WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.647
Volume 6, Issue 9, 281-294 Research Article ISSN 2278 – 4357

COMPARISON BETWEEN PHENOTYPIC AND GENOTYPIC METHODS FOR THE DETECTION OF METALLO BETA LACTAMASES PRODUCING *PSEUDOMONAS AERUGINOSA*

Prof. Eman A. E. Abushady*[1], Asmaa M. M. Elgendy[2], Sabah M.M. Alkhawagah[3], Haneya A.A. Ali[4]

[1,2]Professors, Department of Microbiology and Immunology, Faculty of Medicine (for girls), Al-Azhar University Cairo – Egypt.

[4]Assistant professor, Department of Microbiology and Immunology, Faculty of Medicine (for girls), Al-Azhar University Cairo – Egypt.

[3]Instructor, Department of Microbiology and Immunology, Faculty of Medicine (for girls), Al-Azhar University Cairo – Egypt.

Article Received on
19 July 2017,

Revised on 09 August 2017,
Accepted on 30 August 2017,

DOI: 10.20959/wjpps20179-10066

***Corresponding Author**
Prof. Eman A. E. Abushady
Professors, Department of Microbiology and Immunology, Faculty of Medicine (for girls), Al-Azhar University Cairo – Egypt.

ABSTRACT

Background: *Pseudomonas aeruginosa* is a frequent nosocomial pathogen that causes severe diseases in many settings. Carbapenem resistance may be conferred through various mechanisms, including the expression of carbapenemases. MBLs constitute the most clinically important group of carbapenemases, rapid and specific detection of the MBLs is crucial for the optimum treatment and control the spread of resistance. **The aim of this study:** was to compare between phenotypic and genotypic methods for the detection of MBLs-producing *P. aeruginosa* and to evaluate MBL E-test in comparison with MBLs-gene detection by PCR. **Subjects and Methods:** fifty *P. aeruginosa* isolates were collected from 138 patients in the intensive care units, different wards and outpatients. **MBLs producing** *P. aeruginosa* were identified based on conventional methods and antimicrobial susceptibility testing, MBL E-test, IPM-EDTA combined disk method and IPM-EDTA double disk synergy test in addition to detection of blaVIM-2 and blaIMP-7 genes by PCR. **Results:** 40% of *P. aeruginosa isolates* were resistant to ceftazidime, 30% of them were resistant to carbapenems and 10% were susceptible. Eleven isolates were MBL-VIM-2 positive by PCR. Eight of them were resistant to carbapenems and ceftazidime and detected

phenotypically as MBL-producers. All *P. aeruginosa* isolates were negative for blaIMP-7 gene. Eight isolates were positive by MBL E-test, ten by IPM- EDTA combined disk method and three by IPM-EDTA double disk synergy test. **Conclusion:** MBL E-test is a fast and accurate test for detection of MBL-production among the resistant strains, while PCR is useful in prevention of horizontal interspecies spread of hidden MBLs.

KEYWORDS: *P. aeruginosa*, metallo-beta-lactamases, phenotypic and genotypic methods.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic organism associated with a broad spectrum of infections in humans. It is frequently involved in infections of immunosuppressed patients and also causes outbreaks of hospital acquired infections.[1] The pathogenicity of this organism is mainly due to the ability of the organism to produce a variety of toxins and proteases as well as the ability to resist phagocytosis. Bacterial infection caused by *Pseudomonas aeruginosa* is usually associated with difficulties in treatment attributed to the emergence and spread of drug-resistant strains.[2] The most potent antimicrobial agents for the treatment of these infections are carbapenems which include imipenem and meropenem.[3]

Carbapenem resistance may be mediated by production of certain β-lactamases called metallo-beta-lactamases (MBLs),[2] which have the ability to hydrolyze a wide variety of β-lactam agents. These enzymes require zinc for their catalytic activity and are inhibited by metal chelators, such as an ethylene diaminetetraacetic acid (EDTA) and thiol-based compounds.[4] MBLS are considered the most clinically significant mechanism of carbapenem resistance in *P. aeruginosa*.[5] *P. aeruginosa*, producing metallo-beta-lactamases (MBLs), was first reported from Japan in 1991[6] and since then has been described from various parts of the world including Asia, Europe, Australia, South America, North America[7] and Egypt.[8]

