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MOLECULAR DETECTION OF MULTI-DRUG RESISTANT *P. AERUGINOSA* ISOLATED FROM NEONATAL INFECTIONS

AMAL TALIB ATIYAH AL-SA'ADY[1] & NAHER H. S[2]

[1]Department of Clinical Laboratory Sciences, College of Pharmacy, University of Babylon, Hillah, Iraq

[2]Department of Microbiology, College of Medicine, University of Babylon, Hillah, Iraq

ABSTRACT

Throughout this study, multi-drug resistant *P. aeruginosa* was isolated from neonatal infections in order to detect the genes being responsible of antibiotic resistance in these bacteria by molecular technique (PCR). Antibiotic sensitivity test results demonstrated that *P. aeruginosa* have (100%) resistance against each Carbenicillin, Amoxyclave, Cephalothin and Rifampicin, and high level resistance ≥70% for most other antibiotics. While 0% resistance (100% sensitivity) was shown against each IPM, MRP. The results of minimum inhibitory concentration,MIC, by using HiComb test showed that *P. aeruginosa* has 100% resistance for Amoxyclave (4-240µg/ml), 70% for Cloramphenicol(4-120 µg/ml), 60% for Cefepem(16-128 µg/ml), 50% for Ciprofloxacin(0.5-120 µg/ml), 30% for Amikacin(0.032-128 µg/ml), 20% for Piperacillin(5-120 µg/ml) and 10% for Ceftazidime(8-64 µg/ml). PCR results demonstrated that among 35 isolates of *P. aeruginosa*, highest incidence 51.4% have *arr-2* gene followed by 49% have *aac(3)I* and 37% have ^{bla}*CARB*.

KEYWORDS: Neonates, Infections, Multi-Drug Resistant *P. aeruginosa*

INTRODUCTION

P.aeruginosa represents an unusual phenomenon of antimicrobial resistance among prokaryotes since practically all known mechanisms of resistance be found in this organism including decreased outer membrane permeability, pincillin binding protein modification, increased expression of efflux pumps system, alginate and enzymetic inactivation of antibiotics[1]. These various mechanisms of resistance in *P. aeruginosa* often lead to cross-resistance among different antimicrobial classes resulting in multidrug-resistant *P. aeruginosa* strains were first reported in patients with cystic fibrosis[2] and rendering many currently available antipseudomonal antimicrobials ineffective[3]. Nosocomially *P .aeruginosa* showed high resistance to disinfectants and antiseptics used in the hospital leading to wound and hospital contamination. On the other hand, this organism has an extraordinary capacity for the development of antimicrobial resistance to virtually all antipseudomonal agents through the selection of mutations in chromosomal genes leading to the conferring resistance to penicillins, cephalosporins and fluoroquinolones,in addition to the determining resistance to carbapenems,so, intrinsic and acquired resistance makes *P. aeruginosa* as one of the most difficult organisms to be treated and eradicated[4,5]. Some other studies have reported that, the acquisition of resistant strains to ceftazidime, imipenem, piperacillin, or ciprofloxacin is significantly associated with longer hospital stay and an increased rate of secondary bacteremia with *P. aeruginosa* infection[2].

Although forethrmore it is sensitive to some antibiotics, e. g. ceftazidime, imipenem, ciprofloxacin and ofloxacin, but resistance to these antibiotics has also emerged[6,7].

Rifampin is derived from rifamycin which inhibits bacterial DNA-dependentrna synthesis by inhibiting bacterial

DNA-dependent RNA polymerase. *arr-2* gene was responsible of rifampin resistance in *P. aeruginosa* and it is located on a gene cassette within a class I integron. Resistance to rifampicin arises from mutations that alter residues of the rifampicin binding site on RNA polymerase, resulting in decreased affinity for rifampicin[8,9].

Gentamicin is an aminoglycoside antibiotic, it is used to treat many types of bacterial infections, particularly those caused by gram-negative rods and *Staphylococci*. The mechanism of gentamicin action represents by irreversibly binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis[10]. *aac(3)I* gene determines resistance to gentamicin[1].

Carbenicillin is a bacteriolytic antibiotic belonging to the carboxypenicillin subgroup of the penicillins. It has gram-negative coverage but limited gram-positive coverage. The carboxypenicillins are susceptible to degradation by beta-lactamase enzymes, four types of carbenicillin hydrolysing b-lactamases of Pseudomonas specific enzyme (PSE or CARB) were found in *P. aeruginosa* which include PSE-1(CARB-2), PSE-4(CARB-1), CARB-3 and CARB-4. ^{bla}*CARB* gene determines resistance to Carbenicillin[11].

