Effectiveness of Antipseudomonal Antibiotics and Mechanisms of Multidrug Resistance in *Pseudomonas aeruginosa*

MOHAMED E. EL ZOWALATY 1* and BÉLA GYETVÁI 2

1 Biomedical Research Center, Qatar University, Doha 2713, Qatar
2 Department of Pharmacology and Toxicology, Faculty of Veterinary Science, Szent István University, Budapest, Hungary

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**Abstract**

*Pseudomonas aeruginosa* is a leading human pathogen that causes serious infections at various tissues and organs leading to life threatening health problems and possible deadly outcomes. Resistance patterns vary widely whether it is from hospitals or community acquired infections. Reporting resistance profiles to a certain antibiotics provide valuable information in a given setting, but may be extrapolated outside the sampling location. In the present study, *P. aeruginosa* isolates were screened to determine their susceptibilities against antipseudomonal antimicrobial agents and possible existing mechanisms of resistance were determined. Eighty-six isolates of *P. aeruginosa* were recovered. Isolates representing different resistance profiles were screened for the existence of three different resistance mechanisms including drug inactivation due to metallo-β-lactamases, drug impermeability by outer membrane proteins and drug efflux. All tested isolates showed uniform susceptibility (100%, n = 86/86) to piperacillin, meropenem, amikacin, and polymyxin B. A single isolate was found to be imipenem resistant (99%, n = 85/86). The possible mechanisms of resistance of *P. aeruginosa* to imipenem involve active drug efflux pumps, outer membrane impermeability as well as drug inactivating enzymes. These findings demonstrate the fundamental importance of the *in vitro* susceptibility testing of antibiotics prior to antipseudomonal therapy and highlight the need for a continuous antimicrobial resistance surveillance programs to monitor the changing resistance patterns so that clinicians and health care officials are updated as to the most effective therapeutic agents to combat the serious outcomes of *P. aeruginosa* infections.

**Key words:** *Pseudomonas aeruginosa*, antibiotics, antimicrobial, carbapenems, efflux pump, mechanisms, metallo-β-lactamases, polymyxins, multidrug, resistance

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**Introduction**

*Pseudomonas aeruginosa* is a ubiquitous opportunistic Gram-negative non fermentative bacterium of clinical significance and preferentially causes severe infections in patients with diseases including cancer, diabetes, cystic fibrosis, deliberate immunosuppression, and major surgery (Osman et al., 2010). The bacterium can colonize implanted devices, catheters, heart valves, ventilators or dental implants resulting in device-associated hospital acquired infections which are of major concern globally (El-Kholy et al., 2012). *P. aeruginosa* is associated with different types of infections which cause morbidity and mortality (Driscoll et al., 2007; Suárez et al., 2010). The high prevalence of *P. aeruginosa* in developing countries and resource-limited parts of the world as well as other parts of the world owes much to its battery of secreted virulence factors as well as to its high resistance to antimicrobial and various chemical agents (Van Delden and Iglewski, 1998).

Much evidence on its prominence and emergence as a life threatening pathogen is attributed to its high intrinsic and acquired resistance to diverse classes of antimicrobial agents including antipseudomonal agents (Wolter et al., 2009). The resistance rates of *P. aeruginosa* are escalating globally posing a serious public health threat (Jones et al., 2003). *P. aeruginosa* is characterized by increased resistance to antipseudomonal agents (Strateva et al., 2007). *In vitro* sensitivity tests are used as a guide for appropriate antimicrobial therapy prior to antibiotic treatments.

Geographical variations and differences in the resistance rates of *P. aeruginosa* usually correlate with the prescription patterns of antimicrobial agents prescribing habits, overuse of antimicrobial agents in different parts of the world, and the selective pressure of certain...
antibiotics (El Zowalaty et al., 2015). The literature is rich in surveillance studies from all over the world reporting varying resistance rates among P. aeruginosa against different antimicrobial agents. Recently, studies have focused on the decreased susceptibility of P. aeruginosa to currently used antipseudomonal agents, including β-lactams, aminoglycosides, and fluoroquinolones (Al-Tawfiq, 2007) since resistance of P. aeruginosa to carbapenems, piperacillin, and other highly active antibiotics has emerged and is increasing which makes treatment of P. aeruginosa infections troublesome (Strateva et al., 2007).

