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Research Article

Prevalence of Multidrug-Resistant *Pseudomonas aeruginosa* in Patients with Nosocomial Infections at a University Hospital in Egypt, with Special Reference to Typing Methods

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Abstract

Multidrug resistant (MDR) *P.aeruginosa* had emerged in Egypt and seen mainly in nosocomial infections due to the selective pressure by overusage of antibiotics. This study was planned to delineate prevalence of MDR *P.aeruginosa* in nosocomial infection patients, and to screen for ESβLs producing *P.aeruginosa* with typing of *P.aeruginosa* isolates in Menofia University Hospitals (MUH), Egypt. Our study included 287 inpatients admitted to Menoufia University Hospital and having different nosocomial infections. Samples from medical staff and from hospital environment were collected. Antibiotyping of *P. aeruginosa* isolates were determined. MDR and ESBLs *P. aeruginosa* were detected. Plasmid DNA analysis and pyocin typing were done. In our study, a total of 57 *P. aeruginosa* strains were isolated. *P. aeruginosa* and its MDR phenoype accounted for 19 % and 9.5% respectively of nosocomial infections. MDR and ESβLs producer *P. aeruginosa* infections were commonly isolated from burn wound specimens followed by urinary tract, respiratory tract and wound infections. We detected high rates of MDR *P. aeruginosa* (52%) and of ESβLs producer strains (45.6%) and those ESBLs strains were all MDR. Amikacin and imipenem were the most effective drugs against *P. aeruginosa.* 23 different resistance patterns were identified, profiles from (1 - 8) were prevalent. The most prevalent antibiotype (2) included 12 MDR isolates, 9 clinical and 3 environmental isolates having same patterns. 61.5% of ESβLs isolates harbor plasmids. Five groups could be demonstrated among our *P. aeruginosa* isolates. Each had the same antibiotype and plasmid profile. In conclusions, our results clarified that threats of MDR and ESBLs *P. aeruginosa* become of major concern in our hospital and implementation of infection control strategies are major concerns to avoid the spread of this threat. Eenvironmental sources may have a significant role in spread of MDR *P. aeruginosa* among hospitalized patients. Combination of

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simple typing methods as antibiotyping with plasmid profiling may be the cheapest and effective method for tracing source of infection.

Keywords: *P. aeruginosa*- MDR and ESBLs -Typing of *P. aeruginosa.*

Introduction

P. aeruginosa is a non-fermentative Gram-negative bacteria widely distributed in nature and can survive on a wide variety of surfaces and in hospital environment, as the wards encourage bacterial growth ***(*Arora et al., 2011*).*** *P.aeruginosa* is responsible for about 10% -20% of nosocomial infections as bacteraemia and sepsis in ICU, cystic fibrosis, pneumonia, urinary tract infections, burn infection and wound infection (**Carmeli et al., 1999).** Multidrug-resistant (MDR) *P. aeruginosa* phenotype is defined as resistant to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes (carbapenems, fluoroquinolones, penicillins /cephalosporins and aminoglycosides) **(Magiorakos et al., 2011 and Maria et al., 2011).** MDR *P. aeruginosa* are particularly problematic because the organism is inherently resistant to many drug classes and is able to acquire resistance to all effective antimicrobial drugs **(Gad et al., 2007*)*.** MDR *P. aeruginosa* develops resistance by various mechanisms like multi-drug resistance efflux pumps, biofilm formation, production of β-lactamases and aminoglycoside modifying enzymes. The risk for acquiring MDR organisms may be related to the number of carriers in the same ward as well as to individual risk factors, such as patient characteristics and in-hospital events (invasive devices and antibiotic treatment) (**Carmeli et al., *2002)***. Extended spectrum beta lactamases (ESβLs) have been described in *P. aeruginosa* only recently. β-lactamases described in *P. aeruginosa* belong to various families as TEM and SHV types which are common among Enterobacteriaceae, PER type, VEB type which have been reported from various parts of the world **(Amutha et al., 2009).** Typing techniques are essential for epidemiological purposes to establish a clonal relationship between different strains isolated from patients and those isolated from surrounding environment and medical team. Phenotypic methods such as biotyping, antibiotyping, pyocin typing and serotyping and molecular methods such as plasmid profile analysis and PCR were used for this purpose. This study was planned to delineate prevalence and resistance patterns of *P. aeruginosa* and MDR phenotype in nosocomial infection patients, and to screen for ESβLs producing *P. aeruginosa* in Menofia University Hospitals (MUH). Also to characterize clinical and environmental isolates of *P. aeruginosa* by plasmid profile analysis, antibioyping and pyocin typing, aiming for a better management, reducing both mortality and costs*.*