MBL-producing *P. aeruginosa* isolates have been responsible for serious infections, treatment failure and several nosocomial outbreaks in different parts of the world resulting in high morbidity and mortality and increased economic burden, so there is an urgent need to establish a strong infection control protocol and also the early detection may help in appropriate antimicrobial therapy and avoid the development and dissemination of these multi drug resistant strains.[9]

There are several phenotypic methods which are available for the detection of MBL-producing bacteria. All these methods are based on the ability of metal chelators, such as EDTA, to inhibit the activity of MBLs. These tests include the double-disk synergy test, Hodge test, combined disk test and MBL E-test. PCR is used as a molecular method for the detection of MBL gene which is the golden and confirmatory test.[10]

The aim of this study was to compare between phenotypic and genotypic methods for the detection of metallo beta lactamases producing *P. aeruginosa* and to evaluate MBL-E, IPM-EDTA combined disk and IPM-EDTA double disk synergy tests as phenotypic methods.

SUBJECTS AND METHODS

Fifty isolates of *Pseudomonas aeruginosa* were isolated from 138 different clinical samples were collected from patients admitted to Nasser Institute Hospital and Al-Zahraa University Hospital, Cairo- Egypt in the period from March 2015 to September 2015. The isolates were collected from various sources; 32 urine samples, 29 sputum samples, 28 wound swabs, 12 pus (abscess) swabs, 5 infected burn swabs, 4 peritoneal fluid samples and 24 BACTIC blood culture bottles that signaled positive for bacterial growth. Patients with diabetes mellitus, malignancies, long-stay patients (more than 15 days), seriously ill patients (ICU or post operation) and patients taking immunosuppressive drugs were included.

Reference strains: *P. aeruginosa* (ATCC 27853) kindly supplied from NAMRU-3 was used as a negative control in PCR [11] and *P. aeruginosa* strain carrying blaVIM-2 gene kindly supplied from genetic engineering and biotechnology department in National Research Center was used as a positive control in PCR.

Methods

Samples and positive Bactec blood culture bottles were subjected to culture on routine laboratory mediaincluding nutrient agar, blood agar plate, MacConkey agar plate. Isolates were identified as *P. aeruginosa* based on colony morphology, characteristic odour, Gram staining, production of blue-green pigment on nutrient agar, reactions (k/k) on triple sugar iron agar slants, positive oxidase reaction. Confirmation was done by the ability to grow at 42°C.Antibiotic susceptibility testing was done using disk diffusion method; procedures were performed following guidelines laid down by the Clinical Laboratory Standard Institute CLSI.[12] The *identified P. aeruginosa* isolates were tested against Ceftazidime (CAZ) 30 µg, Imipenem (IPM)10 µg, Meropenem (MEM)10 µg, Colistin (CL)10 µg, Polymyxin B

(PB)300 units (Bioanalyse, Turkey). Interpretation was done using guidelines laid down in the CLSI manual.

Phenotypic Detection of Metallo Beta Lactamases

MBL-E-test: the kit instruction was followed. The result was positive for MBL when MIC ratio of IP/IPI of ≥ 8. Phantom zone or deformation of the ellipse was also positive for MBL regardless of the IP/IPI ratio.

Imipenem-EDTA combined disk and Imipenem-EDTA double disk synergy methods: Each tested isolate was suspended in normal saline to 0.5 McFarland standards and then inoculated onto Mueller Hinton agar plates .Then the IPM disks with and without EDTA were placed onto the agar surface approximately 30mm apart. The disks were compared after 24 hours of incubation at 37 °C. Organisms were considered MBL producers if the difference between the inhibition zone around the IPM-EDTA (IEH) disk and the inhibition zone around the IPM disk alone was ≥ 7mm.[13] In case of Imipenem-EDTA double disk synergy enhancement of the zone of inhibition in the area between IPM and IPM-EDTA disk in comparison with the zone of inhibition on the far side of the IPM disk alone was interpreted as a positive result for MBL production.[14]