Recently, resistance rates of P. aeruginosa clinical isolates recovered from patients admitted to Zagazig University hospitals in Egypt against different classes of antimicrobial agents were reported (El Zowalaty, 2012). The current study further examined the susceptibilities and possible resistance mechanisms of P. aeruginosa isolates collected from hospitalized patients against selected antipseudomonal agents that are available in the Egyptian pharmaceutical market and are frequently prescribed to patients.

Experimental
Materials and Methods

Study site. The specimens were collected from Zagazig university-affiliated hospitals as well as outpatient clinics. Meropenem, polymyxin B, and piperacillin have not been previously prescribed while imipenem was sometimes prescribed (depending on socioeconomic factors). Other antimicrobial agents including ceftazidime, ceftriaxone, ciprofloxacin, amikacin, gentamicin, cefotaxime, are first line frequently prescribed antibiotics to all patients regardless of the pathogen antimicrobial sensitivity profile.

Ethics statement. Ethical approval to perform the study was obtained from all patients. Consent was obtained from each patient included in the study as well as from Zagazig University hospital and the Department of Microbiology ethical committee. All samples were de-identified and analyzed anonymously.

Bacterial isolates. Eighty-six non-repeat clinical isolates of P. aeruginosa were collected from hospitalized patients with urinary tract infections, respiratory tract infections, cystic fibrosis, wounds, ear infections, and septicaemia. All patients were under antimicrobial clinical protocol treatment consisting of cefotaxime, ceftazidime, ceftriaxone, gentamicin, or ciprofloxacin. Specimens were collected as urine, purulent discharge or sputum according to the type of infection. The isolates were collected, identified, and confirmed to be P. aeruginosa by routine conventional biochemical tests. P. aeruginosa isolates were cultured aerobically in Muller-Hinton broth for 16–24 hours at 37°C. The isolates were Gram stained, and first inoculated into brain heart infusion medium, then cultured on cetrimide agar. Gram-negative bacilli were further confirmed to be P. aeruginosa using conventional biochemical characteristics. The isolates were further tested for the presence of cytochrome oxidase enzyme using oxidase reagent (bioMérieux, Marcy-l’Étoile, France), oxidative fermentation, and ability to grow at 42°C. All isolates were stored in Mueller-Hinton broth (Difco Laboratories, Maryland, USA) with 30% glycerol (Merck, Darmstadt, Germany) at –20°C until additional tests were performed as described below. The standard laboratory reference strain P. aeruginosa ATCC 90271 (Manassas, VA, USA) was used as control in this study.

Antibiotics. The following antibiotics were obtained from the corresponding supplier: amikacin (Bristol Myers Squibb, Cairo, Egypt), imipenem (Merck Sharp and Dohme, Hertfordshire, U.K.), meropenem (AstraZeneca, Cheshire, U.K.), ticarcillin and piperacillin, (Sigma-Aldrich, Saint Louis, Missouri, USA), and polymyxin B (Novo Industry A/S, Copenhagen, Denmark).

Antimicrobial susceptibility testing. The minimum inhibitory concentrations (MICs) (µg/ml) of different antibiotics were determined on Muller-Hinton agar dilution method as previously described (Andrews, 2001) and in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015).