MATERIALS AND METHODS

During a period of March (2012) to February (2013), a total of 666 samples were collected from neonates, mothers and environment in Babylon Hospital for Pediatric and Gynecology in Hilla \Iraq. Those samples included blood, urine, CSF and swabs (oral cavity, umbilical cord, skin, eye, respiratory secretions, nose and surgical site) from neonates. While samples from mothers included amniotic fluid from mothers in delivery room, umbilical cord blood from recent neonate (cord blood) and HVS from pregnant women. Finally, samples from environment included swabs from mask of mechanical ventilator, cannula, nursery, curtage unit, disinfectant, fluid sucker, catheter, floor and stage of delivery room. Bacterial isolates were investigated for identification according to their characteristics and compared with referential references, [12, 13, 14].

Antimicrobial Susceptibility

Disc Diffusion Test was performed according to Bauer[15] method on by using 27 antibiotic discs. The antibiotic discs were placed on an inoculated Muller-Hinton agar plate and incubation at 37ºC for 18-24 hrs. Then, inhibition zone was measured and compared to standard criteria in CLSI[16]

Minimum Inhibitory Concentration (MIC) by HiComb Test: the HiComb strip was applied to an inoculated Muller-Hinton agar plate and was incubated at 37ºC for 18-24 hrs. After incubation an ellipse will appear, that intersects the MIC value scale (in µg/ml) the lowest concentration that will inhibit the growth of a test organism as determined visually by the lack of turbidity over a defined interval related to an organism's growth rate, most commonly after 18 to 24 hrs[17,18].

Moleculler Assays: Polymerase Chain Reaction (PCR) were performed for detection the responsible genes of antibiotic resistance which included ^{bla} *CARB, aac(3)I* and *arr-2* in multi-druge resistant *P. aeruginosa* by using the primers and PCR conditions which detailed in table 1.

Table 1: Primers Sequences and PCR Conditions for Multi-Druge Resistant *P. aeruginosa*

Genes	Primer Sequence (5' → 3')	Product Size bp	PCR Cycle Program	References
bla CARB	F: 5' - AAA GCA GAT CTT GTG ACC TAT TC–3' R: 5' - TCA GCG CGA CTG TGA TGT ATA AAC-3'	588	94°C 5min 1x 94°C 1min 55°C 1min 40x 72°C 1min 72°C 7min 1x	Wang *et al.* (2006)
aac(3)I	F: 5' –ACC TAC TCC CAA CAT CAG CC -3' 5' –-ATA TAG ATC TCA CTA CGC GC- 3' R:	169	95°C 3min 1x 95°C 30sec 60°C 45sec 40x 72°C 2min 72°C 5min 1x	Shervington *et al.* (2001)
arr-2	F: 5'- GCG TGC CTT GTT TCC ACA TT- 3' R: 5'- TCA CAC GCC CCA TAA AAC GA - 3'	466	94°C 5min 1x 94°C 30sec 55°C 30sec 40x 72°C 30sec 72°C 5min 1x	Designed for this study

RESULTS AND DISCUSSIONS

A total of 510 bacterial isolates were identified from different samples revealed positive results for bacterial culture. Among 510 bacterial isolates, *P.aeruginosa* accounted 48(9.41%).

Antimicrobial Susceptibility

Disc Diffusion Test: The percentages of antibiotic resistance in P.aeruginosa are shown in table 2. Based on these data, this bacterium has fully sensitivety (100%) against each of IPM, MRP which can be attributed to the fact that ß-lactam rings of these antibiotics are resistant to hydrolysis by most ß-lactamases[19]**.** However, the result was in accordance with those results being reported by[20, 21].

Table 2: Percentages of Antibiotic Resistance in *P. aeruginosa* Isolates in this Study

Antibiotic	PIP	CB	AMC	KF	CPM	CTR	CTX	CAZ	IMP	MRP	AT	GEN	AK	TOB	TE	CIP	NX	NA	C	RIF
Resistance %	60	100	100	100	90	83	85	31	0.0	0.0	90	96	42	77	75	60	60	85	90	100

PIP=Piperacillin;CB=Carbencillin;;AMC=Amoxiclave;KF=Cephalothin;CPM=Cefepime;CTR=Ceftriaxone;CTX =Cefotaxime;CAZ=Ceftazidime;IMP=Imepenem;MRP=Meropenem;AT=Aztreonam;GEN=Gentamycin;AK=Amikacin;T OB=Tobramycin;TE=Tetracycline;CIP=Ciprofloxacin;NX=Norfloxacin;NA=Naldixicacid;C=Chloramphenicol; RIF=Rifampicin.

