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

MOHAMED E. EL ZOWALATY[1]* and BÉLA GYETVAI[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, mutidrug, resistance

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

* Corresponding author: Dr. M.E. El Zowalaty, Biomedical Research Center, Qatar University, Doha 2713, Qatar; **e-mail address: elzow001@gmail.com**

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 agent. 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'Etoile, 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*-chloromercuribenzoate; *p*-CMB). 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 bufferd 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 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.

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 10^5 cfu/μl. 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 were incubated at 37°C for 15 min. Cells were collected by centrifugation at 1200 × g for 5 minutes and re-suspended in 10 μl of PBS. Five microliters aliquots of cell suspensions were spotted onto the surface of 1% agarose gel and examined over ultraviolet transilluminator. Drug accumulation in *P. aeruginosa* cells was observed as bright fluorescence of ethidium bromide. To further confirm the presence of efflux system of *P. aeruginosa* resistant isolate, the MICs of antimicrobial agents for the resistant isolate were determined in the presence and absence of 100 μM of the efflux pump inhibitor DNP and dicyclohexylcarbodiimide (DCCD, Steinheim, Germany).

Molecular detection of antimicrobial resistance determinants. Chromosomal DNA template was extracted and conventional PCR was performed. Resistant isolates were screened for resistance genes using sets of specific oligonucleotide primers as follows: *bla*$_{IMP-1}$ forward (CTACCGCAGCAGAGTCTT TGC) and *bla*$_{IMP-1}$ reverse (GAACAACCAGTTTTG CCTTACC) (Poirel *et al.*, 2000), *bla*$_{VIM-1}$ forward (TCTACA TGACCGCGTCTGTC) and *bla*$_{VIM-1}$ reverse (TGTGCTTTGACAACGT TCGC) (Poirel *et al.*, 2000), *bla*$_{OXA-50}$ forward (AATCCGGCGCTC ATCCATC) and *bla*$_{OXA-50}$ reverse (GGTCGGCGACTGAGGC GG) (Girlich *et al.*, 2004), *bla*$_{IBC-2}$ forward (CGTTCCATACAGAAGCTG) and *bla*$_{IBC-2}$ reverse (AAGCAGACTTGCCTGA) (Mavroidi *et al.*, 2001), *mex*R forward (AACCAATGAACTACCCCGTG) and *mex*R reverse (ATCCTCAAGCGGTTG CGCGG) (Dubois *et al.*, 2001) were used to amplify *bla*$_{IMP-1}$, *bla*$_{VIM-1}$, *bla*$_{OXA-50}$, *bla*$_{IBC-2}$, and *mex*R genes, respectively. The isolates were inoculated into 5 ml of trypticase soy broth and incubated for 16 hours at 37°C with shaking. Cells from 1.5 ml of an overnight culture were harvested by centrifugation for 10 minutes at 15 000 × g. The supernatant was decanted and chromosomal DNA from cell pellets was extracted. Whole-cell genomic DNA of *P. aeruginosa* isolates was extracted using a QIAamp DNA Mini Kit (Qiagen, Maryland, USA) according to manufacturer's instructions with one hour incubation at 56°C using 20 μl proteinase K solution. DNA was purified using Qiagen DNeasy Mini spin column protocol. DNA was hydrated in 150 μl of DNA elution solution to increase the final DNA concentration in the eluate. Extracted DNA was aliquoted, stored at –20°C until use. PCR analysis was performed using DNA thermal cycler Biometra Tpersonal Combi (Whatman Biometra, Goettingen, Germany) in a reaction mixture of 100 μl volume containing 10 μl (final concentration of 1 μM or 1 picomole per μl) of each upstream primer, 10 μl (final concentration of 1 μM or 1 picomole per μl) of each downstream primer, 5 μl (final concentration of 250 nanogram) of DNA template, 50 μl PCR Master Mix, 2X (containing 50 units/ml *Taq* DNA polymerase, 400 μM deoxynucleotides triphosphate [dATP, dGTP, dCTP, dTTP] and 3 mM $MgCl_2$ and nuclease-free water was added to complete the volume of the reaction to 100 μl. PCR conditions for the amplification were as follows: an initial incubation of 10 min at 37°C and an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for 1.5 min. After the last cycle, the products were stored at 4°C. The PCR amplification products were analyzed and revealed using 2% agarose gel electrophoresis in 1X trisacetate buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]). Ten microlitres of each PCR product were mixed with 2 μl of blue/orange 6X loading dye and were subjected to electrophoresis for 45 min at 80 V using horizontal apparatus. After electrophoresis, the ethidium bromide-stained PCR amplification products were visualized under UV light transilluminator. The size of each