Detection of metallo-β-lactamases (MβLs). Detection of MβLs in imipenem resistant P. aeruginosa isolate was performed using EDTA-disc diffusion synergy test as described previously (Jesudason et al., 2005). An overnight broth culture of the carbapenem resistant isolate was adjusted to 0.5 McFarland opacity standards and was used to inoculate plates of Mueller-Hinton agar. After drying the plates by incubation at 37°C for one to 2 h, a 10 µg imipenem disc (Oxoid Ltd., Basingstoke, Hampshire, England) and a blank filter paper disc were placed 10 mm apart from edge to edge, 5 µl of 0.5 M EDTA disodium. Aqueos solution, prepared by dissolving 186.1 g in 1000 ml of distilled water and adjusting it to pH 8.0 using NaOH and sterilized by autoclaving, was then applied to the blank disc, which resulted in a concentration of approximately 750 µg EDTA per disc. After overnight incubation, the presence of an enlarged zone of inhibition was interpreted as EDTA synergy positive. P. aeruginosa ATCC 90271 was used as negative control microorganism.

β-lactam hydrolysis assays. The β-lactamase activity was determined by spectrophotometric assay using β-lactam antibiotics (ampicillin and imipenem) as substrates in the presence and absence of β-lactamase inhibitors (clavulanic acid and p-chloromercuriben-
The effects of crude β-lactamase extract on various β-lactam antibiotics were determined as previously described (Danel et al., 1999; Ayala et al., 2005). Briefly, the hydrolytic activity of crude β-lactamase extracts of P. aeruginosa isolates to degrade β-lactam antibiotics was assayed using UV spectrophotometry at 37°C in the presence of phosphate buffered saline at pH 7.0. The following wavelengths were used: ampicillin, 235 nm; cefotaxime, 260 nm; ceftazidime, 260 nm; and imipenem, 299 nm. Inhibition of enzymatic activity of crude extract was performed using different concentrations of clavulanic acid, 2 µg/ml; tazobactam, 4 µg/ml; oxacillin, 1 µM; EDTA (2 µM and 5 µM); and sodium p-chloromercuribenzoate (p-CMB), 1 µM and assayed following the incubation of the crude extract for 20 minutes at 25°C in presence of the previously mentioned concentrations of the inhibitor. Each of the crude β-lactamase extracts or cell lysates of isolates, at a fixed volume of 200 µl aliquot of crude extract, was mixed with the antibiotic solution at zero time in 0.1 M phosphate buffer (pH 7.0) at 37°C and the concentration in the urine was monitored by measuring the absorbance at the corresponding wavelength. The crude extract or cell lysate was pre-incubated with the inhibitor for 20 minutes at 37°C. A control without the inhibitor was used.

**Detection of efflux pumps activity.** The existence of efflux mechanism in P. aeruginosa isolates was determined by detection of the accumulation of ethidium bromide in the presence or absence of efflux inhibitors as described previously with modifications (Nishino and Yamaguchi, 2004). Overnight cultures were adjusted to approximately 10⁷ cfu/ml. Washed cells were resuspended in 20 µl of 1 µg/ml ethidium bromide with or without either 100 µM dinitrophenol (DNP, Steinheim, Germany), 0.4% glucose or 0.1% of toluene and sodium p-chloromercuribenzoate (p-CMB), 1 µM and assayed following the incubation of the crude extract for 20 minutes at 37°C in presence of the previously mentioned concentrations of the inhibitor. Each of the crude β-lactamase extracts or cell lysates of isolates, at a fixed volume of 200 µl aliquot of crude extract, was mixed with the antibiotic solution at zero time in 0.1 M phosphate buffer (pH 7.0) at 37°C and the change in the concentration was monitored by measuring the absorbance at the corresponding wavelength. The crude extract or cell lysate was pre-incubated with the inhibitor for 20 minutes at 37°C. A control without the inhibitor was used.

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PCR products was determined by comparing of PCR products with DNA molecular size marker (1 kb/100 bp ladder; Promega, WI, USA).

