were 9(50%) and 7 isolates (38.8%) were AmpC producers. Detection of *blaCTX-M* gene revealed that 13\18 isolates (72.2%) harbored this gene, while prevalence of *blaCMY* gene was 3\18 (16.6%).

Conclusion: Most of the isolates were able to produce more than one type β-lactamases and 3rd generation resistance is more predominant as compared with 4th generation resistance.

Introduction

Pseudomonas aeruginosa is a pathogen commonly implicated in serious nosocomial infections such as pneumonia, cystic fibrosis, urinary tract infection, and sepsis[1]. T is also notably resistant to many classes of antimicrobial agents including β-lactams, aminoglycosides and fluoroquinolones[1]. The development of β-lactams resistance in this opportunistic pathogen can be caused by several mechanisms including: genetic mutations that lead to stable over expression of AmpC, a chromosome-mediated cephalosporinase; acquisition of transferable genes that code for a variety of ß-lactamases; overproduction of efflux systems; and reduced permeability[2]. ESβLs are a rapidly growing group of ß-lactamases that hydrolyze broad-spectrum cephalosporins as well as aztreonam. ESBL expression also confers penicillin and narrow-spectrum cephalosporin resistance. ESBLs are inhibited by β-lactamases inhibitors such as clavulanic acid[2]. More than 200 different ESBLs have been identified in Gram negative bacilli. The fourth generation represents an attempt to maintain good activity against Gram positive as well as Gram negative organisms including *P. aeruginosa*[3]. They also have a greater resistance to β-lactamases resistance than the third generation Cephalosporins and many can cross the brain barrier and are effective in meningitis. Cefepime is considered a fourth generation Cephalosporins[3]. This antibiotic retains its activity against some opportunist Gram negative bacilli that develop resistance to Cefotaxime and its relatives[4]. It has good activity against Enterobacteriaceae, *Staphylococcus aureus* and *Streptococcus pnemoniae*, highly active against *Haemophilus* and *Neisseria*[5] and with enhanced activity against *Enterobacter* and *Citobacter* that are resistance to third generation Cephalosporins[6]. Cefpirome is the other product in this generation with improved activity against Gram positive and negative bacteria[7]. The aims of this study were to detect ESBLs production in some clinically isolated *P. aeruginosa* and to detect the prevalence of Cefepime resistance gene using PCR technique.

Materials and Methods

Collection and diagnosis of Bacterial isolates

This research was performed in the molecular biology lab for higher studies in the department of biology, college of science, Al-mustansiriya university/Iraq. Eighteen isolates of *P. aeruginosa* were isolated from patients submitted to Baghdad teaching hospitals during the period between July 2012 till December 2012. The isolate were obtained from midstream urine from patients suffering from urinary tract infections, bacteraemia, eye infections, and otitis media (4 isolates for each) and 2 isolates from wound infections. Bacterial diagnosis including morphological and biochemical tests were done according to Atlas *et al.*[8] followed by complementary API 20E test.

Genotyping detection for isolates

Rpsl gene (a house keeping gene with accession no. CP006985.1) was used for genotypic diagnosis. Specific primers listed in table (1) were employed and the amplified size was 201 bp. Template DNA was prepared by boiling[9]. Briefly, few isolated colonies of overnight growth bacteria were suspended thoroughly in 1 mL distilled water and boiled in a water bath for 10 min. After centrifugation, supernatant was used as template DNA.PCR mixture f composed from 12.5 of GoTaq ® Green Master Mix (2x), 5 µl template DNA, 1.5 µl primers (for each) final concentration (0.6pmol/µl), and nuclease free water up to 25 µl (4.5 µl). PCR was run under the following conditions starting with a primary

denaturation step at 95°C for 5 min then 30 repeated cycles started with a denaturation step at 94°C for 30sec, then annealing at 57°C for 30sec, and 1 min at 72°C as extension step followed by final extension step at 72°C for 7 min[10].

Rapid Detection of ESβL Production via ESβL Strips

The detection of β-lactamases production was performed using Rapid ESβL detection kit (Mast group.UK). This kit includes four tests: Preliminary screening kit, Metallo β lactamases, ESβLs confirmation and AmpC detection. The test was performed according to the procedure suggested by the manufacturing company. Briefly, one drop of test substrate (approximately 20µl) was dispensed immediately on the filter pad of the strip before testing. Using a loop, several identical colonies were picked up and spread on the filter pad of the test strip .Any change in color (from yellow to red) observed around the streaked line was considered a positive result. The tested strips were observed after 2 to 15 minutes at room temperature, and the result was read after 15 minutes.