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*, *N*9-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).

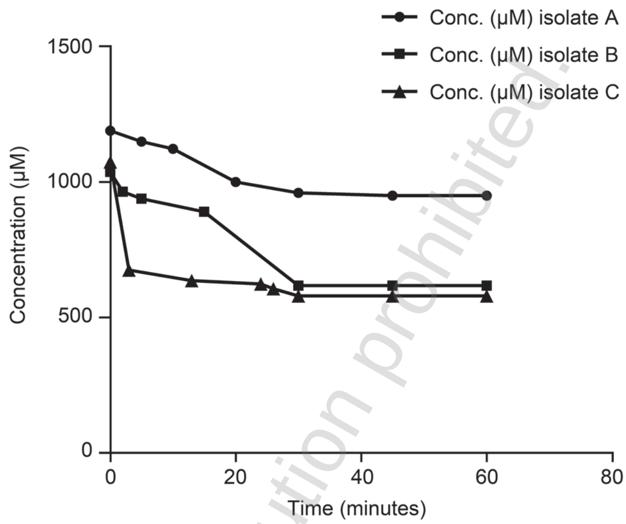

Fig. 1. Effect of crude β-lactamase extracts of *P. aeruginosa* isolates on ampicillin concentration in absence of β-lactamase inhibitors.

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 were 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.

Antibiotic	MIC_{50}	MIC_{90}	Susceptible[a]	Resistant[a]	Intermediate[a]
Meropenem	2	2	100	0	0
Imipenem	4	4	98.9	0	1.1
Piperacillin	8	32	100	0	0
Ticarcillin	64	128	80.9	0	19.1
Polymyxin B	2	2	100	0	0
Amikacin	8	8	100	0	0
Ceftriaxone	32	256	0	70.8	29.2
Ceftazidime	8	32	59.5	28.1	12.4
Cefotaxime	64	256	0	43.8	56.2
Gentamicin	128	512	12.3	7.9	79.8
Ciprofloxacin	1	128	60.7	6.7	32.6

[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.

No. of agents to which isolates were resistant	Frequency	Percent
0	3	3.4
1	20	22.5
2	24	27
3*	23	25.8
4*	14	15.7
5*	4	4.5
6*	1	1.1

* Forty-two out of 89 (47.1%) isolates were resistant to three or more antimicrobials and were defined as MDR.

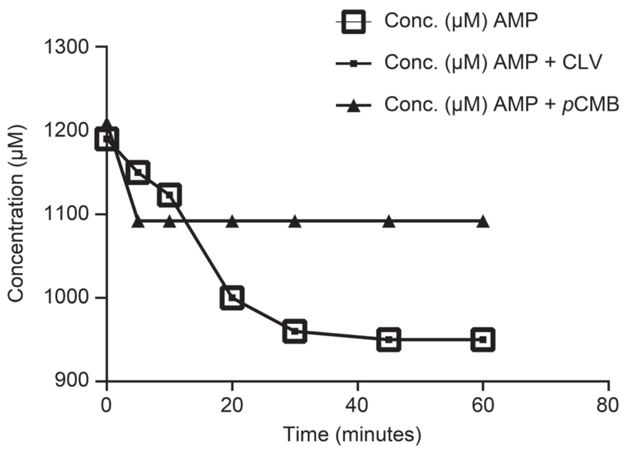

Fig. 2. Effect of crude β-lactamase extract of *P. aeruginosa* (isolate C) on ampicillin concentration in presence and absence of β-lactamase inhibitors (CLV: clavulanic acid, *p*CMB: *p*-chloromercuribenzoate).

