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Occurrence of carbapenem multidrug-resistant *Pseudomonas aeruginosa* carrying bla_{VIM} metallo-β-lactamases and their biofilm phenotypes in Al-Azhar University Hospital

Rehab Mousa[1,2]*, Magdy F. El-Kady[3], Asmaa A. A. Hussein[4], Amr E. Ahmed[2], Ahmed Osama El-Gendy[5]

[1]Pharmacy Department, Al-Azher University Hospital, Assiut, Egypt.
[2]Deparmtent of Biotechnology and Life Sciences, Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Beni-Suef, Egypt.
[3]Department of Poultry Diseases, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.
[4]Department of Zoonoses, Faculty of Veterinary Medicine, and Dean of Molecular BiologyResearches & Studies Institute, Assiut University, Assiut, Egypt.
[5]Department of Microbiology and Immunology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt.

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ABSTRACT

Pseudomonas aeruginosa are quite a common cause of hospital-acquired infections. The goal of this study was to characterize *P. aeruginosa* isolates and identify the prevalence rate of *P. aeruginosa* resistant to imipenem due to metallo-ß-lactamase (MBL) genes. A total of 74 *P. aeruginosa* isolates were obtained between November 2015 and April 2017 from Al-Azhar University Hospital, Assiut, Egypt. The disk diffusion method was applied to determine antimicrobial resistance patterns according to the Clinical and Laboratory Standards Institute, and the microtiter plate assay was managed to explore the biofilm formation. The MBL genes, bla_{IMP} and bla_{VIM}, were finally identified using the polymerase chain reaction (PCR) within imipenem-resistant strains. The rate of resistance to imipenem was 36.5%, and the rates of resistance for *P. aeruginosa* to carbenicillin, gentamicin, ciprofloxacin, ceftazidime, levofloxacin, and amikacin were 94.6%, 70.3%, 68.9%, 66.2%, 62.2%, and 58.1%, respectively. About 69% of the strains were MDR, and 86.27% of the multi-drug resistant (MDR) strains were biofilm producers. PCR showed that eight strains of imipenem-resistant *P. aeruginosa* contained bla_{VIM}, while bla_{IMP} gene was not detected. Possession of nosocomial multi-drug resistant *P. aeruginosa* infections was significantly associated with past antibiotic therapy or catheterization ($p < 0.05$). Moreover, this study demonstrates that the degrees of antibiotic resistance and biofilm production are directly linked. Ongoing antimicrobial susceptibility testing and antibiotic prescription surveillance are critical for enhancing antimicrobial stewardship measures. Additionally, further transmission of nosocomial diseases must be prevented by infection control method.

INTRODUCTION

For hospitalized patients with comorbidities and mortality risk factors, nosocomial infection was considered the leading reason for mortality and morbidity, and it was linked to increased healthcare and financial expenses (Hirsch and Tam, 2010).

Pseudomonas aeruginosa is an opportunistic Gram-negative organism accountable for 51,000 healthcare-associated infections and leads to serious and potentially fatal nosocomial infections, such as urinary tract infections, respiratory tract infections, and bacteremia, in immunocompromised patients (Ruppé *et al*., 2015).

The innate and acquired machineries of antibiotics resistance were leading risk factors for spreading this organism in healthcare places (Landman *et al*., 2002; Lister *et al*., 2009).

Pseudomonas aeruginosa has developed resistance to various antibiotic families, including carbapenems, which are considered trustworthy and efficient antibiotics that are only used as a last resort for the treatment of serious diseases till now (Sekiguchi *et al*., 2005).

