Phenotypic and Molecular Characterization of Imipenem Resistant *pseudomonas* Isolates

Sherin A.S. ElMasry, Rania A. Ammar and Sally M. Saber

Clinical and Chemical Pathology Department, Faculty of Medicine, Ain Shams University.
sallysaber@ymail.com

Abstract: Background: *Pseudomonas* spp. is one of the most frequent nosocomial pathogen that is often difficult to treat due to a multi-drug resistant pattern to a wide range of antibiotics like carbapenems. Imipenem-resistant *Pseudomonas aeruginosa* (IRP) resulting from metallo-b-lactamases has been reported to be an important cause of nosocomial infection and is a critical therapeutic problem worldwide, especially in the case of bacteremia. **Objectives:** In this work we aimed to detect Carbapenemase and MBL production as well as molecular detection of bla IMP gene in impenem resistant nosocomial isolates of *Pseudomonas spp*. **Material and Methods:**A total number of 150 *Pseudomonas* isolates were collected from clinical specimens submitted to Ain Shams University microbiology Laboratory for culture and antibiotic susceptibility testing.We used the Imipenem E-test strips to determine the MIC of the IPM resistant isolates .Screening for Carbapenemase and Metallo-β-lactamase was done phenotypically using modified hodge test (MHT) and Imipenem-EDTA Double disk Synergy test (DDST), also molecular detection of bla_{IMP} gene was performed. **Results:** It was found that 38 out of 150 isolates (25.4%) were resistant to impenem by disc diffusion method which confirmed by E test that showed only 27 out of 150 (18%) were resistant. Ten Out of 27 isolates (37%) were expressed bla_{IMP} gene. However, The Modified Hodge Test Positive isolates were 13 out of 27 E-test resistant isolates (48.1%). While the DDST positive isolates were 21 out of 27 (77.8%) of E-test resistant isolates. **Conclusion**: Metallo-b-lactamases among imipenem-resistant Pseudomonas were detected in 37% by PCR. This number might have been higher if other genes were included. Phenotypic tests could be misleading when testing for metallo-b-lactamases. Polymerase Chain Reaction detection remains the gold standard.

[Sherin A.S. ElMasry, Rania A. Ammar and Sally M. Saber. **Phenotypic and Molecular Characterization of Imipenem Resistant *pseudomonas* Isolates.** *Life Sci J* 2012; 9(2s): 377-383] (ISSN: 1097-8135). http://www.lifesciencesite.com. 42

Keywords: Pseudomonas; imipenem resistance; metallo-b-lactamases; phenotypic tests

1. Introduction

During the past decade, nosocomial outbreaks of *Pseudomonas* spp. have been described with increasing frequency (10-20% of hospital-acquired infections), occurring mostly in surgical wards, intensive care units and internal medicine wards(Fine *et al.;* 2005).

Carbapenems, such as imipenem and meropenem, remain one of the best drugs to treat infections caused by *Pseudomonas spp*. Increasing usage of these drugs and other expanded-spectrum antibiotics has resulted in the development of carbapenem-resistant *Pseudomonas spp*. With treatment failure even with combination therapy (Giamarellos et al., 2006)

MBL, an Ambler class B enzyme, is characterized by its ability to hydrolyze carbapenems, its resistance to all commercially available b-lactamase inhibitors and its inhibition by metal ion chelators as EDTA, Mercapto acetic acid (MAA). The substrate spectrum of this enzyme is quite broad, as it can hydrolyze penicillins,cephalosporins and carbapenems, but it lacks the ability to hydrolyze aztreonam (francoetal. , 2010).

Clinical Laboratory Standard Institute (CLSI) documents do not yet contain a phenotypic method for detection of metallo-β-lactamase production in clinical isolates, and hence methods can be standardized and could be of use as it contributes towards the optimal treatment of patients and control of the spread of resistance and infection control (Jesudasonetal., 2005 and francoetal. ,2010).