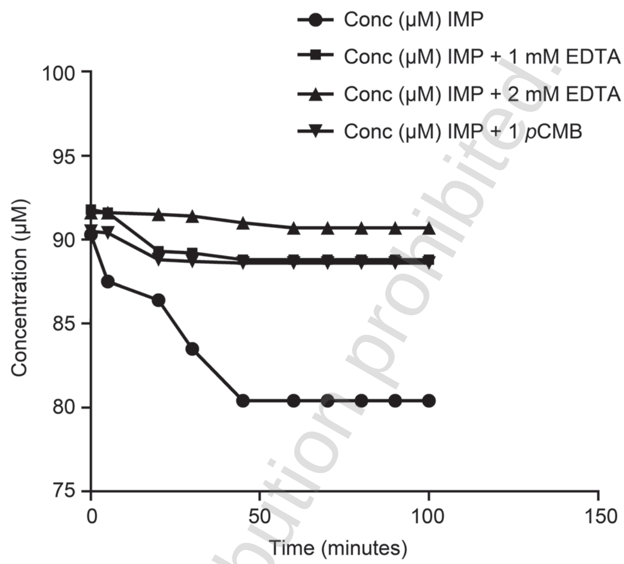

Fig. 4. Effect of crude β-lactamase extract of *P. aeruginosa* isolate C (imipenem resistant) on imipenem concentration in presence and absence of enzyme inhibitors (EDTA and *p*CMB).

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 ticarcillin, imipenem,

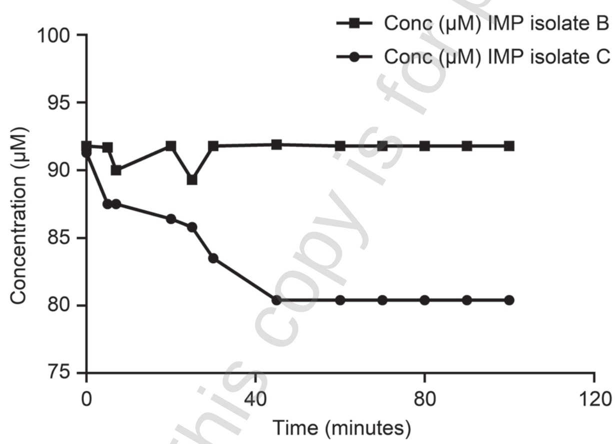

Fig. 3. Effect of crude β-lactamase extracts of *P. aeruginosa* isolates (B: imipenem sensitive and C: imipenem resistant) on imipenem concentration.

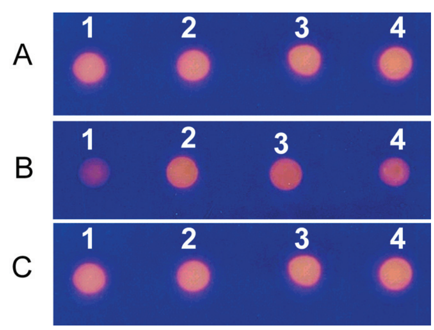

Fig. 5. Detection of efflux pump activity using ethidium bromide accumulation test showing the effect of efflux pump inhibitor (dinitrophenol) and glucose (pump energizer) on the accumulation of ethidium bromide in cells of *P. aeruginosa* (A) *P. aeruginosa* ATCC 90271, (B) *P. aeruginosa* imipenem resistant isolate and (C) *P. aeruginosa* imipenem sensitive isolate. Cells loaded with ethidium bromide in presence of glucose (1), cells loaded with ethidium bromide in presence dinitrophenol (DNP) (2), cells loaded with ethidium bromide in presence toluene (3), and cells loaded with ethidium bromide (4).

