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ORIGINAL ARTICLE

Studying Phenotypically the Role of Plasmid in Transferring Resistance among Multidrµg -Resistant *Pseudomonas*

*[1]Mona AbdelAziz Wassef, [2]Maha M. Gaafar, [3]Dina M. Hassan, [4]Noha Salah ElDein**

[1] Professor of Clinical and Chemical Pathology, Faculty of Medicine-Cairo University
[2] Professor of Clinical and Chemical Pathology, Faculty of Medicine-Cairo University
[3] Lecturer of Clinical and Chemical Pathology, Faculty of Medicine-Cairo University
[4] Assistant lecturers of Clinical and Chemical Pathology, Faculty of Medicine-Cairo University

Key words:

MDR Pseudomonas – carbapenem resistance-Plasmid transfer-E-Coli K12

ABSTRACT

Background*: Multidrµg resistant Pseudomonas is one of the rapidly spreading bacteria causing infections with serious outcomes due to limited therapeutic options. Rapid spread of multidrµg resistant bacteria (MDR) has become an emerging threat and a matter of concern worldwide.* ***Objectives:*** *Our work aimed to screen for carbapenem resistance in 250 isolates of MDR Pseudomonas species and investigate phenotypically the role of plasmid transfer in spread of resistance.* ***Methodology:*** *The antimicrobial susceptibility testing for MDR Pseudomonas was done by Kirby-Bauer disk diffusion method and according to the interpretative criteria of CLSI, 2013. Testing was done phenotypically for plasmid mediated transfer of resistance from MDR Pseudomonas as a donor to a plasmid acceptor E-coli K12 as a recipient by applying conjµgation test.* ***Results:*** *The distribution of the MDR Pseudomonas showed that the majority were encountered from ICU (47.6%) compared to inpatient wards (43.2%). Among different samples, the highest prevalence of Pseudomonas isolates was in sputum (46.8%), followed by urine (26.8%), pus (21.6%) while the samples with the lowest prevalence were blood (4%) and ear discharge (2.8%). The antibiogram for MDR Pseudomonas showed that β-lactams demonstrated the highest percentage of resistance 100%, followed by quinolones (88.8%) and aminoglycosides (86.4%). The Screening for carbapenem resistance showed 68% resistance. Out of 250 MDR Pseudomonas species, 30 (12%) showed transferred resistance demonstrated in the trans-conjµgate E-Coli K12 and 10% showed pigment transfer.* ***Conclusion:*** *our study showed a great increase in the spread of carbapenem resistant MDR Pseudomonas species evidenced by the screening for carbapenem resistance that showed a high percentage. Plasmid plays a role as one of the common methods for horizontal gene transfer among microorganisms; it was shown by the phenotypic demonstration of transferred resistance from MDR Pseudomonas to the trans-conjµgate E-Coli-K12. This necessitates more attention to control infections, by rationalizing the use of antibiotics and adherence to infection control measures.*

INTRODUCTION

The world has become highly concerned with the rapid spread of multidrµg resistant bacteria (MDR) which has become an emerging threat [1]. Multidrµg resistant *Pseudomonas* species is one of the rapidly spreading bacteria causing infections with serious outcomes due to limited therapeutic options. The mortality rate of MDR *Pseudomonas* is significantly higher than those caused by susceptible ones [2].

Carbapenem has always been the first option of empirical treatment of many severe infections[3].

Corresponding Author:
Noha Salah ElDein
Assistant lecturers of Clinical and Chemical Pathology, Faculty of Medicine-Cairo University
E-mail: nsal18@yahoo.com; Tel.: 01016935707

However, misuse of antibiotics in hospitals has been reported to be involved in increasing antibiotic resistance and causing a life threatening problem due to limited therapeutic options [4].

Many bacterial isolates that are multidrµg resistant acquire their genes of resistance throµgh acquisition of additional DNA element chromosomally or throµgh transferable plasmids [5].Testing plasmid transfer is valuable in guiding infection control practice in hospitals [6].