Genotypic Detection of Metallo Beta Lactamases

DNA extraction from fresh culture of the test organism and the control strains wasdone by heat shock method and stored at -70°C till used for detection of blaIMP-7 and blaVIM-2 genes. **PCR amplification**: Amplification of blaIMP-7 and blaVIM-2 genes was performed by PCR technique using primers which were selected from published sequences.[15,10] The PCR total reaction volume was 50 µl containing 12 µl distilled water,2 µl of each primer (Invitrogen, Germany) at a concentration of 20 picomolar, 25 µl of Taq, PCR Master Mix (Qiagen, Germany) and 5 µl of the extracted DNA. Primers were prepared according to manufacturer's instructions. The sequences of primers used for amplification of blaVIM-2 gene were (**Forward: 5'-GTT TGG TCG CAT ATC GCA AC-3') and (Reverse: 5'-AAT GCG CAG CAC CAG GAT AG-3')** which are known to give a PCR product of about **382 base pair** (bp). The sequences of primers used for amplification of blaIMP-7 gene were (**Forward: 5'-GAA GGY GTT TAT GTT CAT AC-3') and (Reverse: 5'-GTA MGT TTC AAG AGT GAT GC-3')** which are known to give a PCR product of about **587 bp** (Y = C or T and M = A or C). Amplification conditions included an initial denaturation step at 94°C for 5 minutes followed by 35 cycles. Each cycle consisted of :denaturation at 94°C for 1

minute, annealing at 54°C for1 minute, extension at 72°C for 1 minute and followed by final extension at 72°C for 7 minutes. The PCR products were separated by 2% agarose gel in Tris- borate EDT stained with 3 µl ethidium bromide. The size of amplified DNA was determined using 100 bp step ladder molecular DNA marker. The expected size for the amplicon was 382bp for blaVIM-2 gene and molecular mass 587bp for blaIMP-7 gene.

Statistical analysis

All the data were entered in Microsoft Excel sheet and the results were analyzed by SPSS (version 18) computer program and the results were presented in tables and charts.Chi-square test was used to compare proportions between two qualitative parameters. Kappa index (statistical measurement of agreement): was used to estimate (evaluate) the level of agreement between the tests.

RESULTS

It was found that out of 50 *P. aeruginosa* isolates, 30 isolates (60%) were susceptible to all tested antibiotic disks while, the remaining 20 isolates (40%) were resistant to ceftazidime but differ in their susceptibility to carbapenems, 15 (30%) isolates were resistant and 5 (10%) isolates were susceptible. Carbapenem-resistant isolates were more prevalent in sputum specimens followed by infected burn swabs and urine specimens but, less prevalent in wound swabs specimens. According to age, carbapenem-resistant isolates were more prevalent in age group 11-30 followed by age group less than 10 years then age group more than 55 years while the lowest rate was found among patients with age group 31-55 but the difference was insignificant. Also carbapenem-resistant isolates were insignificantly more prevalent in male patients than females and significantly in patients with history of antibiotic administration versus other patients. The highest rate of carbapenem resistant *P. aeruginosa* was significantly found in patients underlying invasive procedures and among ICU patients, followed by ward patients and lowest among out-patients (Table - 1).

Table 1: Frequency of distribution of carbapenem sensitive and resistant (CS and CR) *P. aeruginosa* isolates according to demographic data.

Parameter	Total No. of isolates (50)	No. of CS (35)	No &% of CR (15)	Chi-Square	P-value	Sig
Age-group distribution (years)						
<10	2	1	1(50%)	0.632	0.539	NS
11-30	7	2	5(71%)			
31-55	29	23	6(20.7%)			
>55	12	9	3(25%)			
Sex distribution						
Males	26	17	9(34.6%)	0.549	0.459	NS
Females	24	18	6(25%)			
Antibiotic administration						
Administer	**32**	**19**	**13(40.6%)**	**4.778**	**0.029**	***S**
Not administer	18	16	2(11.1%)			
Invasive procedures						
Present	**29**	**17**	**12(41.4%)**	**4.258**	**0.039**	***S**
Absent	21	18	3(14.3%)			
Location of patient						
ICU	**20**	**8**	**12(60%)**	**16.148**	**0.001**	***HS**
Ward	17	15	2(11.8%)			
Out-patients	13	12	1(7.7%)			
Kind of sample						
Urine	14	11	3 (21.4%)	**17.347**	**0.015**	***S**
Sputum	**14**	**5**	**9 (64.3%)**			
Pus swabs	4	4	0			
Burn swabs	2	1	1 (50%)			
Blood	1	1	0			
CSF	1	1	0			
Pus swabs	4	4	0			