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 *mex*R gene as was determined using PCR analysis. In addition, PCR analysis revealed the absence of the screened $bla_{IMP\text{-}1}$, $bla_{VIM\text{-}1}$, $bla_{OXA\text{-}50}$, and $bla_{IBC\text{-}2}$ 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 among isolates 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 carbapenems 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} were 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-Behedy *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 ticarciilin 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_{50} and MIC_{90} 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_{50} and MIC_{90} 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, aztreaonam, 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 MβL-mediated imipenem resistance (Jesudason *et al.*, 2005). Therefore, PCR analysis of the isolate was performed to detect IMP and VIM MβLs, 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 $bla_{VIM1a,b}$ and $bla_{IMP1,2}$ 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 (Strateva 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 machanisms, 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

Abdel R.A.T., S. Hafez, S. Abdelhakam, Z. Ali-Eldin, I. Esmat, M. Elsayed and A. Aboul-Fotouh. 2010. Antimicrobial resistant bacteria among health care workers in intensive care units at Ain Shams University Hospitals. *J. Egypt. Soc. Parasitol.* 40(1): 71.

Al-Tawfiq J.A. 2007. Occurrence and antimicrobial resistance pattern of inpatient and outpatient isolates of *Pseudomonas aeruginosa* in a Saudi Arabian hospital: 1998–2003. *Int. J. Infect. Dis.* 11(2): 109–114.

Andrews J.M. 2001. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* 48(suppl. 1): 5–16.

Ashour H.M. and A. El-Sharif. 2009. Species distribution and antimicrobial susceptibility of gram-negative aerobic bacteria in hospitalized cancer patients. *J. Transl. Med.* 7: 14.

Ayala J., A. Quesada, S. Vadillo, J. Criado and S. Píriz. 2005. Penicillin-binding proteins of *Bacteroides fragilis* and their role in the resistance to imipenem of clinical isolates. *J. Med. Microbiol.* 54(11): 1055–1064.

Barbosa T.M. and S.B. Levy. 2000. The impact of antibiotic use on resistance development and persistence. *Drug Resist. Updat.* 3(5): 303–311.

Benčić I. and D. Baudoin. 2001. Imipenem consumption and Gram-negative pathogen resistance to imipenem at Sestre Milosrdnice University Hospital. *Acta Clin. Croat.* 40: 185–189.

Brown P.D. and A. Izundu. 2004. Antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* in Jamaica. *Rev. Panam. Salud. Publica* 16(2): 125–130.

Casal M., M. Causse, F. Rodriguez-Lopez and M. Casal. 2012. Antimicrobial resistance in clinical patterns of *Pseudomonas aeruginosa* (in Spanish). *Rev. Esp. Quimioter.* 25(1): 37–41.

Clinical Laboratory Standards Institute (CLSI). 2015. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement (M100-S25). Clinical Laboratory Standards Institute. Wayne, Pennsylvania, USA.

Danel F., L.M. Hall, B. Duke, D. Gur and D.M. Livermore. 1999. OXA-17, a further extended-spectrum variant of OXA-10 β-lactamase, isolated from *Pseudomonas aeruginosa*. *Antimicrob. Agents. Chemother.* 43(6): 1362–1366.

Decousser J.W., P. Pina, F. Picot, C. Delalande, B. Pangon, P. Courvalin and P. Allouch. 2003. Frequency of isolation and antimicrobial susceptibility of bacterial pathogens isolated from patients with bloodstream infections: a French prospective national survey. *J. Antimicrob. Chemother.* 51(5): 1213–1222.

Driscoll J.A., S.L. Brody and M.H. Kollef. 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* 67(3): 351–368.