Metallo-ß-lactamase (MBL) assembly is the leading source of extraordinary imipenem resistance in between *P. aeruginosa* isolates (Franco *et al*., 2010). This study aimed to

*Corresponding Author
Rehab Mousa, Al-Azher University Hospital, Assiut, Egypt.
E-mail: rehabsalah2002 @ yahoo.com

isolate and identify *P. aeruginosa* from Al-Azhar University Hospital, identify the measurement of antibacterial activity of different groups of antibiotics on *P. aeruginosa*, determine the incidence of multi-drug resistant (MDR) *P. aeruginosa* in infected patients, and detect MBLs genes among MDR *P. aeruginosa* strains using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Isolation and identification P. aeruginosa

This study included 74 *P. aeruginosa* isolates recovered from 412 clinical isolates taken from patients urine admitted to wards/intensive care units (ICUs) and who acquired nosocomial infections after excluding signs or symptoms of infection at the time of admission. This cross-sectional observational study was carried out from November 2015 to April 2017 in Al-Azhar University Hospital and Molecular Biology Research Unit, Assiut University. All recovered isolates were identified using conventional protocols and validated by growing them on Cetrimide agar and Pseudomonas agar and examining the morphology of their colonies (Govan, 2007).

General information was collected, including age, sex, managing unit, and reason for ICU admission, risk factors as diabetes mellitus, previous operations, catheterizations, total parenteral nutrition and antibiotics used during the hospital stay, and drugs used for prolonged periods.

Antimicrobial susceptibility of P. aeruginosa

Three to five visually identical pure colonies were suspended with 3–4 ml of sterile saline. The turbidity was adjusted to a 0.5 McFarland turbidity standard (approximately 10^8 colony forming unit/ml). A sterile cotton swab was immersed into the bacterial solution, spinning it firmly and evenly distributing bacteria over the whole agar plate. A disk dispenser was used to insert antibiotic disks into the agar plates, and the plates were incubated at 37°C for 18 hours. The zone of inhibition diameters was recorded, and the results were interpreted using Clinical and Laboratory Standards Institute guidelines (Wayne, 2011). For each isolate, a resistance score (R score) was calculated based on the number of antibiotics that were resistant (a score of 0.5 considered intermediately resistant while a score of 1 was resistant).

Exploring biofilm formation using microtiter plate assay technique

Microtiter plate assay was accomplished to evaluate biofilm formation for MDR *P. aeruginosa* urine isolates, according to Stewart *et al*. (2004). Briefly, *P. aeruginosa* was cultured overnight in trypticase soy broth (TSB), and then 200 µl of 1% diluted bacteria was applied to 96-well flat-bottom polystyrene microtiter plates. The inoculated plate was incubated at 30°C for 22–24 hours and phosphate-buffered saline (PBS, pH 7.2) was used in three successive washing steps to allow the non- or loosely attached bacteria to be discarded.

A 100 µl of a 0.1% crystal violet was applied to wells of the microtiter plate, and the plate was then incubated at room temperature for 30 minutes. After several washing steps, 200 µl of 95% ethanol was used to solubilize the crystal violet staining of the biofilm, and 125 µl of the extracted color was transferred to a new microtiter plate. The absorbance was measured at 450 nm using a plate reader. Measurements were carried out in triplicate. According to the criteria of Stepanovic *et al*. (2007), the nonbiofilm producer is indicated when optical density (OD) ≤ ODc, weak biofilm producer is defined as OD > ODc and ≤ 2× ODc, moderate biofilm producer is defined as OD > 2× ODc and ≤ 4× ODc, and strong biofilm producer is indicated when OD > 4× ODc (Stepanovic *et al*., 2007).

Detection of bla_{IMP-1} and bla_{VIM-1} genes by PCR

The bacterial colonies were subcultured into TSB for 24 hours at 37°C. To extract the genomic DNA, the cells grown in suspension were collected by centrifugation for 5 minutes at 8,000 rpm in a 1.5 ml Eppendorf tube. Cell pellets were resuspended in 200 µl PBS, and an equal volume of QIAGEN protease was added. A 200 µl buffer AL was added, followed by vortexing for 15 seconds. After incubating the samples at 56°C for 10 minutes, 200 µl of 96% ethanol was added and mixed by vortexing for 15 seconds. The mixture was centrifuged at 8,000 rpm for 1 minute using the QIAamp Mini spin column. The washing protocol was followed according to the manufacturer's instructions before the final elution step and the collection of the extracted DNA.

PCR amplification was accomplished using specified primers (Table 1), using the thermocycler (Personal Thermocycler Model, Biometra, Germany). An amplification program for the detection of bla_{IMP-1} and bla_{VIM-1} genes was conducted according to Rodrigues *et al*. (2011) and Shibata *et al*. (2003).