***S:** significance**, *HS:** highly significance.

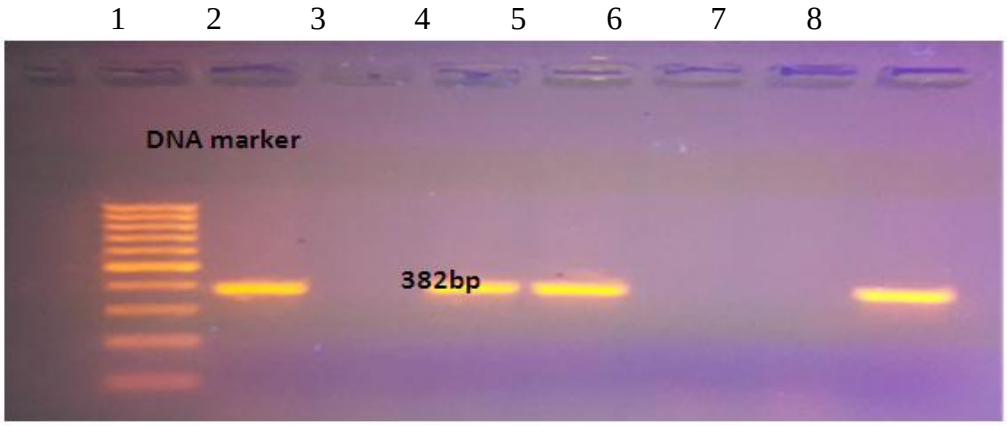

Figure 1: Detection of MBLs *P. aeruginosa* isolates carrying blaIMP-7 and blaVIM-2 genes by PCR.

Lane 1: molecular weight marker (100 bp), Lane 2: positive control for blavim-2, Lane 3: negative control, Lanes 4, 5 and 8: blavim-2 positive isolates, Lanes 6 and 7: negative isolates.

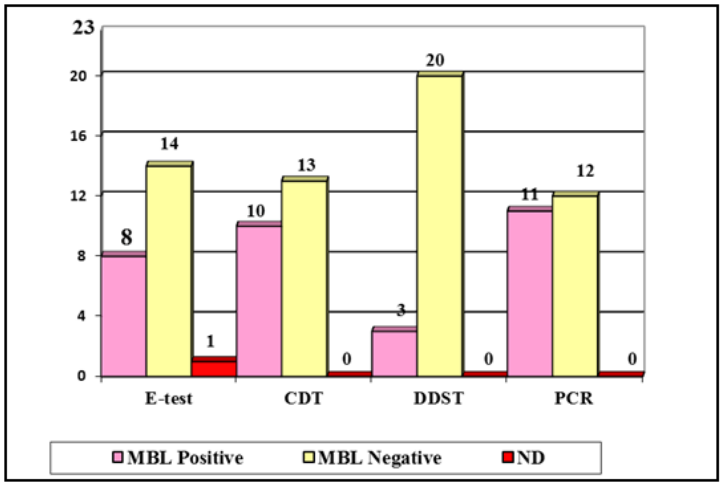

Figure 2: Performance of E-test, CDT and DDST compared to PCR.

Eight (72.7%) out of 11 isolates positive for MBL by PCR were positive by E-test and CDT while, three (27.7%) were positive by DDST (Figure -2).

The statistical correlation between MBL E-test, IPM-EDTA combined disk method and IPM-EDTA double disk synergy test using PCR as the standard test was done to assess the sensitivity, specificity, predictive values of positive, predictive values of negative, diagnostic accuracy and the degree of agreement between the first three methods and PCR(Table -2).

Table 2: Comparison between E-test, CDT and DDST using PCR as the standard test for the detection of MBLs among all tested isolates (23).