The implementation of a simple reliable phenotypic method to detect carbapenemase and metallo-β-lactamase production is useful particularly in situations where carbapenem and other β-lactams are indicated or preferred as therapeutic regimen (francoetal. , 2010).

Objectives To determine the frequency of metallo-b-lactamases and Carbapenemase among imipenem-resistant Pseudomonas isolates by phenotypic and molecular detection methods.

2. Materials and Methods

The current study was conducted on 150 *Pseudomonas spp.* isolated from clinical specimens submitted to Ain Shams University Hospital Microbiology Central Laboratory for culture and sensitivity.

All selected isolates of *Pseudomonas spp* in this study were subjected to:

I) Routine standard culture & sensitivity testing:

- Routine subculture on blood agar and MacConkey agar mediaNo. (Oxoid, England) for 24 to 48 hours incubation at 37 ^{o} C to obtained separate colonies. Identification of the isolates using: Gram stains morphology& Conventional biochemical tests.
- Antibiotic susceptibility test by disc diffusion method according to (CLSI, 2011).For the following antibiotics: Imipenem, Meropenem, Piperacillin+Tazobactam, Cefepime, Amikacin, Ciprofloxacin, Cefotaxime, Ceftazidime.

II) Imipenem resistant isolates were subjected to:

A-Determination of MIC by E-test strips:

The inoculums were prepared and the media was inoculated, with sterile forceps, the E-test strips were placed on the agar plate. Plates were incubated in an inverted position for 16-18 hours at 35-37°c. If MIC ≤ 4µg/ml, the organism was considered susceptible. While if MIC ≥ 16µg/ml the organism was considered resistant (CLSI, 2011).

B-Screening for Carbapenemase and Metallo-β-lactamase according to franco etal.,2010 :

Modified Hodge test (MHT): The surface of a Mueller-Hinton agar plate was inoculated with a culture suspension of quality control reference strain E. coli ATCC 25922 which was susceptible to imipenem. An imipenem disc10 µg was placed at the center and the strains to be tested were streaked from the edge of the disc to the periphery. Plates were incubated at 35-37°covernight. MHT Positive test showed a clover leaf-like indentation of the E.coli indicating that this isolate was producing a carbapenemase, or MBL Fig 1.

Imipenem-EDTA Double disk Synergy test (DDST): To differentiate metalloenzymes from other carbapenemases. Test strains were adjusted to the McFarland 0.5 standard and used to inoculate Mueller-Hinton agar plates. A 10 µg IMP disc was placed on the plate and a 10 µl EDTA solution disc was placed at a distance of 10 mm (edge to edge). After overnight incubation, the presence of a synergistic inhibition zone between both discs was interpreted as a positive result Fig 2.

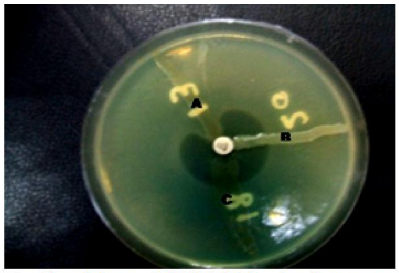

Figure (1): Positive MHT (A) isolates performed on a 100 mm MHA plate

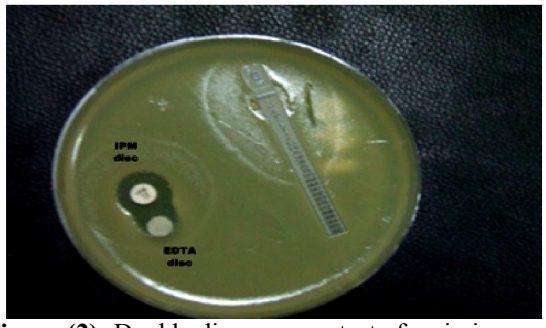

Figure (2): Double disc synergy test of an imipenem resistant *Pseudomonas* isolate

C- Molecular identification of imipenem resistant gene, (MBLs gene IMP type, *bla*$_{IMP}$ gene) using conventional PCR, according to Senda *et al*; (1996).