Statistical analysis

Various data visualization, graphing, statistical calculations, data entry, and data analysis systems and software were used as Statistical Package for Social Science (Inc., Chicago, version 16). Statistical significance was recognized when $p < 0.05$. Using R (https://www.r-project.org) and the ggplot2 package, data visualization was carrieed out. The unweighted pair group technique with the arithmetic mean (UPGMA) clustering (http://genomes.urv.cat/UPGMA) algorithm was employed for constructing a distance matrix and a tree, which was produced with the free software application FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Pseudomonas aeruginosa isolates were found in 18% (74/412) of clinical isolates. Of them, the resistance rate to imipenem, carbenicillin, gentamicin, ciprofloxacin, ceftazidime,

Table 1. Nucleotide sequences of primers used for the detection of *IMP* and *VIM* genes.

Amplicon size (bp)	Nucleotide Sequence (5′–3′)	Primers
587	F (ACCGCAGCAGAGTCTTTGCC) R (-ACAACCAGTTTTGCCTTACC)	IMP
261	F (A G T G G T G A G TAT C C G A C A G) R (-ATGAAAGTGCGTGGAGAC -)	VIM

Adapted from (Rodrigues *et al*., (2011) and; Shibata *et al*., (2003).

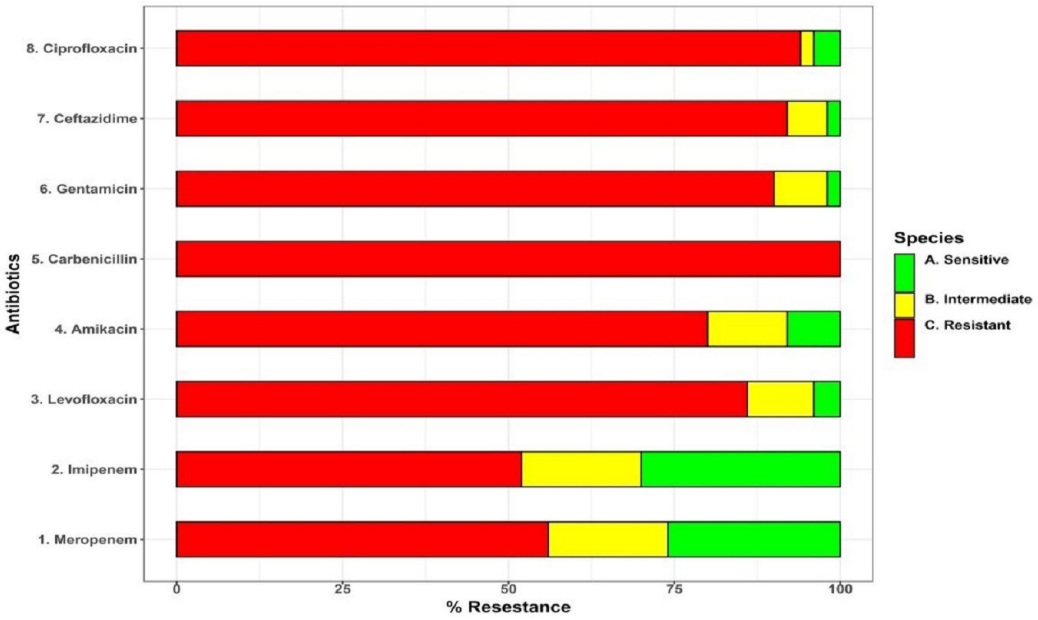

Figure 1. (A) A stacked bar plot that depicts the 74 *P. aeruginosa* isolates' distribution of resistance to eight antibiotics (Y-axis). (B) A heatmap that represents resistance phenotypes of 74 *P. aeruginosa* isolates (red = resistant, yellow = intermediate, and green = sensitive). The X-axis describes isolates, whereas the Y-axis shows antibiotics. The horizontal and vertical trees reflect hierarchical clustering, which is used to order isolates and antibiotics, respectively.

levofloxacin, and amikacin was 36.5%, 94.6%, 70.3%, 68.9%, 66.2%, 62.2%, and 58.1%, respectively (Supplementary Table S1). A heatmap was conducted to compare the antibiotic resistance among 74 isolates of *P. aeruginosa,* depicting the relative resistance of each isolate (Fig. 1A and B). The clustering dendrogram was created using the UPGMA (Fig. 2) to show relations between different isolates phenotypes. However, the tree has the benefit of allowing one to see the more obscure phenotypes, like that of isolate P59 (sensitive to four antibiotics).