	E-test	CDT	DDST
True positive	8	8	3
False positive	0	2	0
False negative	3	3	8
True negative	11	10	12
Sensitivity	72.7%	72.7%	27.3%
Specificity	100%	83.3%	100%
PPV	100%	80%	100%
NPV	78.6%	76.9%	60%
Accuracy	86.4%	78.3%	65.2%
P value	0.000	0.007	0.052
Kappa index	0.736	0.563	0.281
Degree of agreement	Medium	Medium	Weak

Susceptibility of all *P. aeruginosa* isolates to colistin and polymyxin B was done. It was found that, out of 50 isolates, 47 (94%) isolates were susceptible to colistin and 50 (100%) isolates were susceptible to polymyxin B.

DISCUSSION

Antibiotic resistance among Gram-negative pathogens such as *P. aeruginosa* is one of the major problems in treating hospitalized patients. In case of *P. aeruginosa*, multiple mechanisms are involved in β-lactam resistance, one of which is MBL production. Early detection of MBL producing organisms is crucial for optimal treatment of critically ill patients, to permit rapid initiation of strict infection control and to prevent nosocomial spread.[16]

In the present study it was found that 60% of isolates were susceptible to all tested antibiotic disks. The remaining 40% were resistant to the third generation cephalosporin. The imipenem disk diffusion screening divided 50 study isolates into two groups: 15 isolates (30%) of imipenem resistant and 35 isolates (70%) of imipenem sensitive *P. aeruginosa*. Similar results have been described by different authors[10,17,8,18,16] While, lower results were reported by[19] and higher results were published by.[20] So, it was noticed that the prevalence of IPM resistance varies greatly between and within countries and even between hospitals within a community and this could be attributed to the differences in antimicrobial usage and infection control practice in different localities.[16]

IPM-resistant isolates were more prevalent in sputum (64.3%) and infected burn swabs (50%) and less prevalent in urine (21.4%) and wound swabs (14.3%) specimens. This finding was in agreement with.[21,14]

The majority of carbapenem-resistant *P. aeruginosa* isolates were isolated from intensive care unit (ICU) patients (60%), patients with history of antibiotic administration (86.7%) and also from patients undergoing invasive procedures (80%) (table1). Several studies pointed to stay in ICU, prior use of antibiotic and applied invasive procedures either diagnostic or therapeutic as important risk factors associated with CRPA infections.[22,23]

All 15 imipenem resistant *P. aeruginosa* isolates were tested phenotypically for MBL production. The results of MBL production by MBL E-test, IPM- EDTA CDT and IPM-EDTA DDST was (8/15), (10/15) and (3/15) respectively, the 5 isolates which were resistant CAZ and susceptible to IMP were tested and none of them proved to be a potential MBL-producer. This is in complete agreement with two studies performed by[24,16] who showed that MBL-producers were not detected among CAZ-resistant/IPM-susceptible isolates but were only detected among IPM-resistant isolates.

Searching for MBLs genes in all the isolates by PCR technique revealed that out of 50 *P. aeruginosa* isolates, 11 (22%) were positive for bla_{VIM-2} and all of them were negative for bla_{IMP-7}. Accordingly, bla_{VIM-2} gene was the highly prevalent gene among MBL-positive *P. aeruginosa* isolates and this finding was supported by results of many previous studies.[25,26,27,28] Up to our knowledge, there is no report about detection of bla_{IMP-7} gene from Egyptian isolates of *P. aeruginosa* until now. Out of 11 VIM-2 producers, 3 isolates were carbapenem-susceptible. These three isolates were further tested for MBL-production by the phenotypic methods and none of them was detected phenotypically as MBL-producer. This may be due to the fact that the gene is present but not expressed phenotypically. Prevalence of VIM-2 producers among carbapenem resistant *P. aeruginosa* isolates by PCR technique was found to be 8/15 (53.3%). Similar increased prevalence was detected in another three Egyptian studies,[16,25,27] as, they reported that the prevalence of MBL producing *P. aeruginosa* among IMP-resistant isolates was 60%, 64% and 68.7% (33/48) respectively.

