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RESEARCH ARTICLE

Efflux pumps in Colistin Resistant *Pseudomonas aeruginosa* Isolates in Baghdad

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Abstract

One Hundred and Twenty isolates of *Pseudomonas aeruginosa* were collected from different hospitals in Baghdad . The susceptibility of isolates was tested against colistin , results revealed that 27 isolates(22.5%) were resistant to colistin.

The susceptibility of colistin resistant *P. aeruginosa* isolates to different antibiotics was evaluated by disk diffusion method. Results showed that all isolates were resistant to Amoxicillin and carbenicillin. The resistance to Gentamycin and Lomefloxacin were 70.3% and 59.2% respectively. Norfloxacin , Levofloxacin and Ciprofloxacin were found to be the most effective agents against Colistin resistant *P. aeruginosa* isolates.

Sixity isolates (27 Colistin resistant isolates and 33 Colistin sensitive isolates) were choosen for detect efflux pumps by using Ethidium Bromide – Agar Cartwheel Method (EtBr-CW) , the result showed that 46 isolates (%76.6) give positive result .Polymerase Chain Reaction (PCR) technique was used to detect the *mexA* , *oprM* and *mexY* gene . The results showed that *oprM* found in 50% of Colistin resistant isolates , *mexA* found in 56.25% of isolates and *mexY* found in 88.9% of isolates .

Detection of gene expression was performed done by using qRT-PCR technique after RNA extraction from colistin resistant isolates and cDNA synthesis . The result showed that gene expression were high expression in efflux pumps especially in MexXY efflux pumps.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacteria that is capable of infecting a wide range of plant and animal hosts (Fernández et al.,2010). It is responsible for eventually fatal chronic lung infections in patients with cystic fibrosis (CF), as well as serious acute infections in immunocompromised and injured individuals, where it is a serious problem in septic burn wounds and ventilator-associated pneumonia (Bonomo and Szabo,2006).

The polymyxins, a group of cationic lipopeptides antimicrobials synthesized by bacteria, are being used increasingly to treat infections by multiresistant *P. aeruginosa* strains (Li et al.,2006)

Antibiotics are expelled from the cells by membrane transporter proteins, the so-called drug-efflux pumps. Of particular interest are efflux pumps capable of extruding out of the bacterial cell a different of structurally unrelated compounds (Lomovskaya et al.,2001). Efflux pumps contribute to multidrug resistance as they expel different types of antibiotics and chemicals such as dyes, detergents, organic solvents, biocides, molecules needed for the cell–cell communication and metabolic products (Askoura et al.,2011). The major mechanism by which *P. aeruginosa* may readily decrease its susceptibility to Antibiotic consists of production of an resistance nodulation cell division family (RND) efflux pump and MexXY-OprM (Lister et al.,2009)

Ribosome protection experiments have demonstrated that MexXY-OprM contributes to the natural resistance of *P. aeruginosa* to only those exported substrates able to induce *mexXY* operon expression, as a result of protein synthesis impairment (Jeannot et al.,2005 ; Guénard et al.,2014)
Resistance-nodulation-division (RND) are one type of efflux system , are commonly found in gram-negative bacteria. RND family transporters catalyze the active efflux of many chemotherapeutic agents and Antibiotic (Piddock ,2006). *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Burkholderia ssp*. are of clinical significance because of their ability to acquire high-level multidrug resistance and their innate multidrug resistance (Poole, 2001).The aims of this study were to determine the prevalence of efflux pumps in colistin resistant *Pseudomonas aeruginosa* isolates from the patients admitted to some Iraqi hospitals. .

Material and Methods

Bacterial Isolates:
Bacterial isolates were recovered from blood, wound, Burns ,urine, ear and respiratory tract from patients admitted to Some Iraqi medical centers in Baghdad between 15/9/2014 till 15/11/2014 . The isolates were identified by their colony characteristic, gram-stain and confirmed by the pattern of biochemical profiles using Vitek 2-GN system.
Antibiotic susceptibility testing :
A) Susceptibility of *Pseudomonas aeruginosa* isolates was tested by the disk diffusion test for Colistin (CT 10µg) (Bioanalyse, Turkey) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.
B) The Antimicrobial susceptibility of colistin resistant *P. aeruginosa* isolates was done by using Kirby-Bauer disc diffusion technique on Mueller Hinton agar (Oxoid, England) using overnight culture at a 0.5 McFarland standard followed by incubation at 35 oC for 16 to 18 h. following Clinical and Laboratory Standards Institute (CLSI) guidelines (2011)with commercially available antimicrobial discs (Bioanalyse/Turkey). Isolates were tested against the following antimicrobial agents: Ciprofloxacin (CIP 10µg), Norfloxacin (Nor 10µg), Aztreonam(ATM 30 µg), Amoxicillin(AX 25 µg) , Carbencillin(PY 25 µg) , Gentamycin (CN 10µg) ,Nalidixic acid(NA30µg)Tobramycin(TOB 10µg) ,Lomefloxacin(LOM 10 µg) , Ofloxacin(OFX 5 µg) ,Polymyxin B (PB 300 µg and Levofloxacin(LEV 5 µg) .