**Electrophoretic separation of outer membrane proteins.** The outer membrane proteins were analyzed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), as previously reported (Laemmli, 1970), with 10.7% (wt/vol) acrylamide and 0.3% (wt/vol) N, N9-methylenebisacrylamide in the running gel. Samples for SDS-PAGE were treated with 2% SDS w/v – 5% w/v 2-mercaptoethanol at 100°C for 5 min or at 37°C for 10 min, and then subjected to electrophoresis at a constant current of 25 mA at 4°C. The gel was stained using coomassie brilliant blue to visualize the protein bands. The size of the proteins was determined as compared to size of a protein marker (Bio-Rad protein ladder).

**Results**

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility results were interpreted using the CLSI breakpoints (CLSI, 2015). It was reported previously that *P. aeruginosa* isolates were highly resistant to commonly prescribed antibiotics (El Zowalaty, 2012). The resistance rates of *P. aeruginosa* clinical isolates to one or more antimicrobial agents were shown in Table I. The respective MIC\(_{90}\) distributions of different antibiotics for 86 isolates of *P. aeruginosa* were shown. All tested isolates of *P. aeruginosa* were susceptible to the antibiotics piperacillin, meropenem, amikacin, and polymyxin B. A single isolate was found resistant to imipenem. For other antibiotics tested namely ticarcillin, ciprofloxacin, ceftazidime, and gentamicin the susceptibility rates were shown in Table I. *P. aeruginosa* isolated strains were highly resistant to all other antibiotics tested. In addition, all of the 86 clinical isolates of *P. aeruginosa* were resistant to more than three classes and were defined as MDR. The resistance rates of *P. aeruginosa* isolates to one or more antimicrobial agents shown in Figure 1 and Table II. In total, forty-two out of eighty-six isolates were found to be resistant to three or more antimicrobial agents and the rate of multidrug resistant (MDR) *P. aeruginosa* isolates was 47.1% (El Zowalaty, 2012).

In order to explore the possible existing antimicrobial resistance mechanisms in the as-found MDR *P. aeruginosa* isolates, the detection of MβLs, spectrophotometric β-lactamase assays, efflux pump activity, outer membrane protein profiling, and molecular detection of resistance determinants were performed. A single isolate was found to be imipenem resistant as determined using the disk susceptibility testing and had

**Table I**

Susceptibility of *P. aeruginosa* isolates (n = 86) to different antimicrobial agents classes.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC(_{50})</th>
<th>MIC(_{90})</th>
<th>Susceptible(^a)</th>
<th>Resistant(^a)</th>
<th>Intermediate(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem</td>
<td>2</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Imipenem</td>
<td>4</td>
<td>4</td>
<td>98.9</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>8</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>64</td>
<td>128</td>
<td>80.9</td>
<td>0</td>
<td>19.1</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>2</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>32</td>
<td>256</td>
<td>0</td>
<td>70.8</td>
<td>29.2</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>8</td>
<td>32</td>
<td>59.5</td>
<td>28.1</td>
<td>12.4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>64</td>
<td>256</td>
<td>0</td>
<td>43.8</td>
<td>56.2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>128</td>
<td>512</td>
<td>12.3</td>
<td>7.9</td>
<td>79.8</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1</td>
<td>128</td>
<td>60.7</td>
<td>6.7</td>
<td>32.6</td>
</tr>
</tbody>
</table>

\(^a\) Percentage of all isolates. MICs were determined and isolates were defined as resistant, intermediate resistant, and sensitive according to CLSI guidelines.

**Table II**

Profiles of *P. aeruginosa* antibiotic resistance.

<table>
<thead>
<tr>
<th>No. of agents to which isolates were resistant</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>3(^*)</td>
<td>23</td>
</tr>
<tr>
<td>4(^*)</td>
<td>14</td>
</tr>
<tr>
<td>5(^*)</td>
<td>4</td>
</tr>
<tr>
<td>6(^*)</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^*\) Forty-two out of 89 (47.1%) isolates were resistant to three or more antimicrobials and were defined as MDR.
a zone diameter of 10 mm. In presence of EDTA disc, the zone diameter of imipenem increased to 21 mm.