Template DNAsextraction: each isolate were heated in a hot block at 96^{o}C up to 10 minutes, then were placed on ice for 5min

PCR amplification: Reaction mixture contained 1 ml (each) primer, Specific primer designed from Metallo-βLactamase gene (*bla*$_{IMP}$) Forward primer: 5'-CTA CCG CAG CAG AGT CTT TG-3', Reverse primer: 5'-AAC CAG TTT TGC CTT ACC AT-3' (Operon Co., Germany, 200 mM (each)deoxynucleoside triphosphate (Promega Co., Ltd.), 1X reaction buffer containing 1.5 mM $Mg^{++}Cl_2$ (Promega Co., Ltd.), 2.5 U of Promega*Taq*polymerase (Promeg Co., Ltd.), and approximately 25 ng of template DNA. Amplification was performed in a 25-ml volume with the Biozyme PCR system PTC-200 (Perkin-Elmer, USA) thermal cycler. Cycles were designed after an initial denaturation step (2 min at 94^{o}C), 30 cycles of amplification were performed, as follows: denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min, and DNA extension at 72^{o}C for 1.5 min.

Detection of the amplification product on agarose gel electrophoresis

The positive control gave a sharp band at 500bp. Samples that gave a band at the same band of the positive control were considered positive for blaIMP gene as shown in the following figure (3).

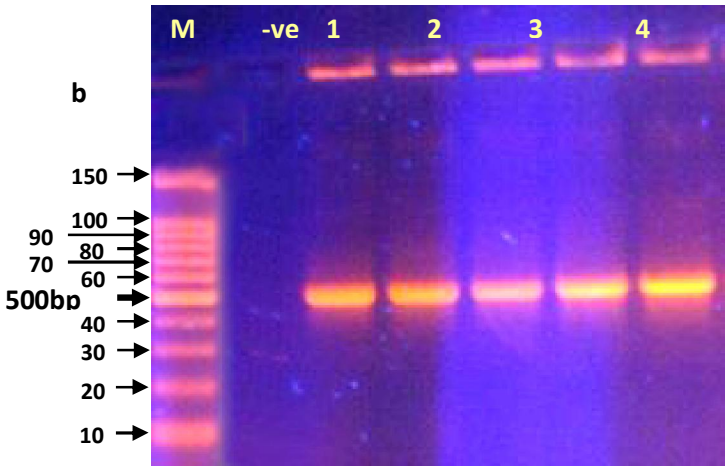

Figure 3: PCR detection of *bla*$_{IMP}$ gene of *IRP* on agarose gel electrophoresis. Lanes (1, 2, 3, 4, and 5) were positive samples for *bla*$_{IMP}$ gene.

3. Results

In this study, 150 *Pseudomonas* spp. isolates were collected and antibiotic resistance were determined .The carbapenems resistant isolates (Imipenem, Meropenem) represented 38 out of 150 (25.4%) by disc diffusion method.

However, using the Imipenem E-test strips, 27 isolates out of 38 (71.1%) was confirmed as resistant to Imipenem 27/150(18%) (MIC ≥ 16 mg/L).The rate of detection among wound swabs was the highest rate (48.2%) as shown in table (1).

Table (1): Distribution of confirmed Imipenem resistant *Pseudomonas* isolates among different clinical specimens.

Types of specimens	No. (%)
Wound swab	13(48.2%)
Sputum	7(25.9%)
Blood culture	3(11.1%)
Pus	3(11.1%)
CSF	1(3.7%)
Total	27

Most of those 27 (IRP) were isolated from the surgical units (51.9%) (Table 2).

Table (2): Distribution of confirmed IRP isolates in our hospital.