PCR Amplification for blaCTX and blaCMY genes

ß-lactamases producers were subjected to molecular screening study using PCR amplification technique.PCR mixture was composed from 5µl template DNA prepared by boiling method as described above, 12.5 µl of GoTaq ® Green Master Mix (2x), 1.5 µl from forward and reverse primers (final concentration 1pmol \ µl) for each gene, then the volume was complete to 25 µl using nuclease free water.PCR was run under the following conditions: for bla_{CTX-M} gene were (according to the current study): 95°Cfor 5 min; 30 repeated cycles of 94°C for 30sec, 55°C for 30sec and 72°C for 1 min then final extension step at 72 °C for 6 min. for bla_{CMY} gene(according to 9) primary denaturation step at 95°C for 5 min; 30 repeated cycles of denaturation step at 94°C for 45sec, annealing at 50°C for 45sec, and 1 min at 72°C as extension step followed by final extension step at 72°C for 7 min. PCR products were electrophoresed in 1% agarose gel and visualized under UV light[11].

Results

In this study, 18 isolates of *P. aeruginosa* were collected from different clinical samples including UTI, septicemia, otitis media and wound infection. Figure (2) shows positive agarose gel electrophoresis results for *rpsl* gene products (amplified size 201bp) used for genotypic detection of *P. aeruginosa* isolates.

Figure (2) shows a positive β–lactemase result by changing the color form yellow to red for one isolate *P. aeruginosa* no.1 as compared with the standard strain (*E. coli* ATCC 35218).

Table (2) shows the results of β-Lactemase screening test. Out of 18 isolates, 15 (83.3%) gave positive results in the preliminary screening of β-lactamases, 13 (72.2%) were ESβLs producer isolates for *P. aeruginosa*. For MβL, the rate was reduced to 9 (50%), finally 7 (38.8%) of the isolates were AmpC producers.

Figure (3) shows the results of the amplified bla_{CTX-M} gene in *P. aeruginosa* isolates (amplicon size 550bp). The prevalence of bla_{CTX-M} gene reached 72.2% when it was detected in 13\18 isolates.

Detection of cefepime resistant gene (bla_{CMY}) showed that only 3 isolates (U1, U2 and B6) out of 18 (16.6%) gave positive results with amplified size 1014bp (fig 4).

Discussion

P. aeruginosa is responsible for 10–15% of the nosocomial infections worldwide[14]. Often these infections are hard to treat due to the natural resistance of the species, as well as to its remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents with production a verity of β–lactemase enzymes[15,16]. Because standard phenotypic methods are time consuming and most have inherent limitations, genotypic detection depending on certain housekeeping gene such as 16s rRNA or other genes are used as confirmatory test and provide a rapid diagnostic technique for the identification of bacteria specially, *P. aeruginosa*. In the current study *rpsl* gene was used[17]. Four types of β-lactamases were detected including: preliminary screening for β-lactamases, ESβLs type, Metallo-β-lactamases and AmpC enzyme. Detection of bla_{CTX-M} gene responsible for 3rd generation cephalosprine resistance revealed that (72.2%) were positive to this gene and this result goes in line with Al-Grawi[18] in Baghdad who found that 80% of *P. aeruginosa* isolates harbor bla_{CTX-M} gene, while the result of current study didn't agree with Shacheraghi *et al.*[19] who found that the percentage was low when it reached only 20.43%.The members of gram negative bacteria can acquire resistance to extended spectrum beta-lactams by a different mechanisms; the most important one being the plasmid encoded extended spectrum β-lactamase (ESβL) and AmpC beta lactamases[20]. Recently, bacterial pathogens are more complicated than those isolated before a decade or two ago. They don't only have new resistance mechanisms represented by boarder extended spectrum β-lactamase (ESβLs), but such isolates also produce multiple β-lactamases causing serious therapeutic problem in many parts of the world[21]. In a study performed by Polotto *et al.*[16] they verify that genes encoding ESBLs were detected in 23.2% of isolates and the $bla_{CTX-M-2}$ was the most prevalent ESBLs gene (19.6%) among their isolates. This percentage is consumes low as compared with other resistance. Co-existence of bla_{CTX-M} and bla_{CMY} were reported in 3 isolates (UI, U2 and B6) in the current study.

