Prevalence of Metallo-β-Lactamaseproducing *Pseudomonas aeruginosa*isolated from different clinical samples in Baghdad province

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الخلاصة

بكتريا الزوائف الزنجارية المنتجة لانزيم Metallo-β-lactamase مثبته بانها من المسببات المهمة للاصابات والالتهابات المختلفة في المستشفيات. بكتريا الزوائف الزنجارية لها قابلية عالية على مقاومة العديد من المضادات الحياتية المستخدمة حاليا من خلال ميكانيات مختلفة منها داخلية او مكتسبة واهم الميكانيكيات هو انتاج انزيم Metallo-β-lactamase

تم جمع 75 عزلة من بكتريا الزوائف الزنجارية من مختلف العينات السريرية من بعض المستشفيات والمختبرات الحكومية والخاصة في محافظة بغداد للفترة من نيسان ولغاية اب لعام 2011. تم تشخيص البكتريا باستخدام مختلف الطرق سواء كانت فحوص كيميائية او عن طريق تشخيص البكتريا في اوساط زرعية مختلفة،كذلك تم تشخيص العزلات البكتيرية باستخدام جهاز VITEK2 وتم قياس الحساسية الدوائية للعزلات البكتيرية باستخدام disk diffusion وكذلك تم قياس اقل تركيز مثبط من العقار (MIC) باستخدام جهاز VITEK2 وكذلك طريقة E-Test لعقاريImipenem و Meropenem وتم اجراء فحص (CDST) كفحص تاكيدي للعزلات المقاومة لعقاري Imipenem ,Meropenem للتاكد من انتاج انزيم MBL .وتم التوصل للنتائج التالية:نسبة انتشار العزلات المقاومة لعقارات الكاربابينيم هي 8% وعدد العزلات البكتيرية المنتجة لانزيم MBLباستخدام الطرق المظهرية هو 4 وبنسبة 5.3%.

Abstract

Background: Metallo beta lactamase(MBL) producing *Pseudomonas aeruginosa*have been reported to be an important nosocomialinfections.*Pseudomonas aeruginosa* is a leading cause of nosocomial infections, giving risetoa wide range of life-threatening conditions. Its intrinsic & acquired resistance to many antimicrobial agents and its ability to developmultidrug resistance imposes a serious therapeutic problem.

Materials and Methods: A total of 75 *P.aeruginosa*isolates were isolated from different clinical samples in some public & private hospitals in Baghdad city during the period from April to August 2011.Bacterial identification was done using conventional cultural & chemical methods &and VITEk 2 cards for identification (GN), while the minimum inhibitory concentration (MIC) testing was performed using disk diffusion,E-test for Imipenem& Meropenem(oxoid, UK) & (AST-GN30)cards in VITEK 2 automated system(bioMérieux, , France).

Each *P.aeruginosa* isolates showed resistance to Carbapenems(Imipenem& Meropenem) were subjected to Imipenem-EDTA combined disc synergy test (CDST) to investigate the production of MBL(confirmative test)

Results:Out of 75 *P.aeruginosa* isolates,16 (21.3%) were grow on MacConkey agar supplemented with Meropenem4mg/L (MMAC),this method used as screening test, The MIC of different antibiotics was performed on these isolates using different methods(VITEK2AST-GN30,Imipenem&Meropenem E-test) showed that 6 (37.5%) isolates were Carbapenem resistant MIC ≥16µg/ml,while 4(25%) pseudomonas isolates appear to be MBL producer usingImipenem-EDTA combined disc synergy test(CDST).

Discussion: MBL mediated carbapenemresistance in *P. aeruginosa* is a cause for concern in thetherapy of critically ill patients. The MBL producing *P. aeruginosa* isolates were more resistant to various antimicrobial agents. This result suggests that MBL producing isolates in hospitals may cause serious infections that illustrated when these strains were responsible for a nosocomial outbreak.The findings strongly suggest that there is a need to track the detection of MBL producers and that judicious use of carbapenems is necessary to prevent the further spread of these organisms.

Conclusion: The prevalence of multi-drug resistant *P.aeruginosa* isolates especially Carbapenem resistant bacteria was increased in Baghdad province.Phenotypic characterization of MBLs provide information about the prevalence of MBLs producing *P. aeruginosa* in Baghdad.