Out of the 74 *P. aeruginosa* isolates, 51 (69%) strains were MDR (resistance to at least three antipseudomonal antibiotic groups including β-lactams, aminoglycosides, and fluoroquinolones), and 23 (32%) strains were non-MDR strains. Isolates were classified into four classes according to the OD of the bacterial biofilms (ODc was calculated as 0.064): nonbiofilm forming (if OD ≤ 0.064), weak biofilm forming (if OD > 0.064, but ≤ 0.128), moderate biofilm forming (if OD > 0.128, but ≤ 0.256), and strong biofilm forming (if OD > 0.256).

A 44/51 (86.27%) of MDR strains were biofilm producers; 33.33% of them were strong biofilm producers, 29.41% of them were moderate, and 23.53% of strains were weak biofilm producers.

There was a relationship between biofilm formation and R score, with significant biofilm formation associated with isolates with high R scores contrasted to poor biofilm formation associated with isolates with low R scores, as seen in (Fig. 3).

PCR showed that eight strains of imipenem-resistant *P. aeruginosa* contained bla_{VIM}, while bla_{IMP} gene was not detected, as shown in (Fig. 4).

Patients positive for MDR *P. aeruginosa* (n = 51) were compared with patients positive for non-MDR *P. aeruginosa* (n = 23) to determine factors associated with the acquisition of multidrug-resistant phenotype. Several variables were studied, including age, gender, prior antibiotic treatment, catheterization, central line in ICU, parenteral nutrition, renal diseases, and diabetes mellitus. p-value was calculated for each variable, and significance was determined when the p-value was lower than 0.05.

Table 2 illustrates the risk factor for the nosocomial multi-drug resistant P. aeruginosa (MDRPA) and non-MDRPA infection. Possession of nosocomial MDRPA infections was significantly associated with past antibiotic therapy or catheterization (p < 0.05). The other risk factors, including age, sex, mechanical ventilation, central line in ICU, parenteral nutrition, and diabetes mellitus, were not significant with MDRPA infection.

DISCUSSION

During the study period, nosocomial *P. aeruginosa* accounted for 18% of all isolated uropathogens. This finding agreed to some extent with that reported by Gad *et al*. (2007), who identified *P. aeruginosa* isolates in 18.2% of the clinical samples, and Wassef *et al*. (2015), who identified *P. aeruginosa* isolates in 20.7% of different clinical specimens. Furthermore, it was higher than that obtained in Iran, where the rate of incidence of *P. aeruginosa* in UTI was 13.2% (Aminizadeh and Kashi, 2011). However, these results were less than those found by Mansour *et al*. (2013), who reported that the frequency of isolation of *P. aeruginosa* from clinical specimens in Egypt and Saudi Arabia was 32.8% and 30.0%, respectively.

In the current study, urinary isolated *P. aeruginosa* had a resistance rate of 94.59% to carbenicillin and 66.2% to ceftazidime. The same was reported by Kotsakis *et al*. (2010), who revealed that 65.4% of *P. aeruginosa* were resistant to ceftazidime. Our result was lower than that of Arora *et al*. (2011), who reported that 73.2% of *P. aeruginosa* were ceftazidime-resistant. At the same time, Manjunath *et al*. (2011) reported higher rates (69.3%) of carbenicillin.