Distribution of isolates	No. (%)
Surgical units	14 (51.9%)
ICU	11 (40.7%)
Internal medicine wards	2 (7.4%)
Total	27

The IRP isolates with MIC ≥ 16 mg/L were subjected to modified Hodge test (MHT) and double disc synergy test (DDST). The Modified Hodge Test Positive isolates were 13 out of 27 E-test resistant isolates (48.1%). While the Double Disc Synergy Test Positive isolates were 21 out of 27 E-test resistant isolates (77.8%) . As regards the association between the results of two tests, it was found that 11/27(40.8%) were positive by the two tests (MBL positive), while 10/27 where positive only by DDST (MBL positive), however, 2/27 (7.4%) where positive only MHT Carbapenemase production due to non MBL production (table 3).

Table (3) the association between results of MHT and DDST.

Modified Hodge test	Double disc synergy test
	Positive / **Negative**
Positive	11(40.8%) / 2(7.4%)
Negative	10(37%) / 4(14.8%)
21(77.8%)	6(22.2%)

As regards the results of (PCR), it was found that10 out of 27(37%) isolates of *IRP* expressed bla$_{IMP}$ gene. Most of these isolates were from wound 6 out of 10 (60%) and sputum: 4 out of 10 (40%).Interpretation of antibiotic resistance pattern of the confirmed IRP isolates was summarized in table (4). PCR confirmed IRP isolates were resistant to Ceftazidime, cefepime,cefotaxime, tazocin and Meropenem.

Table (4): Interpretation of antibiotic resistance pattern of the confirmed IRP isolates

Antibiotic	IRP No. 27	bla_{IMP} Isolates No. 10
Piperacillin+Tazobactam	17 (63%)	10 (100%)
Cefepime	19 (70.4%)	10 (100%)
Amikcin	18 (66.7%)	7(70%)
Ciprofloxacin	20 (74.1%)	9(90%)
Cefotaxime	21 (77.8%)	10 (100%)
Ceftazidime	27 (100%)	10(100%)
Meropenem	27 (100%)	10(100%)

As regards the diagnostic performance of phenotypic tests in detection of MBL gene , DDST was more sensitive while MHT was more specific table (5).

Table 5 : Diagnostic performance of phenotypic methods for detecting IMP gene (PCR gold standard):

Phenotypic test	Sensitivity	Specificity	PPV	NPV	Accuracy
MHT	70%	64.7%	53.8%	46.1%	66.6%
DDST	100%	35.2%	47.6%	100%	59.2%

3. Discussion

Imipenem-Resistant *Pseudomonas* (IRP) is a current and significant concern, especially because of the limited therapeutic options for this pathogen. MBL enzymes demonstrated that a large proportion of MBL genes are associated with one or more aminoglycoside- or b-lactam resistant genes, partially explaining multi-drug-resistant Cases. (Agrawal et al., 2008 and Franco et al., 2010).

In this study a total number of 150 *Pseudomonas* isolates were collected from clinical specimens submitted to Ain Shams University Microbiology Laboratory for culture and antibiotic susceptibility testing. The resistance was (25.4%) to both Imipenem and meropenem by disc diffusion. In a study by Altoparlak et al., (2005) in Turkey, the resistance rates of *Pseudomonas* strains collected only from burn wounds were as: (30.8%) to imipenem, (32.5%) to meropenem. In anothera study by Sarkar et al., (2006) in India showed resistance (36.36%) to Imipenem. On the other hand,Carvalhoet al., (2005) in Portugal. The antibiotic susceptibility tests revealed high resistance to most antibiotics tested, Imipenem (96.3%) and Meropenem (84%). Astudy by Behera et al. (2008) in Indiashowed (69%) to Imipenem. This may be due to the wild use of those antibiotics regardless of the severity of infection.

A study conducted in India by Agrawal et al., (2008), the resistance pattern was (8.05%) to Imipenem . Another study by Hui Wang et al. (2010) from China, The resistance was (13.7%) to Imipenem and (13.9%) to meropenem. The resistance to carbapenems in our study was higher than the previous two studies. This may be due to the extensive use of carbapenems in our hospital.