METHODOLOGY

The present study was conducted on 250 clinical isolates of multi-drµg resistant *Pseudomonas* species that were collected from hospitalized patients at Kasr

El-Aini Hospital during the period from April 2013 to July 2014.

Inclusion criteria:

Multi-drµg resistant *Pseudomonas* species from any clinical specimen defined as resistant to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes (carbapenems, fluoroquinolones, penicillins/cephalosporins and aminoglycosides) [7].

Exclusion criteria:

This study excluded *Pseudomonas* organisms not reaching the level of defined criteria for multi-drµg resistance.

a. Isolation:

Pseudomonas isolates were obtained after culture of urine specimens on CLED (Oxoid Co. England), while pus, blood and sputum specimens were cultured on blood agar, MacConkey and chocolate agar plates (Oxoid Co. England) and then incubated aerobically at 37C° for 24 hours while chocolate agar plates were incubated at 37C° in CO_2 for overnight [8].

b. Identification:

Isolates were identified by conventional methods such as culture characteristics, oxidase and biochemical reactions. Triple sµgar iron agar (TSI), Lysine iron agar (LIA), motility, ornithine decarboxylase activity, and indole production (MIO), Simmons citrate agar and Urea agar base (Oxoid Co. England).

c. Susceptibility testing:

It was done by Kirby-Bauer method where an inoculum density equivalent to 0.5 McFarland is inoculated on Mueller-Hinton agar plates (Oxoid Co. England) and the susceptibility of isolates was tested to different antimicrobial agents: ceftazidime (CAZ) (30µg/disk), piperacillin (100µg), cefepime (CPM) (30 µg/disk), cefoxitin (FOX) (30µg/disk), amikacin (AK) (30µg/disk), gentamicin (GM) (l0 µg/disk), piperacillin-Tazobactam (TZP), flouroquinolones (levofloxacin and ciprofloxacin) each (5µg/disk), norfloxacin (10µg/disk-only for urine samples, polymixin (PB) (Oxoid Co. England). Also, meropenem, and imipenem susceptibility were determined by standard disk diffusion (SDD) using commercially available disks (Oxoid Co. England) and all were categorized as sensitive, intermediate and resistant as per Clinical Laboratory Standard Institute guidelines CLSI. [9] as shown in table 1.

d. Screening for carbapenem resistance:

It was done by disk diffusion method using imipenem and meropenem commercially available disks and all *Pseudomonas* isolates were categorized as sensitive, intermediate and resistant per CLSI guidelines[9].

Table 1: **Zone diameter interpretative standards for susceptibility of *Pseudomonas aerµginosa CLSI, 2013***

Antibiotic Disks	*Disk content*	*Susceptible*	*Intermediate*	*Resistant*
piperacillin	100µg	≥21	15-20	≤14
piperacillin-tazobactam (TZP)	100/10µg	≥21	15-20	≤14
ceftazidime (CAZ)	30µg	≥18	15-17	≤14
cefepime (CPM)	30µg	≥18	15-17	≤14
imipenem (IPM),meropenem (MEM)	10µg	≥19	16-18	≤15
azteronam (ATM)	30µg	≥22	16-21	≤15
gentamicin (G)	10µg	≥15	13-14	≤12
amikacin (AK)	30µg	≥17	15-16	≤14
ciprofloxacin (CIP)	5µg	≥21	16-20	≤15
levofloxacin (LEV)	5µg	≥17	14-16	≤13
polymixin (PB)	300 unit	≥12	-	≤11
norfloxacin	10µg	≥17	13-16	≤12

e. Plasmid mediated conjugation:

The isolates of MDR *Pseudomonas* were incubated with a totally sensitive plasmid recipient *E-coliK12* on glycerol broth for 48 hours at 37°C. Then, a purity subculture plate was done to separate *E-coli K 12* [10]. The plasmid mediated transfer of resistance was demonstrated by applying anti-microbial susceptibility testing following the interpretative criteria of CLSI. [9] as shown in table 2.