Key words: *Pseudomonas aeruginosa* ,MBL,Carbapenems ,ESBL, CRPA

Introduction

Pseudomonas aeruginosa producing metallo-β-lactamase (MBL) was first reported fromJapan in 1991 and since then has been describedfrom various parts of the world including Asia,Europe, Australia, South America , andNorth America (1). Metallo-β-lactamases belong to Ambler class B and have the ability to hydrolyze a wide variety of β-lactam agents, such as penicillins, cephalosporins, and carbapenemsand consist of five groups of enzymes, namely IMP, VIM, SPM, GIM and SIM These enzymes require zinc for their catalytic activity and are inhibited by metal chelators, such as EDTA and thiol-based compounds (2). The genes responsible for the production of MBLs are typically part of an integron structure and are carried on transferable plasmids but can also be part of the chromosome .Therefore, because of the integron-associated gene cassettes, *P. aeruginosa* isolates producing MBL are often resistant to different groups of antimicrobial agents, which can be transferred to various types of bacteria(3).

Carbapenems (Imipenem& Meropenem) have a potent antipseudomonal activity and are often used as a last resort for the treatment of infections due to multi resistant Pseudomonas isolates. The introduction of carbapenems into clinical practice marked a great advance for the treatment of serious bacterial infections caused by beta-lactam-resistant bacteria(4).

*Pseudomonadsaeruginosa*can develop resistance to carbapenems through diminished permeability, stable derepression of chromosomal AmpC β-lactamases, or over-expression of the up regulating efflux system. During the last decade, carbapenem-resistance among hospital-acquired pseudomonads has been sporadically attributed to the production of the metallo-β-lactamase (5).

The most common and widespread acquired MBLs are those of the IMP and VIM types, both exhibit a worldwide distribution and for which several allelic variants are known. Acquired drug resistance is frequent in nosocomial isolates of *P. aeruginosa* and often involves more than one antibiotic class (6,7).

Infection with the metallo-beta-lactamase (MBL) producing organisms is associated with higher rates of mortality, morbidity, and health care costs.The international epidemiology of MBL-producing *P. aeruginosa* is still unknown in most countries (8), which is at least partly due to thelack of proper screening recommendations. For some countries, such Korea and Brazilthe proportion of MBL-producers among imipenem-resistant *P. aeruginosa* has beenestimated to 11.4 and 20%, respectively (8)Nosocomial infection involving multi-resistant *Pseudomonas aeruginosa*is a

growing problem worldwide.

Rapid detection ofmetallo-β-lactamases(MBLs) is crucial for patient managementand appropriate infection control procedures. the MBL enzyme detection in carbapenemresistant *P. aeruginosa* was doneby using twoMBL screening tests: the imipenem/EDTA combined disc test (MBL-CD) and the imipenem&meropenem (E-test), both ofwhich are based on the ability of EDTA to sequester zinc ionsand to inactivate the metalloenzymes.. Some studies of regional epidemiologyhave tried to address the issue of clonal dissemination, and although certain diversitycan be observed in many areas there is still evidence supporting that clonal expansion isan important mode of spread (9).

Materials and Methods

Bacterial Isolates

Seventy five isolates of *Pseudomonas aeruginosa* were isolated from different clinical samples in Baghdad/Iraq during the period from April to August 2011.These isolates were as follows: burn (22), ear (14), sputum (13), wound (7), urine (5), blood (5), nasal swab (4), eye (3), biopsy (2). Clinical samples were collected from Teaching laboratories of medical city, Al-Yarmouk Hospital, Al-Waseti hospital, Al-Jadriya Hospital, in addition to some private laboratories. Bacteria were cultured on MacConkey and Nutrient agar in aerobic condition at 42 C for 24-48 h. Then identified by conventional biochemical tests and by using of VITEK 2 Automated system using (GN) cards.

Antibioticsusceptibility testing

All *pseudomonas aeruginosa* isolates were cultures on MacConkey agar supplemented with a Carbapenem (Meropenem) 4mg/L (selective media) to check the susceptibility of the 75 *P.aeruginosa* isolates, only 16 pseudomonas isolates grow on this media & antibiotic susceptibility testing was performed as recommended by the Clinical & Laboratory Standards Institute (CLSI, 2011) using disks containing containingceftazidime (CAZ:30 µg), ceftriaxone (CRO:30 µg), cefotaxime (CTX:30 µg), piperacilin/tazobactam (TZP:110 µg), gentamicin(GM:10 µg), amikacin (AN:30 µg), imipenem (IPM:10 µg), ciprofloxacin (CIP:5 µg), polymyxin B (PB:300 unit), meropenem (MEM:10 µg) (Himedia, India).Isolates were considered to be imipenem resistant when the zone around imipenem was = 13 mm, intermediate 14-15 mm and sensitive =16 mm (10).