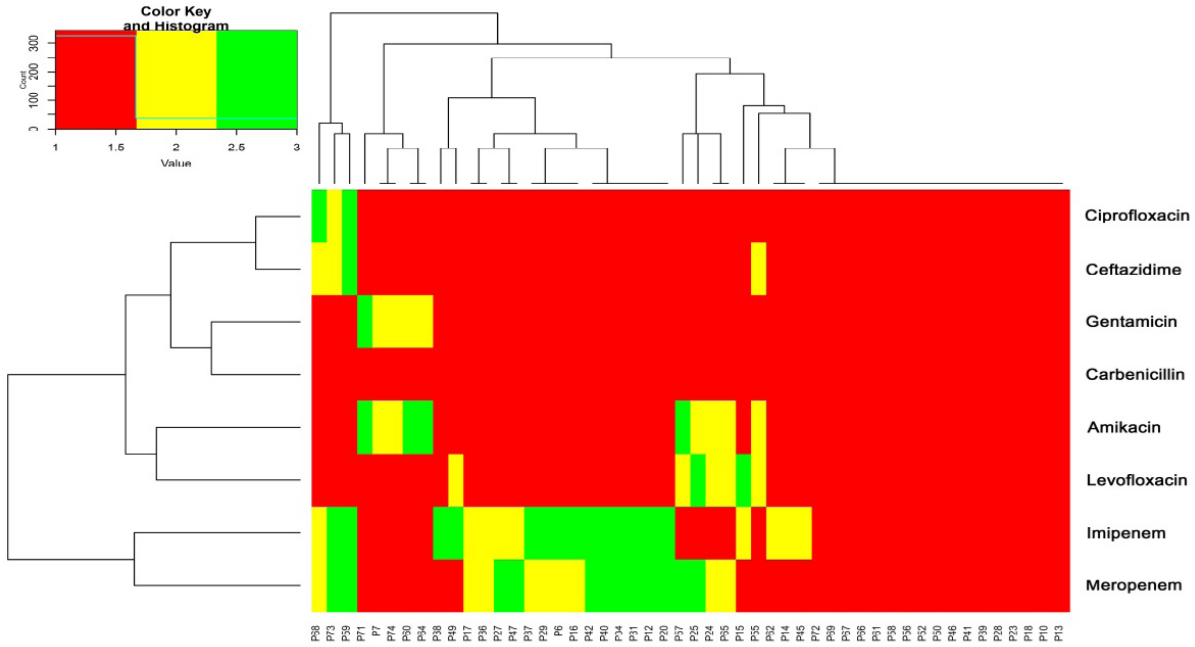

Figure 2. Circular tree depicting the relationships between 74 *P. aeruginosa* isolates based on antibiotic susceptibility phenotypes.

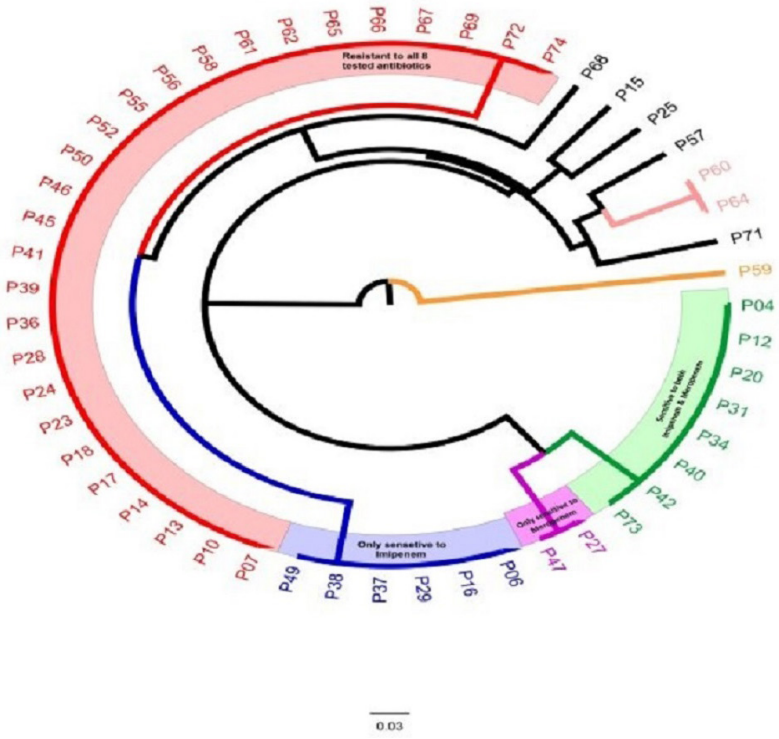

Figure 3. Boxplots depicting the R scores of *P. aeruginosa* isolates with relationship to their biofilm formation.