The spectrophotometric β-lactamase assays showed a decrease in the concentration of ampicillin due to the effect of the crude β-lactamase extract activity Figure I. The crude β-lactamase extract activity was not inhibited by clavulanate, tazobactam or oxacillin while in presence of p-chloromercuribenzoate (p-CMB) the crude β-lactamase extract activity was inhibited as shown in Figure 2. The crude β-lactamase extract activity had no effect on the concentration of cefotaxime and ceftazidime.

As shown in Figure 3, the crude β-lactamase extract activity of IMP-sensitive isolate (B) had no effect on the concentration of imipenem while there was a decrease in the concentration of imipenem that might be attributed to the effect of the crude β-lactamase extract activity of IMP-resistant isolate (C). The crude β-lactamase extract activity of IMP-resistant isolate (C) was inhibited in presence of either EDTA or p-CMB as shown in Figure 4.

Resistance through the efflux pump. It was found that IMP-resistant isolate was positive for efflux pump activity as shown in Figure 5. The reduction in fluorescence intensity was observed in the absence of efflux pump inhibitor and in the presence of glucose which is an efflux pump energizer. In the presence of efflux pump inhibitor or toluene, the latter is a membrane permeabilizer; there was an increase in the fluorescence intensity. *P. aeruginosa* ATCC 90271 was used as negative control. The effect of efflux pump inhibitors (DNP, and DCCD) on the MIC of imipenem resistant isolate was determined. The MICs of ticarillin, imipenem,
meropenem, and norfloxacin in the presence and absence of the efflux inhibitors was determined. The reduction in MIC of a certain antibiotic with DNP or DCCD is indicative of resistance against this antibiotic mediated by an efflux system.

The addition of DNP and DCCD enhanced the activities of selected antibiotics by lowering the MIC as observed in the reduction of MIC. In the presence of DNP and DCCD, the largest effect was observed with ticarcillin and norfloxacin (a 32-fold decrease in MIC) followed by aztreonam (16-fold decrease in MIC). An intermediate effect was obtained with meropenem (8-fold decrease in MIC). These results emphasized the existence of efflux-mediated resistance in the tested isolates.

**Polymerase chain reaction.** The tested isolates carried the mexR gene as was determined using PCR analysis. In addition, PCR analysis revealed the absence of the screened bla_{IMP-1}, bla_{TIM-1}, bla_{OXA-58} and bla_{IBC-2} genes in the tested isolates; however this does not exclude the presence of other resistance determinants.

**Analysis of outer membrane proteins.** The outer membrane protein profiles of *P. aeruginosa* isolates representing different resistance profiles showed the presence of a protein band of approximate weight of 50 kDa, in addition to several bands of approximate weights of 17, 23, 35, 38 and 49 kDa.

**Discussion**

*P. aeruginosa* gains specific concern among health care officials especially in resource limited settings (RLS) and developing countries. There are only few recent reports on the antimicrobial resistance of *P. aeruginosa* isolated from patients in Egypt (Abdel et al., 2010). The present study reported the *in vitro* activity of antipseudomonal drugs against *P. aeruginosa* clinical isolates. Antibiotic treatment guidelines recommended for *P. aeruginosa* are not similar due to different resistance profiles from different sources.

The current study showed that all tested *P. aeruginosa* clinical isolates were uniformly susceptible to meropenem, piperacillin, imipenem, amikacin, and polymyxin B. In this study, antimicrobial susceptibility testing of eighty-six clinical isolates of *P. aeruginosa* was performed using the agar dilution method according to the guidelines of the CLSI (CLSI, 2015). The MIC$_{50}$ and MIC$_{90}$ were 2 and 2 µg/ml for polymyxin and meropenem; 8 and 8 µg/ml for amikacin and 8 and 32 µg/ml for piperacillin, respectively.