We used the Imipenem E-test strips to determine the MIC of the IPM resistant isolates and to confirm their resistance to IPM. 27 out of 38 (71.1%) were confirmed as resistant with MIC $\geq$ 16 mg / l.

In a study by Japoni et al. (2006) from Iran, (37%) of resistant isolates by antibiotic susceptibility test was confirmed as resistant by E-test. Another one by Behera et al., (2008) in India, (100%) of resistant isolates by antibiotic susceptibility test was confirmed as resistant by E-test. In another study by Hemalatha et al. (2008) 87.5% of resistant isolates by antibiotic susceptibility test was confirmed as resistant but they used the agar dilution method. It has been established that although all MBLs hydrolyze imipenem, they vary considerably in their rate of hydrolysis which may or may not correlate with the bacterium's level of resistance to carbapenems resulting in imipenem sensitive phenotypes (Agrawal et al., 2008)

In our study, the distribution of confirmed IRP isolates according to the type of specimens were (48.2%) from wound, (25.9%) from sputum, (11.1%) from pus and blood culture and (3.7%) from CSF. They were collected from surgical units (51.9%). The high percentage of resistance in surgical units explains the high percentage of resistance from wound.

In a study by Bisiklis et al., (2005), the confirmed resistant isolates were distributed as: sputum (47.8%) which was higher than our results, pus (13%) which was close to our results, wound (17.4%) which was lower than our results, blood

culture (8.7%) and CSF (4.4%). Those confirmed resistant strains were collected from ICU (61%), surgical units (26%) and internal medicine wards (13%). The high percentage of resistance in ICU explains the high percentage of resistance from sputum.

In another study by Pitout et al., (2008) from Kenya, the confirmed resistant isolates were distributed as: sputum (53%), pus (7%), wound (30%) and blood culture (7%).The majority of them were collected from ICU (58%).

In our study, the MHT detected (48.1%) as carbapenemase and MBL producers. The DDST detected (77.8%) as MBL producers. These results are close to results of a study by Jesudason et al., (2005) from India as the modified Hodge test detected (56%) as carbapenemase and MBL producers. The double disc synergy test detected (72%) as MBL producers. Another study by Noyal et al., (2009) from India found that the modified Hodge test detected (28.1%) as carbapenemase and MBL producers and the double disc synergy test detected (50%) as MBL producers. A study by Lee et al., (2003) from Korea, used the modified Hodge test which detected (83.7%) as carbapenemase and MBL producers and the double disc synergy test detected (91.8%) as MBL producers. The DDST in the previous studies detected more positives than MHT with their results similar to our results. This may be due to false negative results frequently produced by the MHT.

On the other hand, Sevillano et al., (2006) from Spain used the MHT and detected (15.15%) as carbapenemase and MBL producers. However, the double disc synergy test detected (0%) as metallo-β-lactamase producers, they may be resistant to carbapenems due to mechanisms other than metallo-β-lactamase production.

In a study by Mendiratta et al., (2005), the modified Hodge test did not give an appreciable distorted zone of inhibition and therefore was not found suitable when compared to DDST which detected (93.3%) as metallo-β-lactamase producers.

In a study performed in Portugal by Pena et al., (2005), they used only the DDST with 52.8% detected as metallo-β-lactamase producers. Similar studies byIrfan et al., (2008), Behera et al., (2008) and Renata et al., (2008).They used only the DDST to detect metallo-β-lactamase producers with 100%, 57.14%, 96.4% respectively as metallo-β-lactamase producers.

As regards the performance of phenotypic tests in detecting MBL in our study, the DDST detected additional 10 isolates which were negative by MHT. This was similar to studies by Jesudason et al., (2005) and Noyal et al., (2009)in which the DDST detected 8 and 9 additional MβL producing isolates respectively not detected by MHT .In the current study, DDST was more sensitive than MHT(100%, 70%) respectively, this result was in accordance to Franco etal,2010. They reported that the DDST was a better method for screening MBL detection than MHT , the sensitivity was (100%, 82,4%) respectively. They reported that they don't rely on MHT as a good test for carbapenemase production due to the high rate of false negative results. However, the evaluation of the performance of DDST depends mainly on inhibitor-substrate combinations being better in mercapto acetic (MAA) or propionic acid (MPA)than EDTA – Imepenem combinations.