Table 2: Zone diameter interpretative standards for susceptibility of *E-Coli CLSI, 2013*

Antibiotic Disks	*Disk content*	*Susceptible*	*Intermediate*	*Resistant*
piperacillin (PIP)	100µg	≥21	18-20	≤17
piperacillin-tazobactam (TZP)	100/10µg	≥21	18-20	≤17
ceftazidime (CAZ)	30µg	≥21	18-20	≤17
cefepime (CPM)	30µg	≥18	15-17	≤14
imipenem (IPM),meropenem (MEM)	10µg	≥23	20-22	≤19
azteronam (ATM)	30µg	≥21	18-20	≤17
gentamicin (G)	10µg	≥15	13-14	≤12
amikacin (AK)	30µg	≥17	15-16	≤14
ciprofloxacin (CIP)	5µg	≥21	16-20	≤15
levofloxacin (LEV)	5µg	≥17	14-16	≤13
norfloxacin	10µg	≥17	13-16	≤12

RESULTS

Our study was done on 250 clinical isolates of MDR *Pseudomonas* species isolated from samples sent to microbiological laboratory of Kasr El Aini hospitals. The isolates were subjected to screening for carbapenem resistance, testing invitro effect of (β-lactam/ aminoglycoside) combination and plasmid mediated transfer of resistance from *Pseudomonas* (donor) to *E-coli K12* (recipient) by conjugation.

a. Types and sources of samples:

MDR *Pseudomonas* was isolated from different samples in the form of sputum 117 (46.8%), urine 62 (26.8%), wound or pus 54 (21.6%), blood 10 (4%), ear discharge 7(2.8%). These samples were collected from different sources as shown in table 3 and fig 1 and 2 with the highest percentage was recovered from ICU.

Table 3: Types and sources of samples from which MDR *Pseudomonas* were isolated

Sample	*No. (%)*	*Source*		
		ICU	**Inpatient**	**Outpatient**
Sputum	117(46.8%)	80	37	-
Urine	62(26.8%)	20	30	12
Wound swab (pus)	54(21.6%)	15	35	4
Blood	10(4%)	4	6	-
Ear discharge	7(2.8%)	-	-	7
Total	**250**	**119(47.6%)**	**108(43.2%)**	**23(9.2%)**

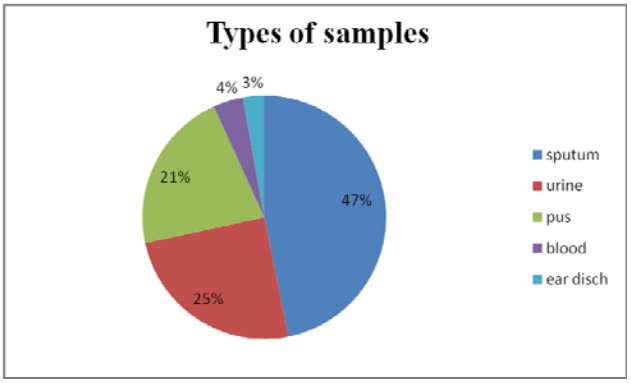

Fig. 1: Types of samples from which MDR *Pseudomonas* were collected

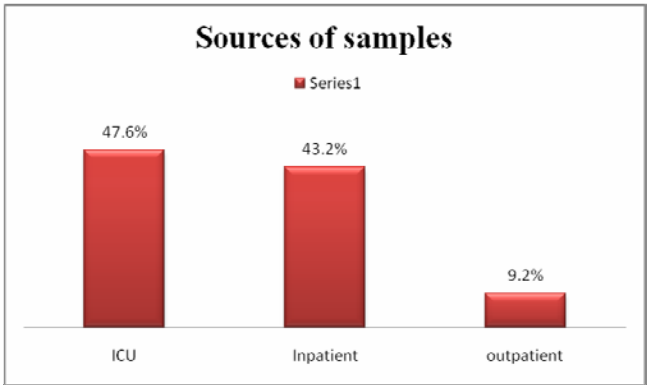

Fig. 2: The sources of isolates of MDR *Pseudomonas* species

b. Results of screening for Carbapenem resistance:

Imipenem and meropenem were used to screen for carbapenem resistance by disk diffusion method. According to the interpretative criteria of CLSI 2013, it was shown that out of 250 MDR *Pseudomonas*, 170 (68%) were resistant to both imipenem and meropenem as shown in table 4 and fig 3

Table 4: Results of screening for carbapenem resistance among MDR *Pseudomonas* species:

	Carbapenems	
	IMI	**MEM**
Sensitive no.(%)	80 (32%)	80 (32%)
Resistant no.(%)	170 (68%)	170 (68%)

IMI: imipenem, MEM: meropenem

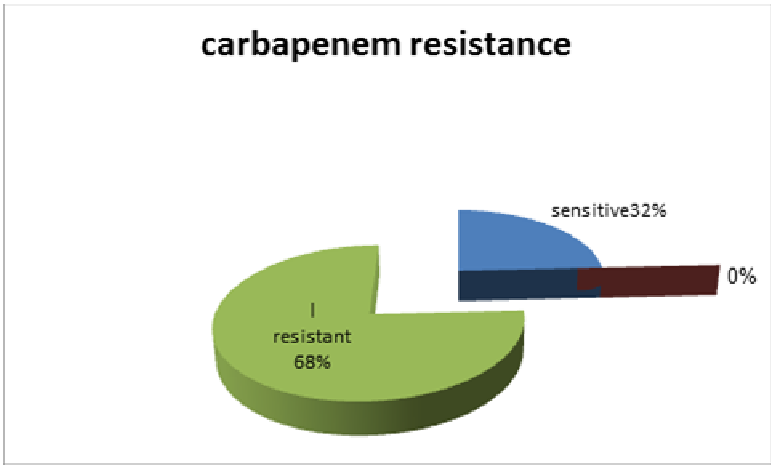

Fig. 3: Results of carbapenem resistance in MDR *Pseudomonas*

The carbapenem resistant *Pseudomonas* was isolated most commonly from sputum 79(46.4%), followed by other samples**,** urine 50(29.4%), pus 32(18.8%), blood 9(5.2%). Also, the ICU was recorded as the major source 89(52.3%) in comparison to other hospital wards 81(47.6%) as shown in fig 4 and 5.