On the contrary, carbapenem resistance among *P. aeruginosa* have been increasing in other parts of the world posing a continuous threat and possible looming emergence of highly serious pandrug resistant *P. aeruginosa*, which may be explained in parts by several factors including the intensive use of carbapenem which enhanced the emergence of carbapenem-resistant isolates (Walsh, 2010).

It has been reported that extensive use and consumption of carbapenems forced the emergence of resistance to these antimicrobial agents (Benčić and Baudoin, 2001). This probably will present a particular challenge and could results in a major global problem since carbapenems are the final choice in the treatment of the difficult-to-treat pseudomonal infections and they are often the last resort for treating infections due to multidrug resistant isolates (Nordmann, 2010). The emerging carbapenem resistance will be very dangerous and of serious complications resulting in pan drug resistant strains leading to increased mortality rates (Hong et al., 2015; Liu et al., 2015).

In the present study, the resistance rate to imipenem was relatively low and accounted for only 1%. Except for a single isolate which was found to be imipenem resistant with an MIC of 16 µg/ml, all isolates were sensitive to imipenem with MIC of 4 µg/ml, MIC$_{50}$ and MIC$_{90}$ equal to 4 µg/ml. Contrary to the present findings, higher resistance rate to imipenem were reported where it was found that 29% and 14.3% (Hassan et al., 2010) of *P. aeruginosa* clinical isolates were resistant to imipenem. In another study from Egypt, 11.9% out of 261 clinical isolates of *P. aeruginosa* isolated from Zagazig University hospital between 2003 and 2004 were resistant to imipenem as determined by disc diffusion method (El-Beheyd et al., 2002).

Contrary to the current study, the overall resistance rates of *P. aeruginosa* to imipenem are on continuous increase globally. In a Saudi Arabian hospital between 1998 and 2003, rates were 2.6% and 5.8%, respectively (Al-Tawfiq, 2007). In a study from California, USA, the annual imipenem resistance rates against *P. aeruginosa* isolates increased from 2% in 1996 to 18% in 1999 (Huang et al., 2002).

The susceptibility rate to imipenem in clinical isolates of *P. aeruginosa* in a study in Spain was 89.7% from 2005 to 2010 (Casal et al., 2012). The susceptibility and resistance rates of *P. aeruginosa* to imipenem in USA were reported to be 24% and 70%, respectively.

In the present study, all tested isolates of *P. aeruginosa* were sensitive to meropenem with MIC$_{50}$ and MIC$_{90}$ of 2 and 2 µg/ml. Contrary to the present results, a study in Egypt reported a resistance rate of 37.7% to meropenem among *P. aeruginosa* isolated from hospitalized cancer patients (Decousser et al., 2003). This is explained by the differences in the pattern of antibiotic prescription and usage between the two studies. The susceptibility rate to meropenem in clinical isolates of *P. aeruginosa* in a study in Spain was 92.98% from 2005 to 2010 (Casal et al., 2012).
According to our findings, the susceptibility rate of ticarcillin was found to be 80.1%. Similarly, the susceptibility rate to ticarcillin was reported to be 81% in a study in France (Decousser et al., 2003). The resistance rate of P. aeruginosa in the present study to ticarcillin was found to be 19.9% which was much lower than the resistance rate reported in a study in Egypt, where the resistance rate of P. aeruginosa isolated from hospitalized cancer patients to ticarcillin was found to be 91.7% (Ashour and El-Sharif, 2009) which is much higher than the resistance rate in the present study which was found to be 19.9%.

All P. aeruginosa isolates, in the present study, were susceptible to piperacillin with MIC<sub>90</sub> and MIC<sub>99</sub> of 8 and 32 µg/ml, respectively. On the other hand, only 53% of 303 clinical isolates of P. aeruginosa collected from patients in five hospitals in the greater Cairo region between July 1999 and June 2000, were susceptible to piperacillin (El Kholy et al., 2003).