Detection of Efflux pumps
Ethidium Bromide-Agar Cartweel Method (EtBr-CW) was used to detection of efflux pump in *P. aeruginosa* isolates according to Martins et al.(2011).

Molecular Detection of MexAB-OprM and MexXY efflux pumps using PCR technique :
All of colistin resistant isolates were submitted to PCR technique to detection for *mexA* , *oprM* and *mexY* genes; DNA amplification was carried with a Gradient PCR System (TechNet-500 /USA) . PCR was performed with a final volume of 25 µl. Each reaction contained 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 0.2 mM each deoxynucleoside triphosphate; 1.5 mM MgCl2; 1.5 µl each primer (table 1) ; 1.25 U of *Taq* DNA polymerase . Template DNA (2 µl). Amplified PCR products were detected by agarose gel electrophoresis. A DNA marker (Promega/USA) was run with each gel, and the genotype was determined by the size of the amplified product.

RNA isolation and quantitative RT-PCR

RNA isolation and cDNA preparation from cultures were performed .Total *P. aeruginosa* RNA was prepared by extraction from cells grown to the exponential or post exponential phase at 37°C, using the RNeasy mini kit supplemented with DNase (Geneaid, Tailand). The reaction was submitted to the PCR condition initial denaturation at 95°C for 15 min, denaturation at 95°C for 15 sec,Annealing 60°C for 20 sec. polymerization at 72°C for 20 sec , 40 cycles and final Polymerization at72°C for 10 min.

Table 1. Primers used for detecting efflux pumps genes among colistin resistant *P. aeruginosa* isolates.

Gene	Sequence of forward Primer(5´- 3´)	Sequence of reverse primer (5´- 3´)	Reference
mexY	mexY1 -TGG TCA ACG TCA GCG CCAGCT AT	mexY2TCGACGATCTTCAGGCGTTCTG	Hocquet *etal*.,(2006)

mexA	mexA1-CGACCAGGCCGTGAGCAAGCAGC	mexA2-GGAGACCTTCGCCGCGTTGTCGC	Dumas *et al* .,(2006)
OprM	OprM1 GATCCCCGACTACCAGCGCCCCG	OprM2 ATGCCGGTACTGCGCCCGGAAGGC	Dumas *et al* .,(2006)
mexB	mexB1 ATCCGCCAGACCATCGCCA	mexB2 CATCACCAGGAACACGAGGAGG	Vettoretti*et al*.,(2009)
mexX	mexX1 TGAAGGCGGCCCTGGACATCAGC	mexX2 GATCTGCTCGACGCGGGTCAGCG	Dumas *et al* .,(2006)
rpsL	rpsl-F GCAAGCGCATGGTCGACAAGA	rpsl-R CGCTGTGCTCTTGCAGGTTGTGA	Dumas *et al* .,(2006)

Results and Discussion

A total of 120 clinical isolates of gram negative bacteria primary identified as *Pseudomonas aeruginosa* were collected from different sources.The source of these isolates were as follows : 33 isolates from urine , 25 isolates from Burns , 25 isolates from sputum , 17 isolates from Wounds , 12 isolates from Blood , 5 isolates from ears ,and the last 3 isolates from Catheters (Table 2) . All isolates were identified through morphological , cultural and some biochemical tests and using Vitek-2-GN system.

Table 2 :No. of *Pseudomonas aeruginosa* isolates according to source of samples.

Sources of samples	No. of *P. aeruginosa* isolates
Wound	17
Blood	12
Urine	33
Burns	25
Ears	5
Sputum	25
catheter	3
Total	120

The susceptibility of isolates was tested against colistin by using the agar diffusion method ;results showed that 27 isolates (%22.5) were resistant to Colistin ,then Colistin resistant *P. aeruginosa* isolates were tested against twelve antimicrobial agents by measuring inhibition zone around disk , Result showed that all isolates were resistant to Amoxicillin and Carbenicillin , While 70.3% of isolates were resistant to Gentamycin, 77.7% to Nalidixic acid , 55.5 % to Tobramycin and 59.2 % to Lomefloxacin **.**The most effective antibiotic against Colistin resistant isolates were Ciprofloxacin , Norfloxacin and Levofloxacin (Tabl 3).

Table : 3. Susceptibility of Colistin resistant *P. aeruginosa* isolates to the Antibiotics.

Antibiotic	Resistance%
Amoxcillin	100
Carbenicillin	100
Aztreonam	18.5
Gentamycin	70.3
Tobramycin	55.5
Polymyxin B	18.5
Norfloxacin	14.8
Lomefloxacin	59.2
Ciprofloxacin	7.4
Levofloxacin	11.1
Ofloxacin	29.6
Nalidixic acid	77.7

Infections caused by *P. aeruginosa* are difficult to treat, as the majority of isolates exhibit intrinsic resistance to several antimicrobial agents, due to poor outer-membrane permeability, constitutive expression of various efflux pumps and production of antibiotic inactivating enzymes(Lambert,2002). *P. aeruginosa* is a multidrug resistant bacteria (MDR). This is demonstrated by different types of antibiotic resistance that are presented by this bacteria (Askoura et al., 2011) .Increased antibiotics resistance among clinically important Gram-negative bacilli has renewed interest in colistin as a therapeutic option (Falagas et al., 2010).