Materials and Methods

This study was conducted during the period from May 2009 to July 2011 and included 287 inpatients (group I) (165 males and 122 females) admitted to different wards and units of MUH and having different nosocomial infections, their ages ranged from 0 to 90 years. A total of 287clinical specimens were examined; 78 urine specimens, 46 ear swabs, 25 wound swabs and 65 burn swabs. 51 hand, throat and nasal swabs were collected and processed from 17 medical staff (3 swabs each) (group II) working at MUH. 40 environmental samples were taken from surgical instruments, dressings, bath, suction devices, floors, walls, beds, commodes, sinks and antiseptic solutions. Samples were inoculated onto blood agar, nutrient agar, CLED agar and MacConkey agar (Oxoid, England). Colonies grown on different media were subjected to further morphological and biochemical identification. Suspected *P. aeruginosa* colonies were identified according to standard microbiological methods ***(Koneman et al., 2006)***. Antibiotic

sensitivity and antibiotyping of *P.aeruginosa* clinical and environmental isolates were determined by disc diffusion method of Kirby Bauer on Muller-Hinton agar (Oxoid, England) and interpreted according to the method described by the Clinical and Laboratory Standard Institute (***CLSI, 2009)***. In our work MDR *P. aeruginosa* was detected as resistant to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes **(*Magiorakos et al., 2011* and *Maria et al., 2011).*** Strains having the same resistance patterns (antibiotype) considered to be from the same clone. Phenotypic confirmatory tests recommended by the CLSI for ESβLs detection were carried to assess the prevalence of ESBL. The double – disc synergy test was done, organisms were swabbed onto a Mueller – Hinton agar plate (Oxoid, England). Disc containing amoxicillin – clavulanate (20 µg / 10µg respectively) was placed in the center of the plate. Discs containing one of the oxyimino –β– lactam antibiotics (cefotaxime, ceftazidime, ceftriaxone and aztreonam) were placed 30 mm (center to center) from amoxicillin – clavulanate disc and incubated at 35°C for 24 hours. A clear extension of the edge of the oxyimino β-lactam inhibition zone towards the disc containing clavulanate was interpreted as synergy indicating a positive result. Another phenotypic confirmatory test, cephalosporin/clavulanate combination discs this requires use of both ceftazidime - clavulanic acid (30 µg/10 µg) and cefotaxime -clavulanic acid (30 µg/10µg) discs. The discs were applied to the plates using a sterile forceps and incubated at 35°C for 18 hours. An increase in the zone diameter of ≥5 mm in the presence of CA, compared to ceftazidime or cefotaxime alone, is taken to be a phenotypic confirmation of ESβL production ***(CLSI, 2009*)**. Plasmid DNA analysis for all *P. aeruginosa* isolates were done**.** Plasmids were extracted as previously described by alkaline lysis method **(Sambrok et al., 2001 and Ranjbar et al., 2007*).*** Extracted plasmids were then separated by electrophoresis, stained with ethidium bromide and visualized under UV illumination. The strains were grouped depending on the size and number of the plasmid DNA bands. Pyocin typing was done by Gillies and Govan technique **(Fyfe et al., 1984)**. Each isolated strain was streaked diametrically across the surface of tryptone soya agar to give an inoculum width of approximately 1 cm. The growth was exposed to chloroform for 15 minute. The plate was then opened exposing the medium to air for few minutes. Five standard indicator strains were grown in nutrient broth were then streaked on to the medium at right angles to the line of the original inoculum. The plate was then incubated at 37°C for 18hrs. Any pyocin produced by the original tested strains diffused into the medium during the first period of incubation and then exerted their inhibitory action on the indicator strains during the second incubation. Plates were observed for growth inhibition of indicator strains.