In line with the literature (Landman et al., 2008), the present data revealed that polymyxin B had in vitro activity against the isolates tested, with susceptibility rates of 100% for P. aeruginosa. In contrast to the present findings recent studies showed resistance of P. aeruginosa to polymyxin B. While P. aeruginosa are typically susceptible to polymyxins, resistance has been known to occur as polymyxin usage increases, the emergence of resistance to this agent of last resort becomes an obvious concern (Landman et al., 2005).

All P. aeruginosa isolates in the present study were susceptible to amikacin with MIC<sub>90</sub> and MIC<sub>99</sub> of 8 and 8 µg/ml, respectively. In agreement with the current amikacin susceptibility results were data in studies in Turkey where the susceptibility rate of P. aeruginosa strains to amikacin was 100% (Gerçeker and Gürler, 1995) and in Jamaica (Brown and Izundu, 2004).

To determine the possible mechanisms of resistance of P. aeruginosa isolates to antibiotics, the isolates were tested for β-lactamase production and efflux pumps-mediated resistance. P. aeruginosa is known to possess β-lactamase-mediated resistance to antibiotics (Walsh, 2010). In the present study, 48.8% of isolates showed β-lactamase production activity. The reduction in MICs of ticarcillin, aztreonam, and meropenem in the presence of an efflux pump inhibitors (DNP or DCCD) suggested the contribution of an efflux-mediated mechanism in tested P. aeruginosa isolates to different antibiotics. This finding was consistent to other reports which showed a major contribution of efflux as the major resistance mechanism in P. aeruginosa (Drissi et al., 2008).

The possible mechanisms of low-level imipenem resistance in the imipenem resistant isolate were investigated. First, the effect of EDTA on the zone of inhibition by imipenem disc was performed. The addition of EDTA increased the inhibition zone from 11 mm to 21 mm, which might suggest a MBL-mediated imipenem resistance (Jesudason et al., 2005). Therefore, PCR analysis of the isolate was performed to detect IMP and VIM MBLs, which was supported by the full sensitivity of the isolate to meropenem. Although, the present PCR results excluded the presence of the presence of the aforementioned metallo-β-lactamase genes, several types of MBL enzymes including IMP-type, VIM-type, SPM-1, GIM-1, SIM-1 – have been reported in P. aeruginosa (Queenan and Bush, 2007). In the present study, imipenem resistance may be explained by the presence of efflux pump-mediated mechanism using the constitutively expressed MexAB-OprM efflux system which extrudes most β-lactams in its broad substrate spectrum including imipenem (Quale et al., 2006) and the MexEF-OprN system although not contribute to β-lactam efflux, its overexpression indirectly affects the efficacy of carbapenems through a concomitant reduction of the carbapenem-specific OprD porin protein (Rodriguez-Martinez et al., 2009). Another possibility is the overproduction of chromosomal AmpC β-lactamase as shown in the spectrophotometric hydrolysis of imipenem. The inducible effect of some β-lactamases slowly hydrolyses imipenem as shown in several studies which demonstrated the role of cephalosporinase in imipenem resistance among P. aeruginosa (Farra et al., 2008).

Other mechanisms of carbapenem resistance have been identified such as class Clavulanic acid inhibited ESBLs with hydrolytic activity that encompasses imipenem such as GES-2 (Poirel et al., 2001). Thus, imipenem resistance in the present study is probably due to several interplay mechanisms including AmpC overproduction, efflux pumps, and loss of OprD rather than due to the production of specific MBLs, although a novel MBLs may be involved (Shehabi et al., 2011). In agreement to the present study, P. aeruginosa isolates were reported negative to bla<sub>VIM</sub> and bla<sub>IMP</sub> genes, however isolates were found positive to class 1 integrons (Kouda et al., 2009). Contrary to the absence of integron mediated MBLs in the present study, class 1 integron containing MBL-mediated resistance was reported elsewhere (Tawfik et al., 2012). P. aeruginosa can very often accumulate different resistance mechanisms leading to increased resistance to carbapenems as well as other antimicrobial agents (Farra et al., 2008).