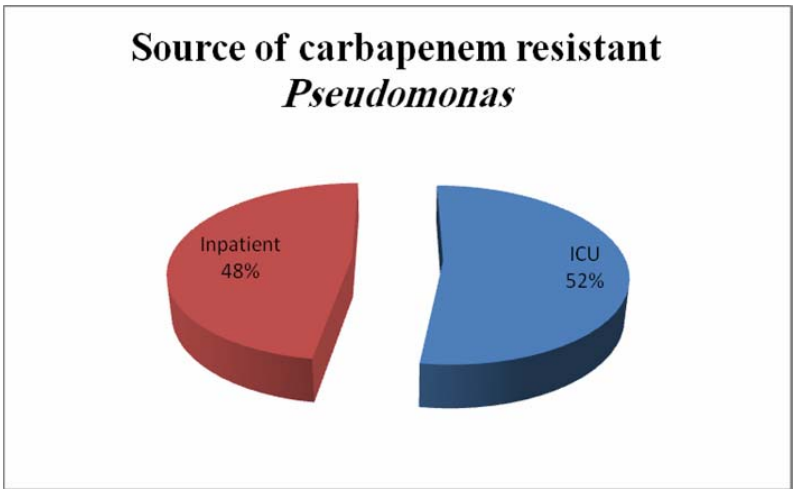

Fig. 4: Source of carbapenem resistant *Pseudomonas*

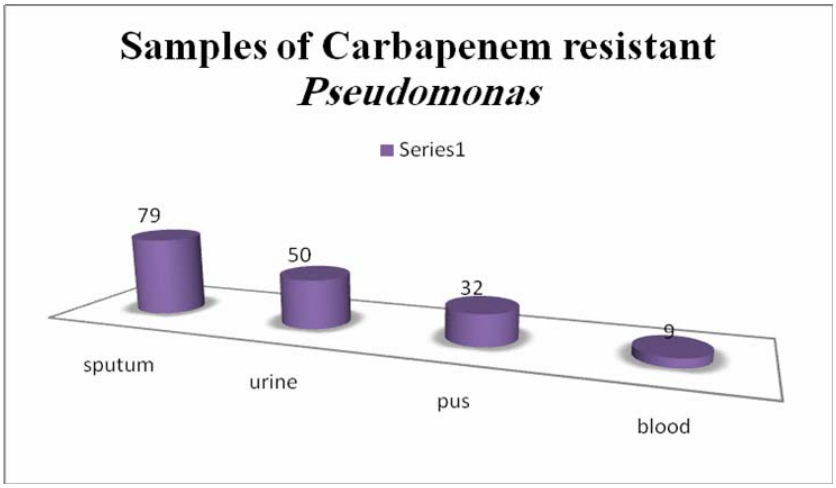

Fig.5: The samples recovering carbapenem resistant *Pseudomonas*

c. Results of susceptibility testing to other groups of antibiotics:

By testing the susceptibility of *Pseudomonas* to other groups of antibiotics using Kirby-Bauer disk diffusion method and according to the interpretative criteria of CLSI, 2013, among the 250 MDR *Pseudomonas* species, the highest percentage of resistance was recorded for β-lactams (100%) and quinolones (88.8%), followed by aminoglycosides (86.4%) and the least was for carbapenems (68%) as shown in table 5 and fig 6.

Table 5: Results of susceptibility testing of MDR *Pseudomonas* species to different groups of antibiotics other than carbapenems

	B-lactams				***Aminoglycosides***		***Quinolones***		***PB***	***fox***
	CAZ	**CPM**	**TZP**	**PIP**	**AK**	**GM**	**CIP**	**LEV**		
S no%	-	-	-	-	34 (13.6%)	34 (13.6%)	28 (11.2%)	28 (11.2%)	250 (100%)	-
R no %	250 (100%)				216 (86.4%)	216 (86.4%)	222 (88.8%)	222 (88.8%)	-	250 (100%)

CAZ: ceftazidime, **CPM:**cefepime, **TZP:**piperacillin-tazobactam,, **AK:** amikacin, **GM:** gentamycin, **CIP:** ciprofloxacin, **LEV:** levofloxacin, **PB:** polymixin B, **FOX:**cefoxitine. **PIP:** piperacillin**S:** sensitive, R: resistant

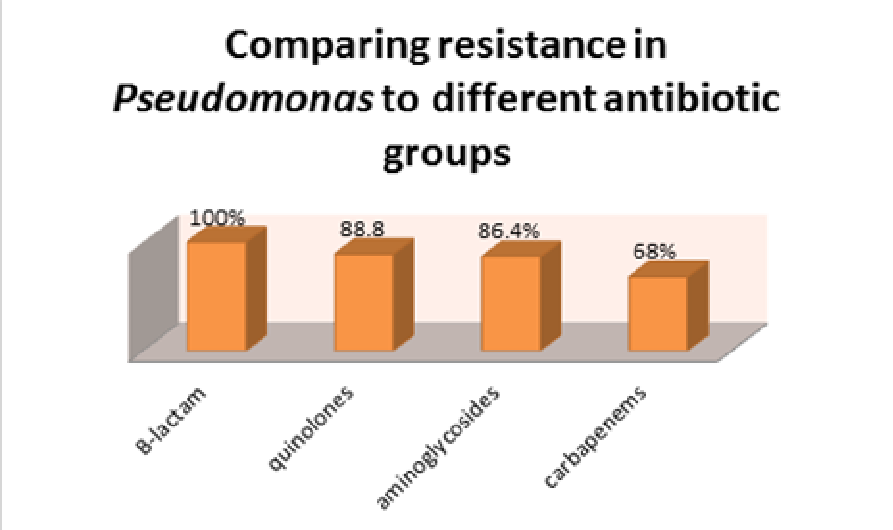

Fig. 6: Comparing percentage of resistance among different antibiotic groups

d. Results of conjµgation experiments:

Out of 250 clinical isolates of MDR *Pseudomonas*, 30 (12%) which were isolated from ICU isolates showed plasmid mediated transfer of resistance to various antibiotics demonstrated phenotypically in trans-conjµgate *E-coliK12* as shown in tables 6 and 7**.**

Table 6: Phenotypic demonstration of antimicrobial susceptibility for the transferred resistance in trans-conjµgate *E-coliK12*:

No. of isolate	B-lactams		Aminoglycosides		Quinolones		Carbapenems	
	P	T	P	T	P	T	P	T
1	R	*	S		R	*	R	*
2	R	*	S		R	*	R	*
3	R	*	S		R	*	R	*
4	R		R	*	R	*	S	
5	R		R		R	*	S	
6	R		R		R	*	S	
7	R	*	S		R	*	R	*
8	R	*	S		R	*	R	*
9	R	*	S		R	*	R	*
10	R	*	S		R	*	R	*
11	R	*	S		R	*	R	*
12	R		R	*	R	*	S	
13	R	*	S		R	*	R	*
14	R	*	S		R	*	R	*
15	R	*	S		R	*	R	*
16	R	*	S		R	*	R	*
17	R		R	*	R	*	S	
18	R	*	s		R	*	R	*
19	R		R		R	*	S	
20	R	*	S		R	*	R	*
21	R		R	*	R	*	S	
22	R		R	*	R	*	S	
23	R		R	*	R	*	S	
24	R	*	S		R	*	R	*
25	R	*	S		R	*	R	*
26	R		R	*	R	*	S	
27	R		R	*	R	*	S	
28	R		R	*	R	*	S	
29	R		R		R	*	S	
30	R		R		R	*	S	

* : transferred resistance to trans conjugate *E-coli K12*

P: original *Pseudomonas* isolate (donor), S: sensitive, R: resistant

T: trans conjugate *E-coli K12* (recipient)

Table 7: Patterns of transferred resistance to the 30 trans-conjµgate *E-coliK12*

Number (%)	***Pattern of resistance***
16/30 (53.3%)	R to B-lactams, quinolones, carbapenems
9/30 (30%)	R to quinolones and aminoglycosides
5/30 (16.6%)	R to quinolones

R: resistant

Also, out of 100 (40%) blue green pigment (pyocyanin) producing *Pseudomonas*, 10 (10%) showed plasmid mediated pigment transfer to the trans conjugate *E-coli K12*.

DISCUSSION

The worldwide emergence of multidrug resistant strains in hospitals and community continues to be a problem of due scientific concern. The emergence of MDR *Pseudomonas aeruginosa* is a challenging clinical problem worldwide associated with increase in rates of morbidity and mortality [11].

Classic agents that used to treat MDR *Pseudomonas* have become outdated. Of the new drμgs available, many have already become targets for bacterial mechanisms of resistance [12].

Over the last few years, carbapenem drugs have become important therapeutic resources for the control of *Pseudomonas aeruginosa* infections. However, growing resistance to imipenem and other carbapenems has been observed and multidrug resistance has become more common. The present study was conducted on 250 isolates of MDR *Pseudomonas*, among which we screened for carbapenem resistance by disk diffusion method using imipenem and meropenem disks and tested plasmid mediated transfer of resistance to *E-coli K12*.

In our study, the samples that showed the highest prevalence of MDR *Pseudomonas* were sputum (46.8%), followed by urine (26.8%), pus (21.6%) while the samples with the least prevalence were blood (4%) and ear discharge (2.8%).

A study done in King Saud University by Shaikh et al.[11] demonstrated a sample distribution close to our study, where it encountered *Pseudomonas* exhibiting multidrμg-resistance with the highest prevalence among sputum (41.67%), followed by pus (28.36%), urine (20.45%), CSF and other body fluids (21.74%) and the least prevalence was in the blood (13.79%).