MBL E-test showed perfect agreement with PCR assay with 100% sensitivity, specificity and accuracy. Similar results were reported by.[28,30] On the other hand, IPM- EDTA showed strong degree of agreement with PCR assay (Kappa value = 0.800), 100% sensitivity and 83.3% specificity. Similar finding was reported by.[31,32] IMP EDTA CDT method gave a false positive result in 2 isolates; this may be due to the inhibition zone of ≥7 mm in zone diameter in the presence of EDTA may not be considered as a definitive clear cut off[30] and using a breakpoint of ≥8 mm in the presence of 750 µg of EDTA instead[13] also EDTA has membrane-permeabilising properties and could exert a deleterious effect on *P. aeruginosa* resulting in false-positive detection.[33] In the current study, IPM-EDTA DDST showed a medium degree of agreement with PCR assay 37.5% sensitivity and 100% specificity. This finding agreed with studies performed by [34,9]. However, the result of the sensitivity may have increased if the distance between the inhibitor and the substrate was 15 mm instead of 20mm as reported by.[34] In the current study 94% (47/50) and 100% (50/50) of the total *50 P. aeruginosa* isolates were sensitive to colistin and polymyxin B respectivelly. This supports the evidence that polymyxin B has increasingly become the last viable therapeutic option for MDR *Pseudomonas* infections. This result agreed with studies done by[35,36] they found that all isolates were sensitive to polymyxin B.

CONCLUSIONS AND RECOMMENDATIONS

The prevalence of MBL producers among carbapenem-resistant isolates was very high (53.3%) by both phenotypic and genotypic methods. MBL E-test can be used as an alternative method to monitor the emergence of MBLs. PCR is useful in prevention of horizontal interspecies spread of hidden MBLs. Finally, strict infection control measures to prevent co-transmission among patients and continuous surveillance for MBL production is needed in institutions with endemic or epidemic carbapenem-resistant *P. aeruginosa* in an attempt to avoid the intra-hospital dissemination of MBL-producers.

ACKNOWLEDGEMENT

The team work express their deep thanks and appreciation to **professor Soha A.R. Elhadi** professor of Microbiology and Immunology, Faculty of Medicine , Ain Shamas University Cairo – Egypt for her help and facilitation of the collection of pathological samples.

REFERENCES

1. Burstein, D., Satanowe,r S., Simovitch, M., Belnik, Y., Zehavi, M., Yerushalmi, G., Ben-Aroya, S., Pupko, T. and Banin, E.: Novel type III effectors in *Pseudomonas aeruginosa*. mBio, 2015; 6(2): e00161-15.
2. Khosravi, Y., Vellasamy, K.M., Tay, S.T. and Vadivelu, J. Molecular detection and characterisation of metallo-β- lactamase (MBL) genes and integrons of imipenem resistant *Pseudomonas aeruginosa*. In: "Microbial pathogens and strategies for combating them: science, technology and education", Méndez-Vilas A. (ed). Formatex Research Center, 2013; 515-521.
3. Lee, H., Ko, K.S., Song, J.H. and Peck, K.R. Antimicrobial activity penems against Gram-negative pathogens from Korea of doripenem and other carbaMicrob Drug 1Resist, 2011; 17(1): 37-45.
4. Akya, A., Salimi, A., Nomanpour, B. and Ahmadi, K. Prevalence and Clonal Dissemination of Metallo-Beta-Lactamase-Producing *Pseudomonas aeruginosa* in Kermanshah. Jundishapur Journal of Microbiology, 2015; 8(7): e20980.
5. Picão, R.C., Carrara-Marroni, F.E., Gales, A.C., Venâncio, E.J., Xavier, D.E., Tognim, M.C. and Pelayo, J.S. Metallo-β- lactamase production in meropenem susceptible *Pseudomonas aeruginosa* isolates: risk for silent spread. MemInstOswaldo Cruz, 2012; 107(6): 747-751.