Results

The present study included 287 inpatients, out of them 283 clinical isolates were recovered. *P. eruginosa* accounted for 19 % (54 of 283) of nosocomial infections isolates. Only 3 *P. aeruginosa* strains were isolated from environmental sites. In our work, *P. aeruginosa* wasn't detected from medical staff swabs. *P. aeruginosa* infections were common in males (66.7 %) than female (33.3%) cases and their mean age was (44.6±25.8). In our study, *P. aeruginosa* mostly isolated from burn unit (32.3%), ICU (16.7%) then urology department (18.75%) (Table I) and (Figure I).

Table (I): Distribution of *P. aeruginosa* Isolates among Different Departments

Department	Total isolates	*P. aeruginosa*	
		N	%
- Burn Unit	65	21	32.3
- ICU	96	15	15.6
- Urology department	48	9	18.7
- Surgery department	31	4	12.9
- Otolaryngylogy	29	5	17.2
- Paediaterics	7	0	0
- Internal medicine	7	0	0
Total	283	54	19

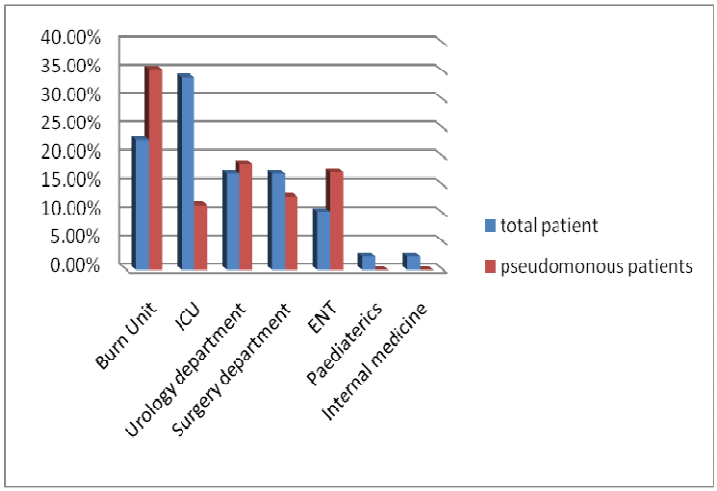

Figure (I): Distribution of *P. aeruginosa* Isolates among Different Departments

Table (II) summarizes the resistance patterns of 57 *P. aeruginosa* strains (54 clinical and 3 environmental isolates). Amikacin was the most effective drug against all *P. aeruginosa* isolates showed maximum sensitivity (80.5%) followed by imipenem (66.7%) and gentamicin (56.1%). On the other hand, *P. aeruginosa* had high resistance rates to cefepime (98%) followed by piperacillin \ tazobactam (94.7%), ceftazidime (91%) and ceftriaxon and amoxicillin- clavulnic acid (87% each). All isolates were totally resistant to carbincillin and tetracycline.

Table (II): Antibiotic Susceptibility Tests of *P. aeruginosa* Isolates, 54 Clinical Isolates from Group I and 3 Environmental Isolates by Disc Diffusion Method

Antibiotics	S		R	
	No	%	No	%
Amoxicillin- clavulinic acid	7	12.3	50	87.7
Piperacillin/ tazobactam	3	5.3	54	94.7
Cefoperazone	15	26.3	42	73.7
Cefotaxime	13	22.8	44	77.2
Ceftiaxone	7	12.3	50	87.7
Ceftazidime	5	8.8	52	91.2
Cefipime	1	1.7	56	98.2
Impineum	38	66.7	19	31.6
Azteronam	10	17.5	47	82.5
Gentamycin	32	56.1	25	43.9
Amikacin	48	80.5	9	15.8
Tobramycin	28	49.1	29	50.9
Tetracyclin	0	(0)	57	100
Ciprofloxacin	25	43.6	32	56.1
Norfloxacin	26	45.6	31	54.4
Cloramphenicol	15	26.3	42	73.7
Carbencillin	0	(0)	57	100