In another study done in Egypt (Assuit) by Afifi et al.[13] also, demonstrated the highest prevalence of *Pseudomonas* in sputum (35%) followed by urine (8%). However, lower frequencies were found previously in Egypt, sputum was (24%) and urine was (10%) [14]. Moreover, other studies encountered lower prevalence in sputum and urine as in Iraq (4.61%) and (9.8%) respectively and in Nigeria, the prevalence was (11.3%) and (51.1%) respectively [14,15,16].

In all previous studies, the high prevalence of MDR *Pseudomonas* in sputum and urine was explained by the correlation with the use of ventilators and urinary catheters as the *Pseudomonas* is one of the bacteria that commonly contaminate ventilators and urinary catheters with biofilm formation that confers multiple resistances in *Pseudomonas* to various drμgs [13].

In our study, the majority of MDR *Pseudomonas* isolates were encountered from ICU (47.6%) compared to inpatient wards (43.2%) and outpatient (9.2%). This was close to another study in Japan done by Shrestha et al.that demonstrated MDR *Pseudomonas* with the highest prevalence in ICU (57%) in comparison to other medical wards [17].

As referred to a study done by Boyer et al., the high prevalence of MDR *Pseudomonas* in ICU is related to the patient colonization by *Pseudomonas* which is attributed to both chronological components along with selective antibiotic pressure. The antibiotic selective pressure alone did not influence *Pseudomonas* acquisition. The hypothesis of a complex mechanism involving antibiotic selective pressure and patient colonization pressure should be relevant for *Pseudomonas* acquisition in an ICU. That is why, developing strategies for either decreased antibiotic use "endogenous like" acquisition, or infection control improvement in response to environmental contamination "exogenous like" acquisition are necessarily recommended [18].

As regard screening for carbapenem resistance, we chose to screen for carbapenem resistance by the use of imipenem and meropenem disk diffusion method being a reference method. In comparison to other methods of screening, a study was done to demonstrate the difference in susceptibility testing using E-test versus Microscan in comparison to disk diffusion as a reference method. It was shown that there is a discrepancy in results where E-test showed error for imipenem and meropenem (34.9%-34.2%) respectively while Microscan showed 10% error [19].

The results of screening for carbapenem resistance according to the interpretative criteria of CLSI, 2013 have shown 68% resistance among 250 MDR *Pseudomonas* to both imipenem and meropenem. In a similar study conducted on 261 MDR *Pseudomonas* met an agreement with our results showing 71% carbapenem resistance in MDR *Pseudomonas*[4]. This was in accordance to another study demonstrating high percentage of resistance to Imipenem 87.8% [20].

Our results also, tend to be close to a study done by Babay et al. that recovered 72% resistance to imipenem among *Pseudomonas* [19]. However, it showed a lower resistance 35% to meropenem in comparison to our study and this can be attributed to our high consumption rate of both imipenem and meropenem as empirical therapy in the clinical field. Another study done in India done by Behera et al. demonstrated a matching result of 69% carbapenem resistance in *Pseudomonas*, however, it screened for carbapenem resistance by imipenem E-test [21].

The high percentage of carbapenem resistance among MDR *Pseudomonas* species encountered in our study as well as the previously mentioned studies was almost attributed to the high antibiotic pressure due to prolonged exposure to carabapenems which is recognized as important contributor to the acquisition of MDR *Pseudomonas* species.

On the contrary, several studies experienced significant lower percentage of resistance to carbapenems among *Pseudomonas*,45% in Brazil, 37% in Tunis, 19.25% in Manipal, 13.4% in Kashmir 17.4% in Romania [22,23,24,25,26].

The studies that opposed our results by experiencing a lower percentage of resistance to carbapenems can be explained by i) following a good policy of antibiotic stewardship and limiting the use of antibiotics, ii) applying interpretative criteria different from CLSI, 2013, iii) some studies were applied for single sample type as in VAP pneumonia., iv) other studies conducted their work up on a smaller sample size than our study, vi) screening for carbapenem resistance in *Pseudomonas* species which is not multidrµg resistance because carbapenem resistance is more frequent in MDR *Pseudomonas* as it develops frequently due to the concomitant presence of more than one mechanism [27].

