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Phenotypic detection of metallo- β -lactamase producing *Pseudomonas aeruginosa* isolated from Urmia hospitals

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Detection of metallo- β -lactamase producing *Pseudomonas aeruginosa* is crucial for the optimal treatment of patients; however there are limited studies on metallo- β -lactamase producing *P. aeruginosa* isolates from West Azarbayejan, Iran. This study was designed to detect the metallo- β -lactamase in *P. aeruginosa* isolates. One hundred isolates were collected from clinical specimens submitted to hospital diagnostic laboratories in Urmia/Iran from July to September 2010. The susceptibilities of the isolates to different classes of antibiotics were tested using Müller-Hinton agar disk diffusion method. All isolates of *P. aeruginosa* were subjected to determination of minimum inhibitory concentrations (MICs) against Imipenem. Imipenem non-susceptible isolates were investigated for metallo- β -lactamase production by the combined disk method. The rates of resistances to antibiotics, as were determined, is as follows: kanamycin (91%), Tobramycin (34%), Ciprofloxacin (16%), Colistin (68%), Ticarcillin (46%), Amikacin (16%), Norfloxacin (23%), gentamicin (33%), Ceftazidime (62%), Ceftizoxime (69%), and Cefepime (39%). Seventy nine isolates (79%) were sensitive (MIC $\leq$ 4 mg/L) and 21 isolate (21%) were resistant to Imipenem (MIC $\geq$ 8 mg/L). The rates of resistance to different antibiotics were much higher in Imipenem resistant isolates. Detection of metallo- β -lactamase producing isolates among Imipenem non-susceptible isolates of *P. aeruginosa* revealed that seven isolates (33.3%) were metallo- β -lactamase positive. Metallo- β -lactamase positive isolates showed high resistances to all tested antibiotics. This result suggests that metallo- β -lactamase producing isolates in hospitals may cause serious infections which can lead to failure in patient's antibiotic therapy.

Key words: *Pseudomonas aeruginosa*, metallo- β -lactamase and Imipenem.

INTRODUCTION

Pseudomonas aeruginosa is a major bacterium causing nosocomial infections, and the development of multidrug resistance of this bacterium to different classes of antibacterial agents has become a main problem (Thuong et al., 2003; Lockhart et al., 2007; Algun et al.,

2004).

This opportunistic bacterium can quickly colonize in immunocompromised host and infect the burn and wound sites, disseminate to the bloodstream and induce endotoxic shock, the clinical outcome in these patients can lead to sepsis which is often fatal. As mentioned previously, antibiotics such as β -lactams are generally ineffective against most serious infections caused by *P. aeruginosa* (Dale et al., 2004). Also resistance to extended spectrum β -lactams has been frequently observed in *P. aeruginosa* (Kahan et al., 1983; Saderi

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et al., 2008). Several mechanisms for resistance to extended spectrum β -lactams have been reported before, but the common form of resistance is mediated by hydrolyzing β -lactamases. Based on molecular studies, carbapenem hydrolyzing enzymes are classified into four groups A, B, C and D (Ambler, 1980). The metallo- β -lactamases (MBLs) belong to group B and requiring divalent cations as cofactors for their enzyme activity. The MBLs efficiently hydrolyze all β -lactams, except aztreonam *in vitro* (Bush et al., 1995). *P. aeruginosa* producing MBLs was first reported from Japan in 1991 (Watanabe et al., 1991) and within a short time period has been reported from various parts of the world (Lee et al., 2004a; Yan et al., 2001; Lagatolla et al., 2004; Toleman et al., 2004).

Several phenotypic methods are available for the detection of MBL producing bacteria. All the methods are based on the ability of metal chelators such as ethylenediaminetetraacetic acid (EDTA) to inhibit the activity of MBLs. For example the double disk synergy tests using EDTA with Imipenem (Lee et al., 2004a; Yan et al., 2004). Since MBL producing *P. aeruginosa* is often resistant not only to all β lactams, but also aminoglycosides, and fluoroquinolones, there are often no drugs to treat infection with these bacteria. On the other hand detection of MBL produced *P. aeruginosa* is crucial for the optimal treatment of patients particularly in the cases of critically ill and to control the spread of resistance (Oie et al., 2009). There are limited studies available on MBL producing *P. aeruginosa* isolates from West Azerbaijan, Iran. Therefore, this study was designed to detect the MBL in *P. aeruginosa* isolates obtained from hospitalized patients in Urmia, Iran.

MATERIALS AND METHODS

Bacterial isolates

A total of 100 isolates were collected from clinical specimens (wound, urine, sputum, blood and pleural fluid) submitted to hospital diagnostic laboratories in Urmia/Iran from July to September 2010. Fifty one isolates were obtained from male patients and 49 isolate from female patients, 36 isolates recovered from outpatients and 64 isolates were obtained from in- patients. The isolates were further processed by the following standard methods to identify as *P. aeruginosa*: Gram stain, colony morphology, pigment production, triple sugar iron agar reaction (Himedia laboratories, Mumbai), oxidase test, IMViC tests (Himedia laboratories, Mumbai), ability of growth in 42°C, O/F reaction and growth on cetrimide agar (Himedia laboratories, Mumbai). Isolated bacteria were maintained for long storage on skimmed milk medium, biological laboratories (BBL) by adding 10% glycerol (Merck) in -60°C, cultures were maintained for daily use on nutrient agar (BBL) slants on 4°C. *P. aeruginosa* ATCC 27853 was used as control.