In our study, the resistance rate of *P. aeruginosa* to imipenem was 36.5% and to meropenem was 39.2%. Our results were in agreement with those reported by Mahmoud *et al*. (2013), who showed that 33.3% of *P. aeruginosa* strains were resistant to imipenem. Our results were higher than those described by Fazlul *et al*. (2011), who showed that imipenem and meropenem were found to be more effective agents, where the resistance of the *P. aeruginosa* isolates toward these antibacterial agents was 18.5% and 20.4%, respectively. Carbapenem resistance rates can vary according to local antibiotic regulations, strain origin, and geographic location.

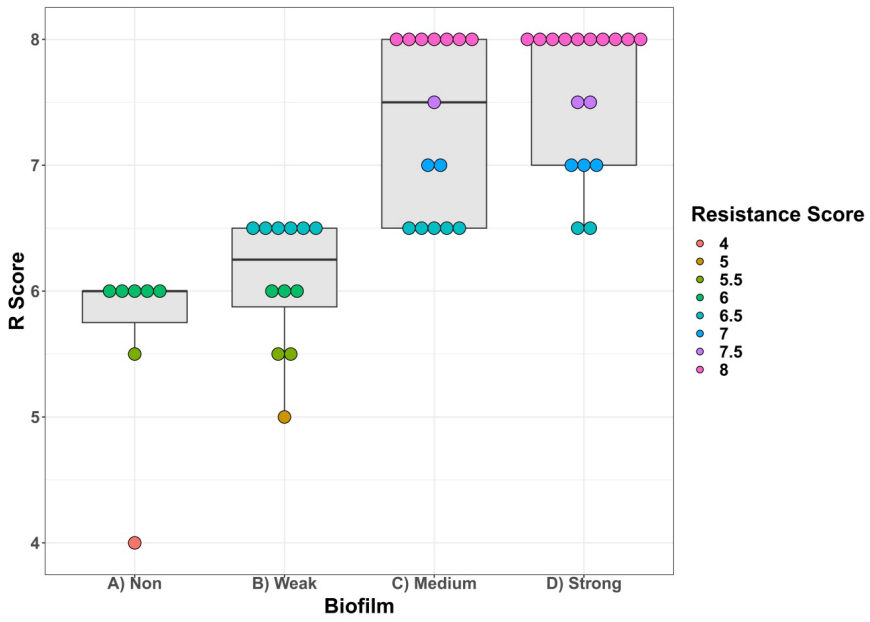

Figure 4. Gel electrophoresis of bla_{VIM-1} gene PCR product. Lane M is the 1 kb ladder; lanes 1, 3,–8 show bla_{VIM-1}-specific band size product.

Table 2. Risk factors for nosocomial MDRPA infection in the 51 case.

Test of significance (*p*-value)	Number of patients positive for NON-MDRPA (*n* = 23)		Number of patients positive for MDRPA (*n* = 51)		Risk factor
	%	No	%	No	
			Age groups		
Mcp = 0.58	8.7	2	5.9	3	5−20 years
	30.4	7	17.6	9	21−40 years
	26.1	6	31.4	16	41−60 years
	34.8	8	45.1	23	61−75 years
			Gender		
$\chi^2 = 0.04$ $p = 0.8$	60.9	14	66.7	34	Males
	39.1	9	33.3	17	Females
			Prior antibiotic treatment		
$\chi^2 = 5.2$ $p = 0.02$*	56.5	13	84.3	43	Yes
	43.5	10	15.7	8	No
			Renal disease		
$\chi^2 = 0.03$ $p = 0.8$	26.1	6	31.4	16	Yes
	73.9	17	68.6	35	No
			Catheterization		
$\chi^2 = 8.1$ $p = 0.004$*	39.1	9	23.5	39	Yes
	60.9	14	15.7	12	No
			Central line in ICU		
$\chi^2 = 1.4$ $p = 0.23$	34.8	8	31.4	27	Yes
	65.2	15	47.1	24	No
			Parenteral nutrition		
$\chi^2 = 0.29$ $p = 0.6$	13	3	21.6	11	Yes
	87	20	78.4	40	No
			Diabetes mellitus		
$\chi^2 = 0.05$ $p = 0.8$	21.7	5	27.5	14	Yes
	78.3	18	72.5	37	No

MCP = Montecarlo test; χ^2 = Chi-square test.