A study was held over an extended period of time 5 years (2004-2008) observed a creep of rising resistance to carbapenems among *Pseudomonas* species which detected 6.06% in 2004, rising to 15.38% in 2005, reached 45% in 2008 [22].

On the contrary, another 3 year study held (2008-2010) observed a decline in the percentages of carbapenem resistance among *Pseudomonas* which detected 27% resistance in 2009 and decreased to 16% in 2010. This can be explained by the recent concern about emergence of antibiotic resistance and the attention that has been recently directed towards restricting the use of antimicrobial agents [28].

In our study, among 250 MDR *Pseudomonas* isolates, B-lactams demonstrated the highest percentage of resistance 100%, followed by quinolones (88.8%) and aminoglycosides (86.4%). This typically matched with a similar study that demonstrated the highest resistance with ceftazidime (95.79%), followed by ciprofloxacin (92.34%) and gentamycin (87.36%) [4]. However, another study showed highest resistance for carabpenems (100%), followed by cephalosporins (91%) and aminoglycosides (21%) [29].

The overall evolution of antibiotic resistance can be attributed to various factors like spread of transposons or R-plasmids to various pathogens mainly because of the selective forces imposed by human due to the overuse of antibiotics. The gene transfer of resistance mediated by plasmid has been implicated in spread of resistance among *Pseudomonas* species which leads to big challenge in the control of multidrµg resistance [30].

In our study, out of 250 clinical isolate of MDR *Pseudomonas* , 30 (12%), all of which from ICU, showed plasmid mediated transfer of resistance to various antibiotics demonstrated in the transconjµgate *E-Coli K12* strain. It was noted that the highest percentage 16 (53.3%) out of 30 transconjµgate *E-coli K12* became resistant to most of the antibiotics used (B-lactams, quinolones, carbapenems) while 9(30%) became resistant to aminoglycosides and quinolones and 5(16.6%) showed resistance only for quinolones. This denotes that most of antibiotic resistance determinants have been transferred from donor cell to recipient cell. This tends to be close to a similar study that demonstrated plasmid mediated transfer of resistance in 11.4% of MDR *Pseudomonas* isolates [10].

However, our results show lower percentages of plasmid transfer in comparison to another study was done in Iran in year 2014 which experienced plasmid mediated transfer of resistance among *Pseudomonas aerµginosa* that reached 60%. In this study, the plasmid DNA was extracted and examined by gel electrophoresis, however, in our study, the plasmid mediated transfer of resistance was demonstrated throµgh antimicrobial susceptibility testing done to the recipient strains phenotypically.This may carry a limitation in that some genes can be transferred but not expressed [31].

A point of interest to mention, that in our study 10% of number 100 pigment producing *Pseudomonas* showed pigment transfer to the transconjµgate *E-Coli K12*which met an agreement with another study that also, experienced pigment transfer from *Pseudomonas aerµginosa* as a donor to recipient transconjµgants [32].

The concern about pigment production is that the pyocyanin (blue-green) pigment produced by *Pseudomonas* is an active metabolite that can be transferred with antibiotic resistance. Also, it has been determined to display antibiotic, antifungal, cytotoxic properties, therefore, contributes to the pathogenesis *of Pseudomonas aerµginosa* and considered to be an infection associated virulence factor [32].

CONCLUSIONS

In our study the screening for carbapenem resistance among MDR *Pseudomonas* showed a great increase in the spread of carbapenem resistant MDR *Pseudomonas*. The antimicrobial susceptibility testing of MDR *Pseudomonas* showed that B-lactams demonstrated the highest percentage of resistance, followed by quinolones and aminoglycosides. Plasmid plays a role as one of the common methods for horizontal gene transfer among microorganisms. It was noted that the highest percentage of transconjµgate *E-coli K12* became resistant to most of the antibiotics used (B-lactams, quinolones, carbapenems). This was shown by the phenotypic demonstration of transferred resistance from MDR *Pseudomonas* to the trans-conjµgate *E-Coli-K12* using conjµgation test. Controlling the high rates of resistance necessitates more attention to rationalization in the use of antibiotics and adherence to infection control measures.

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