Antimicrobial susceptibility testing

The susceptibilities of isolates to different antibiotics were tested using agar disk diffusion method (Bauer et al., 1966) in accordance with national committee for clinical laboratory standards (NCCLS)

incorporating standard strain of *P. aeruginosa* (ATCC 27853). To represents the different classes of antimicrobial agents commonly used for the treatment of *P. aeruginosa* infections, we used Kanamycin (30 mcg), Tobramycin (10 mcg), Ciprofloxacin (5 mcg), Colistin (10 mcg), Ticarcillin (75 mcg), Amikacin (30 mcg), Norfloxacin (10 mcg), Gentamicin (10 mcg), Ceftazidime (30 mcg), Ceftizoxime (30 mcg), and Cefepime (30 mcg) (Hi-media, Mombay, India). The definition of multi drug resistant *P. aeruginosa* was established as isolates resistant to at least three drugs in four different classes including: β -lactams, carbapenems, aminoglycosides, and fluoroquinolones (Obritsch et al., 2005).

Determination of MIC of Imipenem for isolates

All isolates of *P. aeruginosa* were subjected to determine minimum inhibitory concentrations (MICs) against Imipenem. The E-test method has been used for MIC determination according to the manufactures instructions. In brief, bacterial suspensions was prepared from fresh colonies and the concentration has been adjusted to 0.5 McFarland turbidity. Each isolate was inoculated by streaking the bacteria all over a mueller hinton agar (MHA) plate. An Etest strip of Imipenem (AB Biodisk Sweden) (from 0.002 to 32 μ g/mL) was placed on the surface of cultured media, after overnight incubation at $36 \pm 0.5^\circ\text{C}$, MIC has been determined and the sensitive, intermediate and resistant phenotypes were determined according to the European committee on antimicrobial susceptibility testing (EUCAST) guideline (http://www.eucast.org/clinical_breakpoints/).

Detection of MBL producing isolates by Imipenem-EDTA combined disk method

Imipenem non-susceptible isolates were investigated for MBL production by this method. The bacterial suspension with turbidity equivalent to 0.5 McFarland was prepared and cultured on MHA by streaking method. Two Imipenem disks (10 μ g) have been placed on the agar surface and 5 μ l of 0.5 M EDTA solution was added only to one of the disks. After overnight incubation at $36 \pm 0.5^\circ\text{C}$, the inhibition zones of Imipenem disks with or without EDTA were measured and compared. An increase of seven mm or more in the zone diameter for Imipenem + EDTA disk in compare with Imipenem disk was considered as a MBL producing isolate (Lee et al., 2001).

RESULTS

Bacterial isolates

A total of 100 clinical isolates of *P. aeruginosa* were collected from three University Hospitals in Urmia, northwest of Iran. The frequency of specimen's sources of *P. aeruginosa* isolates is shown in Figure 1.

Antibiotic susceptibility testing based on disk diffusion method

Antibiotic susceptibility testing results against 11 antibiotics are presented in Figure 2. *P. aeruginosa* isolates showed high resistances to Kanamycin, Ceftizoxime and Colistin respectively, on the other hand the most effective antibiotics were Amikacin and Ciprofloxacin.

Figure 1. Frequency of *P. aeruginosa* clinical samples source.

Determination of MIC of Imipenem for isolates

Determination of MICs for Imipenem by Etest method revealed that 16 isolates were resistant to all tested concentrations of Imipenem, the mean MIC $\pm$ SD value for the remind 84 isolates was 1.17 ± 1.51 μ g/mL. According to the EUCAST breakpoint for Imipenem, 79 isolates (79%) were sensitive (MIC $\leq$ 4 mg/L) and 21 isolate (21%) were resistant to Imipenem (MIC $\geq$ 8 mg/L).

Prevalence of antibiotic resistance among susceptible and resistant isolates to Imipenem

The rate of resistance to different antibiotics was much higher in 21 Imipenem resistant isolates in comparison with 79 Imipenem non resistant isolates (Figure 3).

Detection of MBL producing isolates by Imipenem-EDTA combined disk method

Detection of MBL producing isolates among Imipenem non-susceptible isolates of *P. aeruginosa* by this phenotypic method revealed that seven isolates (33.3%) were MBL positive (cut off value $\geq$ 7 mm).

Comparison of the rate of antibiotic resistance in MBL positive versus MBL negative isolates

MBL positive isolates showed very high resistances to all tested antibiotics. Five isolates (71.4%) were resistant to all antibiotics; only two isolates (28.5%) were sensitive to Amikacin and one isolate revealed intermediate resistance to Norfloxacin (Table 1).