6. Watanabe, M., Iyobe, S., Inoue, M. and Mitsuhashi, S. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother, 1991; 35(1): 147–151.
7. Mohite, S.L. and Ghorpade, M.V. A Study of Metallo Beta Lactamase Producing Clinical Isolates of *Pseudomonas aeruginosa*. Online J Health Allied Scs, 2014; 13(3): 4-9.
8. Zafer, M.M., Al-Agamy, M.H., El-Mahallawy, H.A., Amin, M.A. and Ashour, M.S. Antimicrobial Resistance Pattern and Their Beta-Lactamase Encoding Genes among *Pseudomonas aeruginosa* Strains Isolated from Cancer Patients. BioMed Research International, 2014; 2014: 101635, 8 pages.
9. Ranjan, S., Banashankari, G.H. and Babu, P.R. Evaluation of phenotypic tests and screening markers for detection of metallo-β-lactamases in clinical isolates of *Pseudomonas aeruginosa*: A prospective study. Medical Journal of Dr. DY Patil University, 2015; 5(8): 599-605.
10. Manoharan, A., Chatterjee, S. and Mathai, D. Detection and characterization of metallo beta lactamases producing *Pseudomonas aeruginosa*. Indian J. Med. Microbial, 2010; 28(3): 241-244.
11. Franco, M.R., Caiaffa-Filho, H.H., Burattini, M.N. and Rossi, F. Metallo-beta-lactamases among imipenem-resistant *Pseudomonas aeruginosa* in a brazilian university hospital. Clinics, 2010; 65(9): 825-829.
12. Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. CLSI document M100-S24. Wayne, PA 19087 USA, 2014.
13. Yong, D., Lee, K., Yum, H., Shin, H.B., Rossolini, G.M. and Chong, Y. Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbio, 2002; 40(10): 3798-3801.
14. Lee, K., Lim, Y., Yong, D., Yum, J. and Chong, Y. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of *Pseudomonas* spp. and Acinetobacter spp. J. Clin. Microbiol, 2003; 41(10): 4623–4629.
15. Pitout, J.D.D., Gregson, D.B., Poirel, L., McClure, J.A., Le, P. and Church, D.L. Detection of *Pseudomonas aeruginosa* Producing Metallo-β-Lactamases in a Large Centralized Laboratory. J. Clin. Microbiol, 2005; 43(7): 3129-3135.
16. EL-Mosallamy, W.A., Osman, A.S., Tabl, H.A. and AL-Tabbakh, A.M. Phenotypic and Genotypic Methods for Detection of Metallo Beta-Lactamase (MβL) Producing

Pseudomonas aeruginosa. Egyptian Journal of Medical Microbiology, 2015; 24(3): 27-35.

17. Mahmoud, A.B., Zahran, A.W., Hindawi, R.G., Labib, Z.A. and Gala, l.R. Prevalence of multidrug-resistant *Pseudomonas aeruginosa* in patients with nosocomial infections at a university hospital in Egypt, with special reference to typing methods. Journal of Virology and Microbiology, 2013; 2013(2): 1-13.
18. Abiri, R., Mohammadi, P., Shavani, N. and Rezaei, M. Detection and genetic characterization of metallo-β-lactamase IMP-1 and VIM-2 in *Pseudomonas aeruginosa* strains from different Hospitals in Kermanshah, Iran. Jundishapur Journal of Microbiology, 2015; 8(9): e22582 .
19. Amer, F., El-Behedy, E.M., Mohtady, H., Zanfaly, H.E., Soud, D.E. and El-Behedy, T.M. Imipenem Resistance and bla IMP Gene among Hospital Strains of *Pseudomonas aeruginosa* at University Hospital in Egypt. Journal of Infection in Developing Countries, 2007; 1(1): 42-47.
20. Mansour, S.A., Eldaly, O., Jiman-Fatani, A., Mohamed, M.L. and Ibrahim, E.M. Epidemiological characterization of *P. aeruginosa* isolates of ICUs in Egypt and Saudi Arabia. Eastern Mediterranean Health Journal, 2013; 19(1): 71-80.
21. Afifi, M.A., Suelam, A., Soliman, M.T. and El-Gohary, M.G. Prevalence and Antimicrobial Susceptibility Pattern of *Pseudomonas aeruginosa* Isolated from Environmental and Clinical Samples in Upper Egypt. International Journal of Biological Chemistry, 2013; 7(2): 47-57.
22. Ghibu, L., Miftode, E., Teodor, A., Bejan, C. and Dorobăţ, C.M. Risk factors for *Pseudomonas aeruginosa* infections, resistant to carbapenem. Rev. Med. Chir. Soc. Med. Nat. Iasi, 2010; 114(4): 1012-1016.
23. Djordjevic, Z., Folic, M., Ruzic, A., Zecevic, D., Ilic, G. and Jankovic, S. Risk factors for carbapenem-resistant *Pseudomonas aeruginosa* infection in a tertiary care hospital in Serbia. The Journal of Infection in Developing Countries, 2013; 7(9): 686-690.
24. Fam, N., Diab, M., Helmi, H. and El- Defrawy, I. Phenotypic detection of metallo beta lactamases and extended spectrum beta lactamases among Gram-negative bacterial clinical isolates. Egyptian. J. Med. Microbiol, 2006; 15(4): 719-730.
25. Diab, M., Fam, N., El-Said, M., El-Dabaa, E., El-Defrawy, I. and Saber, M. Occurrence of VIM-2 Metallo-β- Lactamases in imipenem resistant and susceptible *Pseudomonas aeruginosa* clinical isolates from Egypt. Afr. J. Microbiol. Res, 2013; 7(35): 4465-4472.