In the present study, *P. aeruginosa* have fully resistance (100%) for each CB, AMC, KF and RIF, and high level resistance for other antibiotics: 96%(GEN), 90%(C), 90%(AT), 85%(CTX), 85%(NA), 83%(CTR), 77%(TOB), 75% (TE) and 60% (PIP). This result was in accordance with those results being reported by[22,4] where referred that, *P.aeruginosa* showed a high resistance to most β-lactame antibiotics and cephalosporins. In addition, Strateva[23] pointed out that most *P. aeruginosa* was resistant to carbencillin. Resistance to β-lactams commonly results from drug inactivation by β-lactamases, drug extrusion through efflux pumps, changes in outer membrane permeability, and modification of PBP[24]. Development of antibiotic resistance in Iraq is often related to the availability of antibiotics out of hospitals which encourage self-medication[4].

Minimum Inhibitory Concentration (MIC) TEST: MIC is the lowest concentration of antibiotic that prevents growth of a given organism. The present study used MIC method for testing the susceptibility of *P. aeruginosa* and MIC values were based on break point recommended by CLSI[16] for estimation of the response. Seven antibiotics (CAZ, AK,

CIP, C, CPM, AMC, PIP) against *10* isolates of *P.aeruginosa*. MIC values for the studied antibiotics were detailed in table 3.

As detailed in table 3, the percentages of resistant *P.aeruginosa* isolates for antibiotics according to the MIC values were 10%CAZ(8-64 µg/ml), 30%AK(0.032-128 µg/ml), 50%CIP(0.5-120 µg/ml), 70%C(4-120 µg/ml), 60%CPM(16-128 µg/ml), 100%AMC(4-240 µg/ml) and 20%PIP(5-120 µg/ml). This results partially related with arecent study by[4] who reported that resistant rate of same bacteria for amikacin and ciprofloxacin was 30% (o.o5-256 µg/ml) and 40%(0.004-60 µg/ml) respectively. This variasion may be attributed to that the researchers depend on breakpoints recommended by various international committees which recommended variable values of breakpoints[25].

Table 3: The Values of Minimum Inhibitory Concentration (MIC) for Some Antibiotics against *P. aeruginosa* Isolated in this Study

Antibiotics	CAZ 0.016-256	AK 0.001-256	CIP 0.001-240	C 0.001-240	CPM 0.001-240	AMC 0.001-240	PIP 0.001-240
Standard values ≤S I R≥	8 16 32	16 32 64	1 2 4	4 8 16	8 16 32	4 _ 8	64 _128
MIC ranges µg/ml	8-64	0.032-128	0.5-120	4-120	16-128	4-240	5-120
Percentage of resistant bacteria	10%	30%	50%	70%	60%	100%	20%

Molecular Assays: In the present study, the molecular experiments using PCR assay emphasized on detection of three genes being responsible of resistance for three antibibiotics as indicated in table 4. It is found that among 35 isolates of *P. aeruginosa*, highest incidence was 18(51.4%) for *arr-2* gene followed by 17(48.6%) *aac(3)I* and 13(37.1%) *^{bla}CARB*, table 4. The distribution of these genes varied in respect to the isolation source.

Table 4: Distribution and Percentages of Responsible Genes of Antibiotic Rasistance in *P. aeruginosa* Isolated in this Study

Gene The Source	*aac(3)I*	*^{bla}CARB*	*arr-2*
Blood	2(11.7%)	2(15.4%)	2(11%)
Oral Cavity swab	6(35.3%)	5(38.4%)	6(33.3%)
Urin	1(5.9%)	1(7.7%)	1(5.6%)
CSF	1(5.9%)	---	
Umbilical Cord swab	1(5.9%)	1(7.7%)	1(5.6%)
Skin swab	1(5.9%)	1(7.7%)	1(5.6%)
Respirotary Secretions swab	2(11.7%)	2(15.4%)	2(11%)
Surgical Sites swab	1(5.9%)	---	---
Cannula	---	---	1(5.6%)
Caesarian Section	1(5.9%)	---	1(5.6%)
Floor of Delivery Room	--	---	1(5.6%)
Disinfectant	1(5.9%)	1(7.7%)	1(5.6%)
Catheter	---	---	1(5.6%)
Total	**17(48.6%)**	**13(37.1%)**	**18(51.4%)**
Total Number of Isolates	**35**	**35**	**35**

The molecular detection of *arr-2* gene, which responsible of rifampicin resistance, revealed positive amplification with product size accounted 466 bp, as shown in figure 1. And it was distributed as detailed in table 4. The highest spread

of it was found among isolates from oral cavity 6(33.3%). This finding was in agreement with other studies; where reported that a new rifampin resistance gene, *arr-2*, has been found in *P. aeruginosa*[26] and the emergence of this gene in the American continent as gene cassettes from class I integrons[27].