Because of the widespread use of antibiotics, especially in developing countries, the resistance profile of microorganisms is changing, as evidenced by the increasing occurrence of antibiotic resistance among bacterial populations(O' Brain,1986)

A study done by Al-Marjani et al. (2013) showed that all clinical isolates of *P. aeruginosa* in Baghdad were resistant to carbenicillin . Also, study by Brown and Izundu (2004) in Jamaica revealed that resistance percentage to nalidixic acid were (82.4%) and kanamycin (76.5%). Resistance rates were 25.5% or lower for tobramycin, gentamicin and polymyxin B.

Akingbade et al.(2012) Showed that resistance percentage to Amoxicillin 92.7% and ciprofloxacin (35.5%).

Sixity isolates (27 Colistin resistant isolates and 33 Colistin sensitive isolates) were choosen for detect efflux pumps by using Ethidium Bromide – Agar Cartwheel Method (EtBr CW) , the results showed that 46 isolates (%76.7) give positive result, and all colistin resistant isolates were positive for efflux pumps test.

Polymerase Chain Reaction (PCR) technique was used to detect the *mexA* , *oprM* and *mexY* gene, The results showed that *oprM* gene found in 50% of colistin resistant isolates, *mexA* found in 56.25% of isolates and *mexY* found in 88.9% of isolates, while *mexY* gene not appear in colistin sensitive isolates .

Detection of gene expression was performed done by using qRT-PCR technique after RNA extraction of colistin resistance isolates .The result showed that gene expression were high to efflux pumps especially to MexXY efflux pumps , and the gene expression to MexXY efflux pumps higher than MexAB-OprM efflux pums .

Efflux-mediated resistance has been found in many bacterial genera. Over expression of an efflux system, responsible for reduction in the accumulation of the antibiotic . The Mex efflux pumps of *P. aeruginosa* are of particular interest because of their exceptionally broad substrate specificity. While 12 potential efflux systems of this family have been identified in the *P. aeruginosa* genome(Mesaros et al.,2007) .Four of them (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) are best characterized as antibiotic transporters (Li et al. ,2006). Multidrug pumps, particularly those represented by the clinically relevant AcrAB-TolC and Mex pumps of the resistance-nodulation-division (RND) superfamily, not only mediate intrinsic and acquired multidrug resistance (MDR) but also are involved in other functions, including the bacterial stress response and pathogenicity. Additionally, efflux pumps interact synergistically with other resistance mechanisms (e.g., with the outer membrane permeability barrier) to increase resistance levels (Li et al.,2015).

The intrinsic multidrug resistance in *P. aeruginosa* is caused by synergy between a low-permeability outer membrane and expression of a number of broadly-specific multidrug efflux (Mex) systems, including MexAB-OprM and MexXY-OprM (Schweizer , 2003).

Table 4 : Ct , ΔCt and ΔΔCt for *mexX* and *mexB* in colistin resistant *P. aeruginosa* isolates

sample	Calibrator			Treated			ΔΔCt	Ration (Fold 1)
	mexX	*rps*	ΔCt	*mexX*	*rps*	ΔCt		
1	25.22	15.76	9.46	27.41	22.12	5.29	-4.17	18.0
2	39.99	33.76	6.23	34.63	33.0	1.63	-4.6	24.25
3	25.54	16.11	9.43	26.71	22.3	4.41	-5.02	32.4
4	23.26	16.33	6.93	23.94	18.85	5.09	-1.84	3.6
5	27.54	17.27	10.27	33.49	34.47	-0.98	-11.25	2435.5
6	32.74	33.95	-1.21	29.8	35.82	-6.02	-4.81	28.1
7	27.7	33.5	-5.8	25.5	25.2	0.3	6.1	0.014

8	25.22	15.76	9.46	27.41	22.12	5.29	-4.17	18.0
9	20.78	16.33	4.45	7.13	5.34	1.79	-2.66	6.3
sample	Calibrator			Treated			ΔΔCt	Ration (Fold 1)
	mexB	*rps*	ΔCt	*mexB*	*rps*	ΔCt		
1	20.96	15.76	5.2	18.65	14.63	4.02	-1.18	2.3
2	0	39.99	-39.99	33.6	37.56	-3.96	36.03	1.4
3	26.11	16.11	10	20.25	12.94	7.31	-2.69	6.4
4	20.78	16.33	4.45	7.13	5.34	1.79	-2.66	6.3
5	0	17.27	-17.27	23.98	25.94	-1.96	15.31	0.0
6	0	33.95	-33.95	17.0	15.34	1.66	35.61	1.9
7	39.6	33.5	6.1	27.41	22.12	5.29	-0.81	1.75
8	23.26	16.33	6.93	23.94	18.85	5.09	-1.84	3.6
9	25.22	15.76	9.46	27.41	22.12	5.29	-4.17	18.0

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