Antibiogram categorized 57 *P. aeruginosa* isolates into 23 different antibiotypes, (1-8) antibiotypes including 42 strains are the most prevalent (table III). 3 environmental isolates and 9 clinical isolates were included in antibiotype (2).

Table (III): Groups of *P. aeruginosa* Isolates that Have the Same Antibiotype

Profile	No of isolates	Resistance pattern(100% resistance)
1 (14)	7	Amoxycillin/clavulanic acid – ceftriaxone- ceftazidime - cefepime - cefotaxime - cefoperazone - aztreonam - piperacillin \tazobactam - chloramphenicol –carbencillin - tetracycline- tobramycin- ciprofloxacin – carbencillin- amikacin
2 (15)	12	Amoxycillin/clavulanic acid -ceftriaxone- cefotaxime - cefipime- cefoperazone – ceftazidime- aztreonam - piperacillin \tazobactam - gentamycin- chloramphenicol –tetracycline- carbencillin-norfloxacin- ciprofloxacin – impineum
3 (12)	11	Amoxycillin/clavulanic acid - ceftriaxone- cefotaxime - cefipime- cefoperazone – ceftazidime- ciprofloxacin - chloramphenicol –tetracycline- piperacillin \ tazobactam- aztreonam- carbencillin
4 (10)	2	Amoxycillin/clavulanic acid- cefotaxime - cefipime- cefoperazone – ceftazidime - piperacillin \tazobactam - cefipime- chloramphenicol –tetracycline – carbencillin-
5(5)	3	Amoxycillin/clavulanic acid -tetracycline – carbencillin- cefipime - piperacillin \tazobactam
6 (6)	2	Tetracycline – carbencillin- cefipime- piperacillin \tazobactam- ceftazidime- ceftriaxone
7(4)	2	Amoxycillin/clavulanic acid- tetracycline – carbencillin- cefipime-
8(4)	2	Tetracycline – carbencillin- cefipime- ceftazidime
Total	42	

In our work, 52% of *P. aeruginosa* were MDR and were categorized in antibiotype 1, 2 and 3. Those 30 MDR *P. aeruginosa* isolates were distributed as follow 27 were clinical isolates and accounted for 9.5 % (27\283) of all nosocomial infections and 3 were environmental isolates. In our work 45.6% *P. aeruginosa* (24 clinical isolates and two environmental) were positive for ESβLs production, and all were MDR (26 of 30) 86.6%. In this study, out of total 54 *P. aeruginosa* isolates, 21\54 (38.8%) were from burn wound, 15 (27.7%) from urine, 8 (14.8%) from sputum, 5 isolates from ear discharge, 3 from surgical wound and 2 isolates from blood. MDR - ESβLs producing

P. aeruginosa (24) isolates were isolated from burn wound 12 of 21 (57%), 10 of 15 (66.6%) from urine and 2 of 3 (66.6%) from surgical wound (Table IV).

Table (IV): Distribution of *P. aeruginosa*, MDR and ESBLs Producing Isolates in Clinical Specimens

Type of specimen	***P. aeruginosa***		**MDR**		**ESβLs**	
	No	**%**	**N**	**%**	**N**	**%**
Burn swab	21	38.8	12	57	12	57
Urine	15	27.7	10	66.6	10	66.6
Sputum	8	14.8	3	37.5	0	0
Ear swab	5	9	0	0	0	0
Wound swab (pus)	3	5	2	66.6	2	66.6
Blood	2	3.7	0	0	0	0
Total	54		27\54	50	24\54	44.4

On analysis of plasmid profile, out of 57 *P. aeruginosa* 27 strains (47.4%) harbored plasmids and were categorized into 7 different plasmid profiles (A-G). The sizes of plasmids varied from 1.6 to 140 kb (Figure 3). Two environmental and three clinical isolates had plasmid profile (A). While studying the correlation between plasmid profile and ESβLs production of *P. aeruginosa*, it was found that 16 of 26 (61.5%) ESβLs producer strains had plasmids and were categorized in 4 plasmid profiles (A,B,C and D) (Table V).