ESBLs were reported in P. aeruginosa isolates (Stratева and Yordanov, 2009) and ESBLs and MBLs were detected at high prevalence rate in neighbouring regions (Woodford et al., 2008). In addition, ESBLs are on the rise globally as resistant determinants among P. aeruginosa isolates (Livermore, 2002). A possible resistance mechanism of these isolates could be due to the loss of porin (OprD) (Quale et al., 2006). The discrepancy
between the results of the EDTA-disc diffusion synergy test, spectrophotometric assay of imipenem and the PCR might be explained by the presence of carbapenemases other than IMP- or VIM-type MBLs. This is consistent with the other findings that in the absence of specific carbapenemases, the mechanisms leading to carbapenem resistance are usually multifactorial and it has been recently implicated to involve the interplay among various contributory factors as augmented antibiotic extrusion efflux pumps, increased chromosomal cephalosporinase or AmpC activity, and reduced OprD porin expressions (Rodriguez-Martinez et al., 2009).

In summary, the results of the present study demonstrate the effectiveness of carbapenems against the problematic *P. aeruginosa*. Independent on the geographical location, meropenem, piperacillin, amikacin, polymyxin B, and imipenem were the most active agents against *P. aeruginosa*. Monotherapy with polymyxin B may be adequate to control *P. aeruginosa* infections. Although data presented in this study revealed that no resistance of clinical isolates of *P. aeruginosa* against piperacillin, meropenem, polymyxin B, and amikacin was detected, the importance of the results is indicating that escalating rates of MDR among isolates still pose a clinical problem for patients and health officials. The prevalence of multi-drug-resistant *P. aeruginosa* (MDR-PA) in many parts of the world is concerning and will jeopardize the current antimicrobial agents because efficacious antimicrobial therapeutic options are limited (Song, 2008).

Conclusions. One of the major scientific concerns in the medical community is that the antibiotic clinical protocol for the treatment of bacterial infections in private and governmental clinics and hospitals in developing countries is inappropriate. Antibiotics are prescribed without prior recommendation and knowledge of the in vitro antimicrobial susceptibility testing. In addition, over-the-counter (OTC) antimicrobial prescription among pharmacists and self-antibiotic medication among the public is a present ongoing phenomenon. The patterns of antibiotic usage in developing countries such as overuse, underuse, or inadequate dosing contribute to a great extent to the emergence of antimicrobial resistance in Gram negative bacteria (Barbosa and Levy, 2000; Essack et al., 2008).

The misuse of antibiotics will contribute to the failure of treatment as well as the emergence of new resistant bacterial strains. Furthermore, the present study highlights the importance of improvement or amendment of antibiotic drug policies and antibiotic stewardship in developing countries as well as globally (Essack et al., 2008). In addition, this alarming trend of resistance deserves attention and concern among health care providers and requires the continuation of antimicrobial surveillance studies worldwide and reduction in antibiotic use to control antibiotic resistance (Hamilton-Miller, 2004). Furthermore, search for new antimicrobial agents including nanoantimicrobial antibiotics and alternative therapeutic agents will help control the challenging and spreading resistance of *P. aeruginosa* to antimicrobial agents.

In developing countries, high proportion of patients in hospital and outpatient clinics receive antibiotic without prescription and the inappropriate antibiotic may be prescribed without prior antimicrobial sensitivity testing as well. One more issue is that, little data about the endemic antimicrobial resistance is available from developing countries, where over-the-counter antibiotic usage is a common phenomenon. Further studies are recommended to thoroughly understand the different resistance mechanisms, interactions among bacteria as well as to continue global surveillance studies to monitor the emerging resistance trends. This will help find appropriate and effective measures to restore the balance of coexistence between humans and bacteria. We are currently investigating antimicrobial resistance among zoonotic *P. aeruginosa* isolates.

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Literature