Bacteria that produce ESβL are frequently resistant to other antimicrobial agents, such as aminoglycosides, tetracycline, and trimethoprim-sulfamethoxazole, as many of these additional resistance genes are encoded on the same ESβL-associated plasmid. Fluoroquinolone resistance, which is also frequently associated with ESβL production, is usually chromosomally encoded, however, plasmid-mediated quinolone resistance has been discovered recently[22]. In another study[14] they verified that the resistance against broad-spectrum cephalosporins and monobactames was very high including to: cefepime (97%), cefotaxime (92.5%) ceftazidime (51%), and aztreonam (27%).

Conclusions

Our research are needed to establish antimicrobial resistance surveillance networks for *P. aeruginosa* to determine the appropriate empirical treatment regimens. The high prevalence of multidrug resistance and production of ESβLs in *P. aeruginosa* isolates confirms the necessity of protocols considering these issues in the hospitals.

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Table 1. PCR oligonucleotides primers

Primers name	Primers sequence 5′----------›3′	Origin	Product size (bp)	Reference &accession no.
Rpsl-F	GCAAGCGCATGGTCGACAAGA	Alpha DNA Co. (Canada)	201	Xavier *et al.* (2010) CP006985.1
Rpsl-R	CGCTGTGCTCTTGCAGGTTGTGA			
Bla $_{CTX-MF-}$	CGCTTTGCGATGTGCAG	Alpha DNA Co. (Canada)	550	Nasehi *et al.* (2010) KF971880.1
Bla $_{CTX-M}$R	ACCGCGATATCGTTGGT			
BlaCMY-F	GACAGCCTCTTTCTCCACA	Invitrogene custom primers UK)(	1014	Zhao *et al.* (2003) JX514369.1
BlaCMY-R	TGGAACGAAGGCTACGTA			

Table 2. The percentage of β-lactamase types produced by *P. aeruginosa* isolates

NO. of isolates	Preliminary screening test	ESβLs	MβL	AmpC
P. aeruginosa (18)	15(83.3%)	13(72.2%)	9(50%),	7(38.8%)

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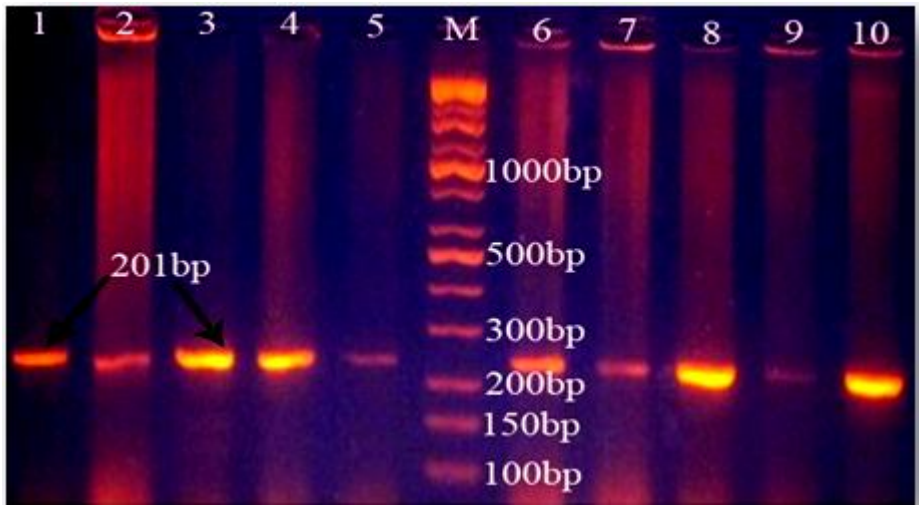

Figure 1. Agarose gel electrophoresis (1% agarose, 7 V/cm for 90min) of *rpsl* gene PCR product (201bp amplicon). Lane M, DNA ladder, lines 1-10 positive result