26. Mathlouthi, N., Areig, Z., Al Bayssari, C., Bakour, S., Ali El Salabi, A., Ben Gwierif, S., Zorgani, A.A., Ben Slama, K., Chouchani, C. and Rolain, J.M. Emergence of carbapenem-resistant *Pseudomonas aeruginosa* and Acinetobacter baumannii clinical isolates collected from some Libyan hospitals. Microb Drug Resist, 2015; 21(3): 335–341.
27. Zafer, M.M., Al-Agamy, M.H., El-Mahallawy, H.A., Amin, M.A. and Ashour, M.S. Dissemination of VIM-2 producing *Pseudomonas aeruginosa* ST233 at tertiary care hospitals in Egypt. BMC Infect Dis, 2015; 12(15): 122-129.
28. Viedma, E., Juan, C., Villa, J., Barrado, L., Orellana, M.A., Sanz, F., Otero, J.R., Oliver, A. and Chaves, F. VIM-2 producing multidrug-resistant *Pseudomonas aeruginosa* ST175 clone, Spain. Emerg Infect Dis, 2012; 18(8): 1235–1241.
29. Khosravi, A.D. and Mihani, F. Detection of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* strains isolated from burn patients in Ahwaz, Iran. Diag Microbiol Infect Dis, 2008; 60(1): 125-128.
30. Khosravi, Y., Loke, M.F., Chua, E.G., Tay, S.T. and Vadivelu, J. Phenotypic Detection of Metallo-β-Lactamase in Imipenem-Resistant *Pseudomonas aeruginosa*. Scientific World Journal, 2012; 12(65): 39-49.
31. Berges, L, Rodriguez-Villalobos, H., Deplano, A. and Struelens, M.J. Prospective evaluation of imipenem/EDTA combined disc and Etest for detection of metallo-beta-lactamase-producing *Pseudomonas aeruginosa*. J AntimicrobChemother, 2007; 59(4): 812-813.
32. Arunagiri, K., Sekar, B., Sangeetha, G. and John, J. Detection and characterization of metallo-beta-lactamases in *Pseudomonas aeruginosa* by phenotypic and molecular methods from clinical samples in a tertiary care hospital. West Indian Med J, 2012; 61(8): 778-783.
33. Chu, Y.W., Cheung, T.K., Ngan, J.Y. and Kai, M.K. "EDTA susceptibility leading to false detection of metallo-β-lactamase in *Pseudomonas aeruginosa* by E-test and an imipenem-EDTA disk method," International Journal of Antimicrobial Agents, 2005; 26(4): 340-341.
34. Qu, T., Zhang, J., Wang, J., Tao, j., Yu, Y.S., Chen, Y.G., Zhou, J.Y. and Li, L.J. Evaluation of Phenotypic Tests for Detection of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* Strains in China .Journal of Clinical Microbiology, 2009; 47(4): 1136-1142.

35. Tawfik, A.F., Shibl, A.M., Aljohi, M.A., Altammami, M.A. and Al-Agamy, A.L. Distribution of Ambler class A, B and D β-lactamases among *Pseudomonas aeruginosa* isolates. Burns, 2012; 3(6): 855–860.
36. Yilmaz, N.O., Agus, N., Bozcal, E. and Uzel, A., Prevalence and molecular characterisation of metallo-beta-lactamase producing strains of imipenem-resistant *Pseudomonas aeruginosa* in Turkey. I.J.M.M., 2014; 32(3): 349-350.