In our work, 10out of 27 IRP isolates (37%) expressed bla_{IMP} gene which was detected by PCR. Our results were close to that in Japan conducted by Zhao et al., (2008) in which bla_{IMP} gene expression was (33%). But in other studies from France conducted by Johann et al., (2005), from Poland conducted by Sacha et al., (2009) and from China conducted by Wang and Huang, (2004) the prevalence ofbla_{IMP} gene was lower than in our study (2%),(28.5%) and (29%) respectively.

Inspite that PCR was the gold standard in detection of MBL, The low bla_{IMP} expression in our study may be explained by the presence of different mechanisms of resistance to carbapenems such as reduced uptake as a result of loss of the porins or the presence of other untested genes, as reported by many previous studies (Hanson et al., 2006, Franco etal., 2010).They all had predominance of another genes bla_{VIM} or bla_{SPMI} in their strains which was not tested in our study.

The main source of bla_{IMP} producing isolates in our study was from wound (60%), sputum (40%), they were mainly from surgical units (70%) and ICUs (30%). In comparison to a study from France conducted by Johann et al., (2005), the isolates were from urine (43%), wound (21%), sputum (9%) and blood (7%), this difference may be due to that most of the isolates were from ICU.

In our study the resistance pattern ofbla_{IMP} expressing *pseudomonas* isolates was (100%) to fortum ,tazocin, cefepime and cefotaxime (70%) to amikin, (90%) to ciprofloxacin. This was in agreement with Franco etal.,2010, they reported that large proportion of MBL gene are associated with one or more aminoglycosides or b lactam resistance gene partially explaining multi drug resistant pattern.

However, in a study from India conducted by Varaiya et al., (2008)the resistance pattern ofbla_{IMP} expressing *pseudomonas* isolates was (34%) to quinolones, (50%) to fourth generation cephalosporins, (67%) to third generation

cephalosporins and(84%) to aminoglycosides. In another study in Canada Gregson et al., (2006)found that the resistance pattern was (59%) to third generation cephalosporins, (64%) to quinolones and (72%) to aminoglycosides.

CONCLUSION:

A high prevalence of IRP is a critical problem representing a practical therapeutic challenge. Even early recognition of MBL or Carbapenemase through routine laboratory testing is desirable; care should be taken when phenotypic tests are interpreted. In such cases, PCR should also be done to validate such results. The best method for MBL screening should be based on inhibitor synergy with respect to local factors such as bacterial type, MBL enzyme prevalence and the technical abilities of relevant facilities. Further genetic investigations for genes responsible for carbapenems resistance are recommended and strict infection control procedures should be followed.

References

Agrawal G., Lodhi R.B., Kamalaker U.P., Khadse R.K. and Jalgaonkar S.V. (2008): Study of metallo-β-lactamase production in clinical isolates of *Pseudomonas aeuroginosa*. Indian J. Med. Microbial.;***26(4):****349-351.*

Altoparlak U., Aktas F., Celebi D., Ozkurt Z. and Mufide N. (2005): Prevalence of metallo-β-lactamase among *Pseudomonas aeruginosa* and *Acinetobacterbaumannii* isolated from burn wounds and in vitro activities of antibiotic combinations against these isolates. Burns; ***31:*** *707–710.*

Behera B., Mathur P., Das A. and Kapil A. (2008): An evaluation of four different phenotypic techniques for detection of metallo-β-lactamase producing *Pseudomonas*spp.Indian J. Med. microbiol.;***26(3):****233-237.*

Bisiklis A., Tsiakiri E. and Alexiou-Daniel S. (2005): Phenotypic detection of *Pseudomonas aeruginosa*producing metallo-beta-lactamases. Clin.Microbiol.and Infect.; ***11(2):****418.*