Table (V): Relation between Resistance Patterns and Plasmid Profiles among 16 of 26 (61.5%) MDR-ESβLs Producer *P. aeruginosa*

Resistance patterns	Plasmid profiles	Number of MDR-ESβLs producers *P. aeruginosa* 16
1	**C (30-7-2**)	1
2	**A (**7- 30- 140)	5
2	**B(**4.5- 30-140)	3
3	**B(**4.5- 30-140)	2
1	**B (**4.5- 30-140)	2
3	**C (30-7-2**)	2
1	**D (**30)	1

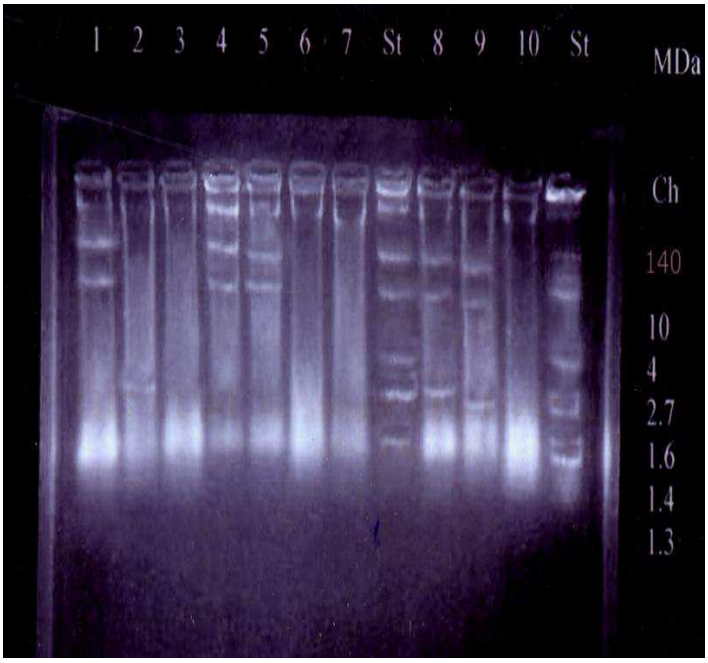

Figure (II): Plasmid Profile of Some of the Studied *P. aeruginosa* Isolates

From left to right: Lane 1, 4, 5 shows 3 plasmids (140-30-7 MDa). Lane 2, 6 shows 1 plasmid (2.2MDa). Lane 9, 10 shows 3 plasmids (30-7-2 MDa). Lane 7, 11 shows no plasmids. Lanes 8 & 12 (standard strain) show 7 plasmids (140-10-4.8-2.7-1.6-1.4-1.3MDa).

In the current study *P. aeruginosa* isolates (57) were typed by pyocine typing method. The results of pyocin typing classified *P.aeruginosa* isolates into 8 groups (I-VIII) (Table VI). Pyocine typing method couldn't discriminate MDR or ESBLs *P.aeruginosa* isolates in unique groups.

Table (VI): Pyocin Typing of *P. aeruginosa* Isolates

Group	No of isolates	A	B	C	D	E
I	9	+ve	+ ve	+ ve	+ ve	+ ve
II	7	+ ve	+ ve	+ ve	- ve	- ve
III	5	- ve	- ve	+ ve	+ ve	+ ve
IV	8	-	-	+	-	+
V	6	-	+	-	+	-
VI	4	-	+	-	-	+
VII	2	+	-	-	-	+
VIII	16	-	-	+	+	-
Total	57	-	-	-	-	-

A, B, C, D, E are indicator strains. +ve means inhibition of growth of indicator strains. - ve means no inhibition of growth of indicator strains.