VITEK 2 system using (AST- GN30) was used & the MIC for these antibiotics was obtained.

MICs for Imipenem& Meropenem also were determined by E-test (Oxoid, UK)method.

Identification of Metallo-β-Lactamase Producing Isolates

1-**Imipenem-EDTA combined-disc test (CDST):** The test organisms were inoculated onMueller Hintonagar as recommended by the CLSIs (NCCLS guidelines). A0.5 M EDTA solution was prepared by dissolving 18.61 g. of EDTA in 100 ml of distilled water and adjusting its pH 8.0 by using NaOH. The Mixture was sterilized by autoclaving. Two imipenem(10µg) discs were placed on the surface of an agar plate at a distance of 30 mmand 4 µl EDTAsolution was added to one of them to obtain a desired concentration of 750 µg. The inhibition zones of imipenem and imipenem-EDTA discs were compared after 16 to 18 h of incubation in air at 37 ºC. In the combined disc test, if the increase in inhibition zone with the imipenem-EDTA disc was >7 mm than thezone of inhibition of imipenemalone, it was considered MBL positive(11).

2-**Modified Hodge Test (MHT):**A 0.5 McFarland dilution of E.coli ATCC 25922 was prepared in 5 ml of tryptic soy broth, a lawn was streaked to the Mueller-Hinton agar plate and allow to

dry (3-5) min, Imipenem disc (10 µg) was placed in the center of the test area. In straight line the test organism streaked from the edge og the disc to the edge of plate , the plates were incubated overnight at 37 C°.MHT positive test has a clover leaf-like identification of *E.coli*ATCC 25922 growing alone the test organism (*Pseudomonas aeruginosa*) growth streak within the disc inhibition zone .MHT negative test has no growth of *E.coli* along the test organism (10).

Results

Out of the 75 *P.aeruginosa* isolates studied,only 16(21.3%)*pseudomonas* isolates growon MacConkey agar supplemented with a Carbapenem (Meropenem) 2mg/L (selective media).The antibiotic susceptibility test was done for these isolates&6(8%) were carbapenemsresistant and 4(5.33%) were MBL producers (Table 1), The 6 imipenem resistant isolates were screened for carbapenem hydrolysis by E-test(Fig 1).

In present study the MIC of 10 antibiotics listed in Table (2) was done using VITEK2-Compact by using AST-GN30 for testing the antibiotic suscepatability of thses isolates. The MIC was done for 16 *P. aeruginosa* isolates that grow on MMAC and the MIC values was interpreted according to the CLSI (10).

The MIC for carbapenems imipenem and Meropenem for the six carbapenem resistance*P. aeruginosa*(CRPA) was also tested using MIC elevator test as confirmative test Table (2).

The Carbapenem resistant *P.aeruginosa* (CRPA) in this study (no. 6) differ in the level of resistance to different antibiotics including the carbapenems as showed in Table (2). Four CRPA isolates showed MIC ≥16 µg/ml for both imipenem and Meropenem respectively , while the remaining 2 CRPA were Meropenem susceptible MIC 4 µg/ml. These two isolates identified as intermediate resistant to meropenem using carbapenem E-test.

The resistance profile of CRPA against the 4th generation of cephalosporins (cefepime) was different, 4 CRPA isolates were resistant to cefepime with MIC 32-≥64 µg/ml, while the other 2 isolates were intermediateresistant tocefepime MIC 16 µg/ml. Only 10 (62.5%) isolates out of 16 were resistant to cefepime.All the *P. aeruginosa* including the CRPA isolates were resistant to cefoxitin ,ceftazidime and ceftraiaxone MIC 32-≥ 64 µg/ml .

The study showed that all CRPA isolates were resistant to ciprofloxacin MIC ≥4 µg/ml.Only one *Pseudomonas aeruginosa* isolate (*Psa*-3W) showed intermediate resistance to aminoglycosides gentamicin MIC 8 µg/ml, while the remaining CRPA *P. aeruginosa* isolates was resistant to gentamicin.