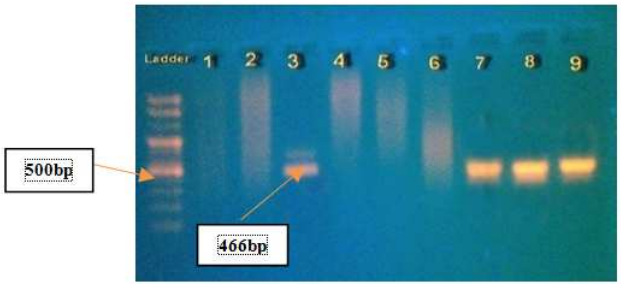

Figure 1: Gel Electrophoresis of PCR Product of *Arr-2* Gene with Product Size= 466bp. The Isolates Numbered (3, 7, 8, 9) Were Positive For *Arr-2* Gene Whereas Isolates Numbered (1, 2, 4, 5, 6) Negative

The molecular detection of *aac(3)I* gene, which responsible of gentamicin resistance, revealed positive amplification with product size accounted for 169 bp, as shown in figure 2. *aac(3)I* gene was distributed as detailed in table 5. The highest freqiunce of it was recorded among isolates from oral cavity6 (35.3%). This finding was in agreement with other study who reported that *aac(3)I* gene is widespread among members of Enterobacteriaceae and other gram negative such as Pseudomonas and Serratia as well as Acinetobecter[1].

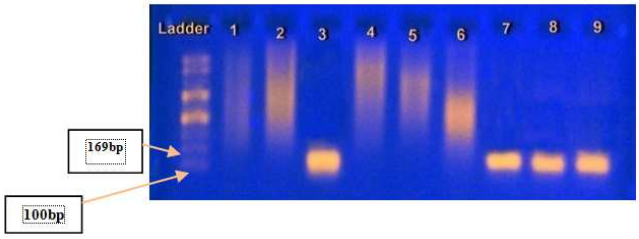

Figure 2: Gel Electrophoresis of PCR Product of *Aac(3)I* Gene with Product Size=169bp. The Isolates Numbered (3, 7, 8, 9) Were Positive for *Aac(3)I* Gene Whereas Isolates Numbered (1, 2, 4, 5, 6) Negative

The molecular detection of ^{bla}*CARB* gene, which responsiblof carbenicillin resistance, revealed positive amplification with product size accounted for 588 bp, as shown in figure 3. ^{bla}*CARB* gene was distributed as detailed in table 5. The highest frequency was found among isolates from oral cavity 5(38.4%). This resistance may attribute to the fact that Carbenicillin is a bacteriolytic antibiotic belonging to the carboxypenicillin subgroup of the penicillins which are susceptible to degradation by beta-lactamase enzymes[11]

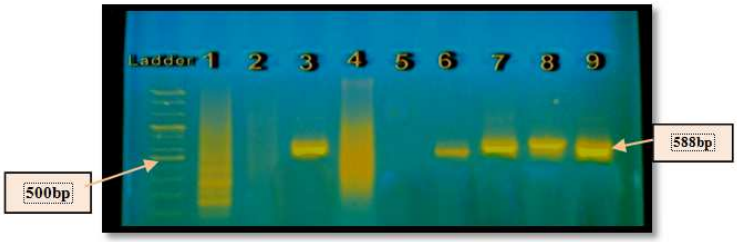

Figure 3: Gel Electrophoresis of PCR Product of ^{Bla}*carb* Gene with Product Size= 588bp. The Isolates Numbered (3, 6, 7, 8, 9) Were Positive for ^{Bla}*carb* Gene Whereas Isolates Numbered (1, 2, 4, 5) Negative

CONCLUSIONS

In light of the results documented in this study, one can conclude that multi-drug resistant *P. aeruginosa* isolated from neonatal infections harbore the genes which render the organisms to resist antibiotic in high rate.

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