DISCUSSION

P. aeruginosa is one of the most important hospital acquired pathogens. Recently obtained hospital isolates of *P. aeruginosa* are often multi drug resistant to many classes of antimicrobial agents, including fluoroquinolones, β -lactams and even aminoglycosides. Carbapenems are the most potent β -lactam antibiotics, which are active even against extended spectrum β -lactamase producing gram-negative bacteria. However, due to acquired MBL production, carbapenem resistance in *P. aeruginosa* and some of the other gram negative bacilli has been reported in many countries (Walsh et al., 2005).

The data obtained in this research indicate that the prevalence of resistance of *P. aeruginosa* isolates to tested antibiotics was relatively high. To our findings the best antibiotics for treatment of *P. aeruginosa* infections were Amikacin and Ciprofloxacin followed by Norfloxacin and Gentamicin (Figure 2). The rate of resistance to Ceftazidime increased in comparison with the previous studies has been done before in the same hospitals (Yousefi et al., 2010). On the other hand the resistance rate to some of antibiotics such as Amikacin, Ciprofloxacin and Gentamicin showed decreases in comparison with the previous studies in the same hospitals. This findings in part may be due to wards where the samples has been taken from them; as in the present study, most of the samples has been obtained from pediatrics ward, but in the previous study has been done by Yousefi et al. (August 2007 to October 2008) many of the isolates has been collected from patients in intensive care unit (ICU), internal or burn wards, also the optimal useage of existing antimicrobial agents in treatment of *P. aeruginosa* infections or the use of alternative treatment options, in the studied hospitals in

Table 1. The rates of resistance to different antibiotics in MBL producing isolates, MBL isolates were resistant to many of tested antibiotics.

Antibiotics isolate No.	Ciprofloxacin	Colistin	Ticarcillin	Cefepime	Amikacin	Norfloxacin	Gentamicin	Ceftazidime	Ceftizoxime	Tobramycin	Kanamycin
4	R	R	R	R	R	R	R	R	R	R	R
6	R	R	R	R	R	R	R	R	R	R	R
36	S	R	R	R	S	I	R	R	R	R	R
45	R	R	R	R	R	R	R	R	R	R	R
77	R	R	R	R	S	R	R	R	R	R	R
83	R	R	R	R	R	R	R	R	R	R	R
91	R	R	R	R	R	R	R	R	R	R	R

r: resistant, s: sensitive and i :intermediate resistant.

Figure 2. The rates of resistance to different antibiotics for 100 clinical isolates of *P. aeruginosa* (r: resistant, s: sensitive and i :intermediate resistant).

the recent years may play a role in decrease of antimicrobial resistance in the study area (Yousefi et al., 2010). Increasing resistance to the various anti-pseudomonas agents has been reported

worldwide and this poses a serious problem in therapeutic management of *P. aeruginosa* infections (Carmeli et al., 1999, Obritsch et al., 2004). Approximately 58% of *P. aeruginosa* isolates in our study were multi-drug resistant

strains, however the prevalence of multi-drug resistance isolates of *P. aeruginosa* in the other studies are much lower than our study (Ohara et al., 2007). In our study 21% of isolates were resistant to

Figure 3. Antibiotic resistance in imipenem resistant (brown colored) versus imipenem non resistant (blue colored) isolates. Cp: Ciprofloxacin, Cl: Colistin, Tic: Ticarcillin, Fep: Cefepime, Amk: Amikacin, Nor: Norfloxacin, Gm: Gentamicin, Caz: Ceftazidime, Ct: Ceftizoxime, Tob: Tobramycin, K: Kanamycin.

Imipenem, this finding is relatively in agreement with previous studies has been done by others which reported the prevalence of Imipenem resistant isolates of *P. aeruginosa* 38.28% (Saderi et al., 2008) and 27.47% (Yousefi et al., 2010), the differences in the reported values between present study and the previous studies may be due to the fewer sample size in the present study. Analysis of the data generated by the participating hospitals in Korea during 1998 till 2004 showed an alarming rise in the imipenem resistance rates of *P. aeruginosa*; from 19 to 24% (Lee et al., 2004b; 2006).

The results of the present study showed that 33% of Imipenem non-susceptible isolates were MBL positive, the previous studies from Korea in 2009 showed that 10.8% of Imipenem non-susceptible isolates were MBL positive (Oie et al., 2009), on the other hand, previous studies from Azarbayejan, Iran showed that 56.73% of Imipenem non-susceptible isolates were MBL positive by Imipenem-EDTA combined disk method (Yousefi et al., 2011). In the present study 95.2 and 100% of Imipenem non-susceptible isolates and MBL producing isolates were multi drug resistant isolates, these findings are in accordance with results obtained by Magalhes et al. (2005) which reported that MBL positive isolates were resistant to main anti-pseudomonas drugs. In conclusion, the clinical significance of *P. aeruginosa* isolates possessing Metallo- β -lactamase enzymes are ultimately judged by their ability to confer *in vivo* resistance to betalactams and even other classes of anti-pseudomonas antibiotics, ultimately lead to the patient fails antibiotic therapy. As shown in the present study, Metallo- β -lactamase producing *P. aeruginosa* isolates is prevalent in Urmia, West Azerbaijan in Iran may cause

serious problems in treatment of infections caused by these strains of *P. aeruginosa*.

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