Five clusters could be demonstrated among our MDR *P. aeruginosa* isolates. Each cluster had the same antibiotic sensitivity pattern and plasmid profile (Table VII), while cluster one isolates had different pyocin type. Two environmental isolates were identical by antibiotyping and plasmid profile to 3 clinical isolates and present in cluster 1 (Table VII).

Table (VII): Clusters of MDR *P. aeruginosa* Isolates that Have the Same Antibiotic Sensitivity Pattern, Plasmid Profile and β-Lactamase Production and Their Relations to Pyocin Groups

Cluster designation	Sources and No of isolates	Plasmid profile	Antibiotype	ESβLS production	Pyocine group
Cluster1	**3 patients**	A	2	present	I
	2 environmental	A	2	present	III
Cluster 2	**3 patients**	B	2	present	IV
Cluster 3	**2 patients**	B	3	present	VIII
Cluster 4	**2 patients**	B	1	present	V
Cluster 5	**2 patients**	D	1	present	VIII

Discussion

P. aeruginosa is a well-recognized nosocomial pathogen that can cause severe infections in hospitalized patients. A total of 57 *P. aeruginosa* strains were isolated (54 clinical and 3 environment isolates). *P. aeruginosa* and its MDR phenoype accounted for 19 % and 9.5% respectively of nosocomial infections in MUH. This is nearer to 18% rate of nosocomial infection reported by ***Gad* e*t al., (2007)*** from three hospitals in Minia, Egypt. Lower incidence reported by ***Khan et al., (2008)*** in Pakistan 6.67%. In our wok *P. aeruginosa* and MDR- ESβLs producer *P. aeruginosa* infections were most common in burn wound infection followed by urinary tract, respiratory tract and wound infections. The highest incidence of *P. aeruginosa* was found in burn unit and ICU. In concordance with our result ***Aly, (2005)*** who founded that *P.aeruginosa* accounted for (44.4%) of infections in burn unit at MUH. Also **Pathmanathan *et al.,* (2009*)*** concluded that *P. aeruginosa* is a nosocomial pathogen often isolated from burn infections. ICUs are generally considered epicenters of (MDR) organisms **(Ramprasad et al., 2010*)***. MDR