The detection of MBLs production among the CRPA isolates was done using combined-disc synergy test (CDST). Only 4 (5.33%) out of 75 *P. aeruginosa* isolates showed a positive result (Table-1). As an inhibition zone with the imipenem-EDTA disc was >7 mm than the zone of inhibition of imipenem alone. These isolates were*Psa*8B, *Psa* 9B, *Psa* 22B, *Psa*12E. while the remaining *Psa*4E, *Psa* 3W were negative as shown in (Fig 2).

The Modified Hodge Test (MHT) was performed for all carbapenem resistant *P. aeruginosa* (CRPA) isolates and the results is resemble to these obtained by CDST. Only 4 isolates (5.33%) were identified as carbapenemase(MBLs) producer.Positive strain shows a ‘cloverleaf shaped’ zone of inhibition due to carbapenemase production, while the negative strain shows an undistorted zone of inhibition. The positive isolates are*Psa*8B, *Psa* 9B, *Psa* 22B, *Psa*12E.

while the remaining *Psa*4E, *Psa* 3W were negative. This result was the same as obtained by CDST, so only 4 of CRPA *P.aeruginosa* isolates out of 75 isolates appear to be MBLs producer Figure (3).

Table-1: Prevalence of MBL producing *P.aeruginosa* isolates

Total number of isolates	Positive for MBLs	
	numbers	percentage
n=75	4	5.3%

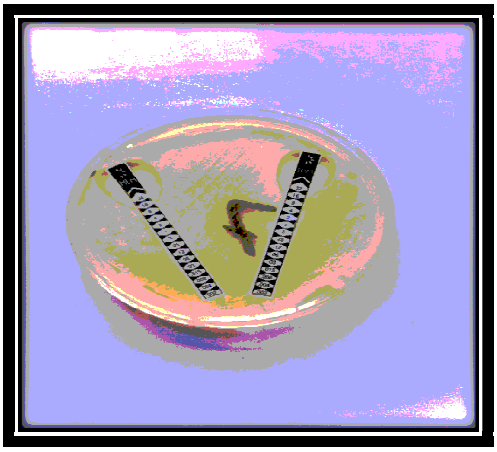

Figure-1: MIC value of carbapenem resistant *P.aeruginosa* isolatesusing Imipenem& Meropenem E-Test

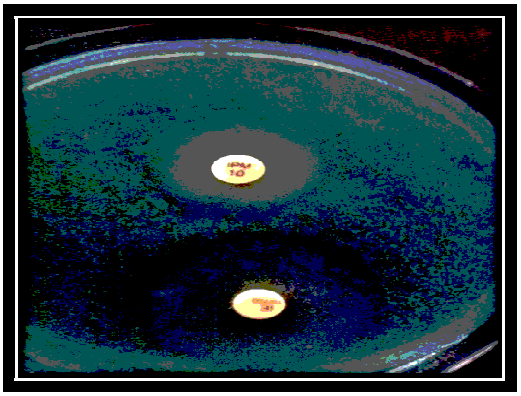

Figure-2:CDST positive for MBL production in CRPA isolate

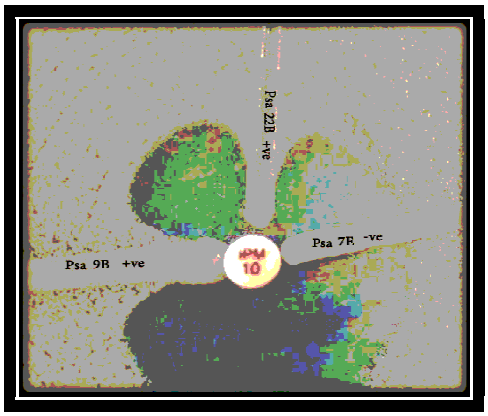

Figure-3: Modified Hodge Test Show positive result for carbapenemaseproduction

Most of the carbapenems resistant *P.aeruginosa* isolates are taken from burned patients(Table2).All the isolates are resistant to Imipenem MIC ≥16, while only 4 isolates are resistant to Meropenem MIC ≥16& these isolates were found to be multi drug resistant (MDR) to the 10 antibiotics (Table 2).