*Statistically significant.

The misuse of antimicrobial agents can explain this rate of carbapenem resistance in the last decade, especially in developing countries which allow the bacteria to modify the resistant mechanisms (Picao *et al*., 2009). *Acinetobacter* and *Pseudomonas* bacteria had the highest resistance levels to imipenem in Egypt (37.03%) (Ashour and El-Sharif, 2009). The emergence of imipenem-resistant strains of *P. aeruginosa* in the Middle East is concerning, particularly in Saudi Arabia, where the resistance rate of *P. aeruginosa* to imipenem has risen to 38.57% (Al-Agamy *et al*., 2011).

The antibiotic resistance pattern in *P. aeruginosa* isolates to aminoglycoside was relatively high (58.1% to amikacin and 70.3% gentamicin). These results are close to those obtained by Mohanasoundaram (2011). Arora *et al*. (2011) found that 79% and 41.5% of *P. aeruginosa* isolates were gentamicin- and amikacin-resistant, respectively.

Fluoroquinolones are most commonly used in urinary tract infections. The resistance to ciprofloxacin and levofloxacin was 68.9% and 62.2%, respectively. These rates were lower than those reported in India, where the rate of ciprofloxacin resistance was 85% (Manjunath *et al*., 2011).

In our study, the prevalence of MDR strains among *P. aeruginosa* urine isolates was 51 (68.92%). Our findings are consistent with previous research, which found that the percentage of MDR in *P. aeruginosa* had grown from 64% in 2008 to 71% in 2011 (Mohanasoundaram, 2011). Our findings were greater than those of Fazlul *et al*. (2011), who reported that the prevalence of MDR in *P. aeruginosa* isolated from catheterized patients' urine was 40%. However, the result was 91.6% lower than that reported by Paranjothi and Dheepa (2010). This correlates with the fact that a growing number of places of the world are witnessing a rise in the MDR of *P. aeruginosa*, which raises a critical treatment challenge.

In our study, biofilm production of nosocomial MDR *P. aeruginosa* strains was estimated by 44/51 (86.27%) of MDR strains.

Our results are comparable to those of Jain and Agarwal (2009) and with a study undertaken by Bendouah *et al*. (2006), who reported that biofilm production was noted in 6/10 *P. aeruginosa* isolates. Yang *et al*. (2005) also supported our results, who declared that biofilm formation is an important phenotype associated with chronic *P. aeruginosa* infections. On the other hand, our results disagree with Delissalde and Amabile-Cuevas (2004), who found that only 14% of their *P. aeruginosa* isolates produced biofilm after 8 hours and 8% after 24 hours incubation.

The appropriate treatment of infected patients and the management of nosocomial spread of resistance are both dependent on reliable detection of the MBL-producing strains (Walsh *et al*., 2005). The prevalence of MBL-producing strains has increased significantly in Latin America (Sader *et al*., 2005). In Brazil, the prevalence of MBL-producing *P. aeruginosa* is ranging from 7.5% to 44% in different hospitals (Mendes *et al*., 2006).

In the present study, 8 out of 27 imipenem-resistant P. aeruginosa isolates were found to harbor bla_{VIM}, while bla_{IMP} was not discovered in any of them. This finding was in harmony with the results of Walsh *et al*. (2005), demonstrating that bla_{VIM} was the most dominant MBL that mediated the imipenem-resistant properties of *P. aeruginosa* and constituted as the greatest clinical threat (Walsh *et al*., 2005). In 2012, 100 *P. aeruginosa* isolates from Shahid Motahari Hospital in Teheran were checked to detect bla_{IMP} and bla_{VIM} and forty-48 out of 83 (57.9%) imipenem-resistant *P. aeruginosa* showed MBL activity while 12% of them had only bla_{VIM} gene (Fallah *et al*., 2013).