P.aeruginosa had emerged in Egypt in recent years and seen mainly in nosocomial infections ***(Ali et al., 2009).*** In this study the isolated *P. aeruginosa* were investigated for MDR and ESβLs production. We found high prevalence of MDR *P. aeruginosa* (52%) and ESβLs producer strains (45.6%) were found in MUH. This high rate of MDR, has been reported elsewhere in previous studies. In Turkey, ***Unan et al., (2000)*** reported that 60% of his *P. aeruginosa* isolates were MDR. In Egypt, ***Gad et al., (2007)*** observed high levels of MDR *P. aeruginosa* and that β-lactamase production is the main mechanism of resistance (36% were MDR and 95% were ESβLs producer) On the other hand, **Zahra (2011)** in Iran detected lower levels as 30% of their isolates were MDR and only 9.2% were ESβLs producer. Egypt is among the countries that reported high rates of antimicrobial resistance ***(El-Kholy et al., 2003).*** MDR *P. aeruginosa* develops resistance by various mechanisms like multi-drug resistance efflux pumps, production of β-lactamases, aminoglycoside modifying enzymes, and decrease outer membrane permeability ***(Maria et al. 2011)***. Amikacin and imipenem were the most effective drugs against *P. aeruginosa.* The effectiveness of amikacin (80.5% sensitive) over imipenem (66.7% sensitive) against *P. aeruginosa* was corroborated by data from recent researches as **Gad et al., (2007)**. Earlier studies reported that imipenem was the most effective antibiotic against *P. aeruginosa* **Gales et al., (2002)**. However, recent studies demonstrated the evolution of imipenem - resistant strains of *P. aeruginosa* ***(Maria et al., 2011)***. Carbapenems were considered to be the treatment of choice against serious ESβLs associated infections, however resistance to carbapenems, especially in *P. aeruginosa*, results from reduced levels of drug accumulation, increased expression of pump efflux or production of β-lactamases ***(Maria et al., 2011).*** Our study revealed moderate activity of ciprofloxacin 43.6%. There were variable data about ciprofloxacin action .Similar results about cipro against *P. aeruginosa* were detected by ***Gad et al., (2007),*** on the other hand ***Zahra (2011)*** reported that ciprofloxacin exhibited high susceptibility pattern (73.6%). In our research, high resistance rates exhibited by *P. aeruginosa* against cefepime (98%), ceftazidime (91%) and ceftriaxon (87%). This may be due to high ESβLs production in our *P. aeruginosa* isolates used β -lactamase-mediated resistance against cephalosporins. In contrast, cephalosporins tested in a study conducted in Nigeria, showed that 90% of the isolates were sensitive to it ***(Oni et al., 2002).*** This discrepancy can be attributed to the continuous development of MDR strains in different parts of the world, also a considerable geographic difference in the prevalence of ESβLs in different countries. In general, in our work high resistance patterns were exhibited by most of *P. aeruginosa* isolates. Typing of nosocomial isolates is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods. Antibiotyping was very discriminative in our work, 23 different resistance patterns were identified among our *P. aeruginosa* isolates. Profiles (1-8) were prevalent and included 42 strains. MDR were categorized in antibiotype 1, 2 and 3. Antibiogram is a sensitive phenotypic marker, however it has the disadvantage of being non reproducible in many instances due to the exchange of R factor among isolates ***(*Ramprasad et al., 2010*).*** In our work, 27 (47.4%) isolates had plasmid with 7 different plasmid profiles (A-G). This coincides with that reported by **Nikbin et al., (2007*)*** in Iran detected plasmids in 39.8% of his *P. aeruginosa* isolates. In our study, there were a correlation between plasmid profile and ESβLs production of *P.aerugionsa* isolates, it was found that 16 (61.5%) of the 26 ESβLs isolates harbor plasmids of different sizes. Nearer results reported by **Ali et al., (2009)** as 55% of his isolates were ESβLs producers. Lower rates were obtained by ***Anjum and Mir, (2009)*** in Pakistan who found that out of 100 isolates, 33 (33%) were found to be ESβLs producers. In our work, 10 strains of the ESβLs producers were plasmidless and β -lactamase production in these strains could be chromosomally mediated. A finding which

was explained by ***Bradford, (2001).*** In our study, pyocin typing didn't differentiate *P.aeruginosa* isolates belonging to different sources. There were 8 pyocin groups in our work but there were no difference between clinical and environmental isolates. Contrary to our result, **Ramprasad et al., (2010*)*** found pyocine typing is a very sensitive method of typing. A possible common source of infection in our study may be hospital environment, as two environmental isolates were identical by two typing method to 3 clinical isolates. Environmental sources may have a significant role in the transmission of *P. aeruginosa* (***Bradford, 2001)***. This study highlighted the need for additional attention to the disinfection of inanimate objects in the hospital environment to limit the transfer of *P. aeruginosa*. A close relationship was found between the presence of certain plasmids and characteristic patterns of antibiotic resistance as five clusters could be demonstrated among our *P. aeruginosa* isolates, each had the same antibiotic sensitivity pattern, plasmid profile and β-lactamase production. These results are nearer to ***Bradford, (2001)*** who reported that combination of multiple typing methods may lead to more precise results and more efficient tracing of the source of infection. In conclusions, our results clarified that threats of MDR and ESBLs *P. aeruginosa* become of major concern in our hospital and implementation of infection control strategies are major concerns to avoid the spread of this threat. Environmental sources may have a significant role in spread of MDR *P. aeruginosa* among hospitalized patients. Combination of simple typing methods as antibiotyping with plasmid profiling may be the cheapest and effective method for tracing source of infection.

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