Table 2- Antibiotic susceptibility of Carbapenem resistant *Pseudomonas aeruginosa* isolates

Isolate	Specimen	E-test		MBL CDST	MIC (µg/ml) of selected antibiotics determined by VITEK 2 system									
		IPM	MEM		IPM	SAM	FEP	FOX	CAZ	CRO	CIP	GM	MEM	TZP
*Psa*3W	Wound	16	8		≥16 (R)	≥32 (R)	≥32 (R)	≥64 (R)	32 (R)	≥64 (R)	≥4 (R)	8 (I)	4(S)	≥128 (R)
Psa 8B	Burn	16	16	+	≥16 (R)	≥32 (R)	16 (I)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)
*Psa*9B	Burn	16	16	+	≥16 (R)	≥32 (R)	≥32 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)
Psa 4E	Ear	16	8		≥16 (R)	≥32 (R)	16 (I)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	4 (S)	≥128 (R)
*Psa*22B	Burn	16	16	+	≥16 (R)	≥32 (R)	≥32 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)
*Psa*12E	Ear	16	16	+	≥16 (R)	≥32 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)

Abbreviation:IPM, imipenem; SAM, ampicillin/sulbactam; FEP, cefepime; FOX, cefoxitin; CAZ, ceftazidime; CRO, ceftriaxone; CIP, ciprofloxacin; GM, gentamicin; MEM, meropenem; TZP, piperacillin/tazobactam

Discussion

Pseudomonas is common pathogen causing nosocomial infection.In present study, MBL positive isolates shows high level of resistance to all β-lactam antibiotics including β-lactamase inhibitor,aminoglycosides and quinolones.Resistance to aminoglycosides was present in most CRPA isolates, but is not a reliable criterion for MBL-screening (12). According to various studies MBL production ranged from 7% to 65%. In Present study, MBL productions was5.3% in isolates of *Pseudomonas aeruginosa*using CDST and MHT. MBL positive isolates leads to serious therapeutic failure because they carry multidrug resistant genes and the only treatment option available is potentially toxic polymyxin B (13).

Out of 75 isolates of Pseudomonas, 6 (8%) were MBL positive by screening tests, 4 (5.3%) were MBL positive by CDST, as the CDST is more sensitive for detection of MBL producing isolates. It is necessary to detect MBL producing Pseudomonas isolates in routine clinical laboratory techniques by using MBL inhibitor (EDTA). Currently, no standardized method for MBL detection has been proposed and despite PCR being highly accurate and reliable, its accessibility is often limited to reference laboratories (14).

In this study all CRPA isolates (6) were found to be resistant to at least 3, 4 and 5 antibiotic classes tested and considered as MDRs isolates (Table 2).

There is an increase in the resistance against the powerful carbapenems antibiotics.MBL positivity is increased dramatically among Imipenem& Meropenem resistant *p.aeruginosa* isolates.Long-term hospitalization, indwelling urinary catheters, and long-term antibiotic use (in particularof carbapenems) are the possible risk factors for colonization and/or infection such pathogens as MBLs positive *Pseudomonas aeruginosa*(15).

In absence of novel antibiotics for the treatment of infections caused by multidrug-resistant gram-negative bacteria in the near future, uncontrolled spread of MBLs producers may lead to treatment failures with increased morbidity and mortality. Appropriate therapeutic protocols and a regular screening/monitoring system should be established to prevent the wider spread of this worrisome resistance determinant (15).The low susceptibility of the CRPA isolates against many antibiotics in this study may be due to extensive using of these antibiotics such as ciprofloxacin and gentamicin in clinical practice in Iraq. Excessive use of broad-spectrum antibiotics in hospitals has lead to the emergence of highly resistant strains of *P. aeruginosa*. To reduce the selection pressure for resistance, it is an important to determine the antibiotic susceptibility pattern of bacteria so that

hospitalized patients can be treated with more narrow-spectrum and target-specific antibiotics (16).

Conclusion

The prevalence of multi-drug resistant *P. aeruginosa* isolates especially Carbapenem resistant bacteria was higher than that which has been found in survey of later years. Phenotypic characterizations of MBLs provideinformation about the prevalence of MBLs producing *P.aeruginosa* in Baghdad. The antibiotic resistance was increased against all third generation cephalosporins.

Although the carbapenem was the drug of choice for ESBL producing *P. aeruginosa* isolates, the emerging of MBLs producing bacteria posses a threat to antibiotic treatment program in Baghdad hospitals.Its highly recommended to track the MBL producing *P. aeruginosa* and using of carbapenem is necessary to prevent the spread of the infection with these bacteria.

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