Carvalho M.J., Saavedra M.J., Correia A., Castro A.P., Duarte A. (2005): Metallo-beta-lactamase in clinical *Pseudomonas aeruginosa* isolate in a Portuguese hospital and identification of a new VIM-2 like enzyme.Microbiol.and Infect.; ***11(2):****409.*

Clinical and Laboratory Standards Institute, 2011: Performance Standards for Antimicrobial Susceptibility Testing. 21th Informational Supplement. CLSI document M100-S19. Wayne, PA

Fine M.J., Smith M.A. and Carson C.A. (2005): "Prognosis and outcomes of patients with community-acquired pneumonia. A meta-analysis". JAMA 275 (2): 134–141.

Franco R G, Caiaffa-Filho´ lH,Burattini M N , RossiF´v(2010): Metallo-beta-lactamases among imipenem-resistantPseudomonas aeruginosa in a brazilian universityhospital, CLINICS ;65(9):825-829

Giamarellos-Bourboulis E.J., Papadimitriou E., Galanakis N., Antonopoulou A., Tsaganos T., Kanellakopoulou K. and Giamarellou H. (2006): Multidrug resitance to antimicrobials as a predominant factor influencing patient survival, Int. J. Antimicrob. Agents; ***27(6):****476–481*

GregsonA.,Bebrone C., Anne C., Galleni M., Frere J.M., and Dideberg O.(2006): Multidrug resistance to antimicrobials as a predominant factor influencing patient survival, Int. J. Antimicrob. Agents; ***27(6):****476–481*

Hanson N.D., Hossain A., Buck L., Moland E.S., and Thomson K.S.(2006): First occurrence of a *Pseudomonas aeruginosa* isolate in the United States producing an IMP metallo-beta-lactamase, IMP-18. Antimicrob. Agents Chemother.;***50:*** *2272-2273.*

Hemalatha V., Uma S. and Vijaylakshmi K. (2008): Detection of metallobetalactamase producing *Pseudomonas aeruginosa* in hospitalized patients. Indian J. Med. Researches ***122:****148-152.*

Hui Wang, Minjun Chena, Yuxing Ni, YudongLiua, HongliSuna, Yunsong Yu and others (2010): Antimicrobial resistance among clinical isolates from the Chinese Meropenem Surveillance Study (CMSS). Int. J. Antimicrob. Agents.***35:****227–234.*

Irfan S., Zafar A., Guhar D., Ahsan T., Hasan R. (2008): Metallo-β-lactamase-producing clinical isolates of *Acinetobacter* species and *Pseudomonas aeruginosa* from intensive care unit patients of a tertiary care hospital. Indian J. Med. Microbiol.; ***26(3):****243-245.*

Japoni A., Alborzi A., Kalani M., Nasiri J., Hayati M. and Farshad S. (2006): Susceptibility patterns and cross-resistance of antibiotics against *Pseudomonas aeruginosa* isolated from burn patients in the South of Iran. Burns; ***32:*** *343–347.*

Jesudason M.V., Kandathil A.J. and Balaji V. (2005): Comparison of two methods to detect carbapenemase &metallo-[beta]-lactamase production in clinical isolates. Indian J. Med. Res.;***121:*** *780-783.*

Johann D., D. Pitout., Daniel B.Gregson., Laurent Poirel., Jo. Am McClure., Phillip Le and Deirdre L.Church (2005): Detection of

Pseudomonas aeruginosa producing metallo-beta-lactamases in a large Centralized Laboratory. J. Clin. Microbiol. 3129-3135.