On the other hand, our results disagree with a study from Iran, which reported that 8 (9.75%) and 10 (12.19%) of MBL-producing *P. aeruginosa* isolates were positive for bla_{IMP-1} and bla_{VIM-1} genes, respectively (Sarhangi *et al*., 2013).

The presence of MBL genes was the widely accepted explanation for carbapenem resistance by hydrolyzing most beta-lactams such as imipenem and meropenem (Lagatolla *et al*., 2004). So, the detection of the MBL-producing strains was necessary for the proper treatment of infected patients and to decrease and prevent the nosocomial spread of resistance strains (Walsh *et al*., 2005).

In the present study, the age of patients with nosocomial MDRPsA infection ranged from 5 years to 75 years with male predominance (66.7%). This means a high prevalence of nosocomial MDRPA infection in old age. These results follow those obtained by Mahmoud *et al*. (2013) and Bashir *et al*. (2011), who reported that 54.8% of MDR *P. aeruginosa* strains were isolated from male patients.

On the other hand, a high prevalence of *Pseudomonas* infections was found in the 35–50 years age group. The incidence of MDR *P. aeruginosa* strains was more elevated in elderly males than elderly female patients (Mohanasoundaram, 2011)

Prior antibiotic treatment and the placement of a urinary catheter were the two most common risk factors for nosocomial MDR-PSA infection. Similar to our findings, previous trials showed that *P. aeruginosa* had a worse prognosis secondary to inappropriate therapy, reaching 53.8%, according to Morata *et al*. (2012). The use of multiple catheters, catheter insertion in emergency conditions, and prolonged use of catheters are the leading causes of nosocomial infections caused by several highly resistant nosocomial pathogens, including *P. aeruginosa* (Özdemir and Dizbay, 2015).

Our recommendations include cleaning our hospital ICUs thoroughly, implementing comprehensive antimicrobial stewardship plans with particular attention to carbapenem use, and using routine infection control methods to protect patients. The standardization of the phenotypic method used to detect MBL manufacturers necessitates more investigations. Despite the high cost of molecular methods used for the recognition of carbapenemase-producing isolates, they could be applied due to their high sensitivity and specificity as the presence of hidden MBL genes that are hard to be detected by phenotypic tests. This can lead to disseminating these MBL genes within workers who could act as transporters for MBL genes in the future.

CONCLUSION

Pseudomonas aeruginosa infections represented 18% of nosocomial infections in studied patients in different ICUs in Al-Azhar University Hospital. *P. aeruginosa* isolates were resistant to imipenem (36.5%) and meropenem (39.2%). The high prevalence of antibiotic resistance observed in our study was significant, leading to the emergence of MDR, extensive drug resistant, and pan-drug resistant isolates with worrisome percentages. Phenotypic methods for detecting carbapenemases and MBLs production in *P. aeruginosa* isolates could not identify the hidden MBL genes. So, molecular confirmation by PCR and analysis of different carbapenemase producers is required for all isolates.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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SUPPLEMENTARY TABLE

Table S1. Antimicrobial susceptibility pattern of 74 *P. aeruginosa* urine isolates.

Antimicrobial drug	Number of resistant isolates (%)	Number of intermediate isolates (%)	Number of sensitive isolates (%)
Imipenem	27 (36.5%)	9 (12.2%)	38(51.3%)
Meropenem	29 (39.2%)	9 (12.2%)	36 (48.6%)
Ceftazidime	49 (66.2%)	9 (12.2%)	16 (21.6%)
Amikacin	43 (58.1%)	19(25.7%)	12(16.2%)
Gentamicin	52 (70.3%)	9 (12.2%)	13(17.5%)
Ciprofloxacin	51 (68.9%)	7 (9.5%)	16 (21.6%)
Levofloxacin	46 (62.1%)	7 (9.5%)	21 (28.4%)
Carbenicillin	70 (94.6%)	1 (1.4%)	3 (4%)