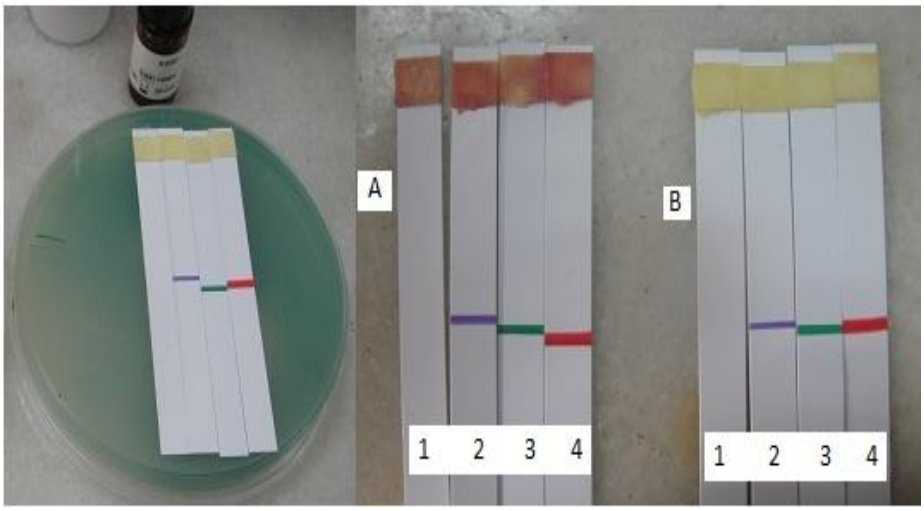

Figure 2. Detection of β-lactemase types using Rapid strips test for *P. aeruginosa* no.1. A-1: positive result for primary screening test, A-2: positive result for ESβLs, A-3: positive result for MβL, A-4: positive result for AmpC. B 1-4: negative results for all above tests for the standard strain (*E. coli* ATCC 35218)

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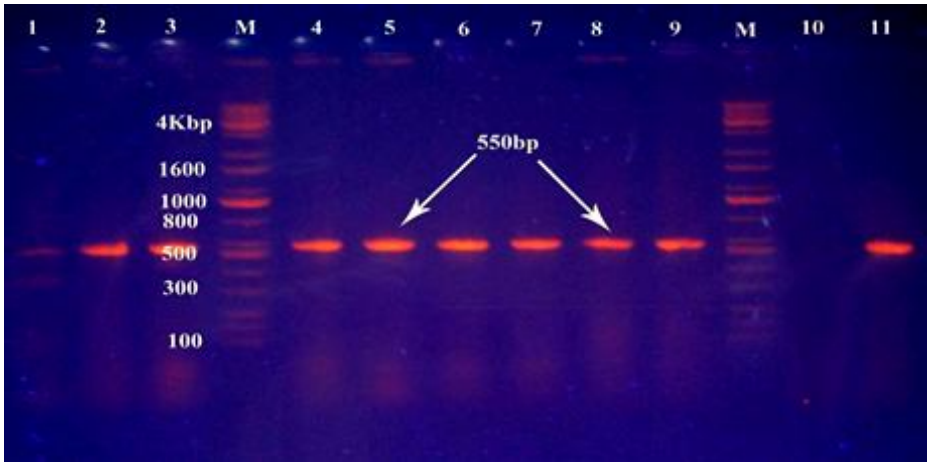

Figure 3. Gel electrophoresis (1%agarose, 7V/cm for 90 min) of *bla*$_{CTX-M}$ gene in *P. aeruginosa* isolates. line M: 100bp DNA ladder, lines (1,2,3,4,5,6,7,9,11) show positive results for *bla*$_{CTX-M}$ gene with 550bp amplicon, while line 10 shows negative results

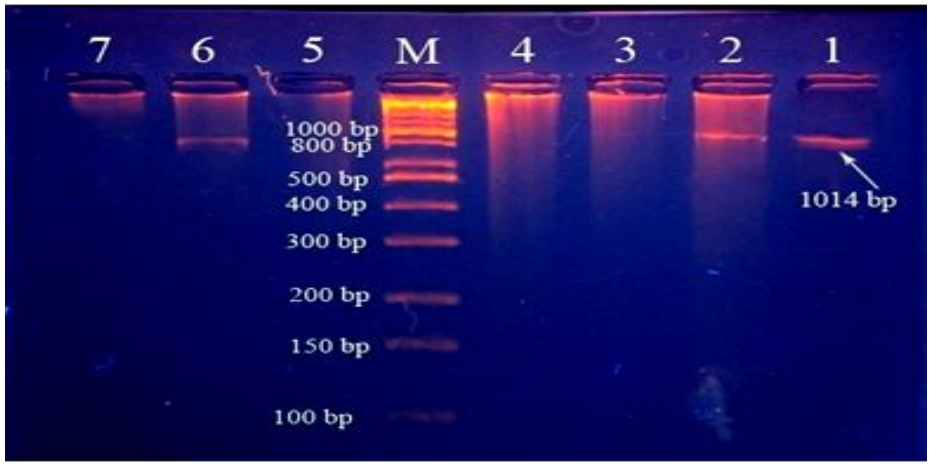

Figure 4. Gel electrophoresis (1%agarose, 7V/cm for 90 min) of *bla*$_{CMY}$ gene in *P. aeruginosa* isolates. line M :100bp DNA ladder , lanes (1,2,6) show positive results with 1014 bp amplicon, while lanes 3,4,5,7 show negative results