Lee K., Lim Y.S., Yong D., Yum J.H. and Chong Y. (2003): Evaluation of the Hodge test and the imipenem-EDTA double disk synergy test for differentiation of metallo- β-lactamases producing clinical isolates of *Pseudomonas* spp. and *Acinetobcter* spp. J. Clin. Microbiol.;***41:*** *4623-4629.*

Mathur P., Behera B., Sharma V., Das A. and Kapil A. (2008): An evaluation of four different phenotypic techniques to evaluate detection of Metallo-β-lactamase producing *Pseudomonas aeruginosa.* Indian J. Med. Microbiol.; ***26(3):****233-237.*

Mendiratta D.K., Vijayshri D. and Pratibha N. (2005): Metallo-β-lactamase producing *Pseudomonas aeruginosa* in a hospital from a rural area **.**Indian J. med. Resear.;***121:****701-703.*

Nordman P. and Poirel L. (2006): Emerging carbapenemases in Gram-aerobes. Clin.Microbiol. Infect. Dis. 8:321-331.

Noyal M.J.C., Menezes G.A., Harish B.N., Sujatha S. andParija S.C. (2009): Simple screening tests for detection of carbapenemases in clinical isolates of non fermentative Gram-negative bacteria. Indian J. Med. Res. ***129:****707-712.*

Pena A., Martins J., Donato A., Leita R. and Cardoso O. (2005): Occurrence of metallo-beta-lactamase VIM-2 in *Pseudomonas aeruginosa* clinical isolates resistant to carbapenems in a hospital in central Portugal. Clin.Microbiol.Infec.;***11(2):****431-436.*

Pitout J., Revathi G., Chow B.L, Kabera B., Kariuki S., Nordmann P. and Poirel L. (2008): Metallo-β-lactamase producing *Pseudomonas aeruginosa* isolated from a large tertiary centre in Kenya. Clin.Microbiol.Infect.**;*14:*** *755–759.*

Pollack M. (2000): Pseudomonas Aeruginosa. In: Principles and Practice of Infectious Diseases. Mandell GL, Bennett JE, Dolin R, eds5th ed. New York, NY: Churchill Livingstone; 2310-2327.

Renata C. Picao , Soraya S. Andrade , Adriana GianinniNicoletti , Eloiza H. Campana , Gabriela C. Moraes , Rodrigo E. Mendes and Ana C. Gales (2008): Metallo-β-Lactamase Detection: Comparative Evaluation of Double-Disk Synergy versus Combined Disk Tests for IMP, GIM, SIM, SPM, or VIM-Producing Isolates. J. clinic.microbial.; ***46:*** *2028–2037.*

Sacha, P., M. Zorawski., T. Hauschild., P. Wieczorek., J. Jaworowska., P. Jakoniuk., E. Tryniszewska (2009): The presence of bla_{IMP} gene on plasmid DNA isolated from multidrug resistant Pseudomonas aeruginosa strains at University Hospital in Bialystok(Poland). Vol. 45:405-408.

Sarkar B., Biswas D. and Prasad R.(2006): A clinicomicrobiologicalstudy on the importance of pseudomonas in nosocomially infected ICU patients with special reference to metallo-β-lactamase production.Indian J. Pathol. Microbiol.;***49:****44-48.*

Sevillano E., Valderreya C., Canduela M.J., Umaran A., CalvobF.andGalleg L.(2006): Resistance to antibiotics in clinical isolates of *Pseudomonas aeruginosa.*PathologieBiologie; ***54 :****493–497.*

Tenover F.C. (2006): Mechanisms of antimicrobial resistance in bacteria. Am. J. Med.;***119 (1):****6-10.*

Varaiya A., Kulkami N., Kulkami M., Bhaleka P. and Dogra J. (2008): Incidence of metallo-beta-lactamase producing Pseudomonas in ICU patients. Indian J. Med. Res.; ***54:****398-402.*

Wang C. and Huang Z. (2004): IMP-1 metallo-beta-lactamase producing Pseudomonas in a University Hospital in China.J. Antimicrob.Chemother.1090-1093.

Zhao W. H., Hu Z. Q., Hera Y. and Shimamura T. (2008): Potency of IMP-10 metallo-beta-lactamase in hydrolyzing various antipseudomonal β-lactams. J. Med. Microbiol. 59:974-979.