Drissi M., Z.B. Ahmed, B. Dehecq, R. Bakour, P. Plesiat and D. Hocquet. 2008. Antibiotic susceptibility and mechanisms of beta-lactam resistance among clinical strains of *Pseudomonas aeruginosa*: first report in Algeria. *Med. Mal. Infect.* 38(4): 187–191.

Dubois V., C. Arpin, M. Melon, B. Melon, C. Andre, C. Frigo and C. Quentin. 2001. Nosocomial outbreak due to a multiresistant strain of *Pseudomonas aeruginosa* P12: efficacy of cefepime-amikacin therapy and analysis of β-lactam resistance. *J. Clin. Microbiol.* 39(6): 2072–2078.

El-Behedy E.M., H. Mohtady, N. Mohamed, F. Amer, H. El Zanfaly, D. Soud, S. Khalil, Y. El Hendy, T. El Behedy and E. El Gendy. 2002. Incidence of resistance to imipenem among hospital strains of *Pseudomonas aeruginosa* and surveillance for bla_{IMP} gene. *Egypt. J. Med. Microbiol.* 13(3): 565–572.

El-Kholy A., H. Baseem, G.S. Hall, G.W. Procop and D.L. Longworth. 2003. Antimicrobial resistance in Cairo, Egypt 1999–2000: a survey of five hospitals. *J. Antimicrob. Chemother.* 51(3): 625–630.

El-Kholy A., T. Saied, M. Gaber, M. A. Younan, M. Haleim, H. El-Sayed, H. El-Karaksy, H. Bazara'a and M. Talaat. 2012. Device-associated nosocomial infection rates in intensive care units at Cairo University hospitals: First step toward initiating surveillance programs in a resource-limited country. *Am. J. Infect. Control.* 40(6): e216–20.

El Zowalaty M.E. 2012. Alarming Trend of Antibiotic Resistance in *Pseudomonas aeruginosa* Isolates. *Journal of Pure and Applied Microbiology* 6(1): 175–183.

El Zowalaty M.E., A. Al Thani, T.J. Webster, A.E. El Zowalaty, H.P. Schweizer, G.K. Nasrallah, H.E. Marei and H.M. Ashour. 2015. *Pseudomonas aeruginosa*: arsenal of resistance mechanisms, decades of changing resistance profiles, and future antimicrobial therapies. *Future Microbiology* 10(10) 1683–1706.

Essack S., C. Connolly and A. Sturm. 2008. Antibiotic use and resistance in public-sector hospitals in KwaZulu-Natal. *S. Afr. Med. J.* 95(11): 865–70.

Farra A., S. Islam, A. Strålfors, M. Sörberg and B. Wretlind. 2008. Role of outer membrane protein OprD and penicillin-binding proteins in resistance of *Pseudomonas aeruginosa* to imipenem and meropenem. *Int. J. Antimicrob. Agents.* 31(5): 427–433.

Gerçeker A. A. and B. Gürler. 1995. *Invitro* activities of various antibiotics, alone and in combination with amikacin against *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 36(4): 707–711.

Girlich D., T. Naas and P. Nordmann. 2004. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 48(6): 2043–2048.

Hamilton-Miller J. 2004. Antibiotic resistance from two perspectives: man and microbe. *Int. J. Antimicrob. Agents.* 23(3): 209–212.

Hassan A.M., O. Ibrahim and M. El Guinaidy. 2010. Surveillance of antibiotic use and resistance in Orthopaedic Department in an Egyptian University Hospital. *Int. J. Infect. Control.* 7(1); doi: 10.3396/ijic.V7i1.001.11.

Hong D.J., I.K. Bae, I.-H. Jang, S.H. Jeong, H.-K. Kang and K. Lee. 2015. Epidemiology and Characteristics of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa*. *Infect. Chemother.* 47(2): 81–97.

Huang S.S., B.J. Labus, M.C. Samuel, D.T. Wan and A.L. Reingold. 2002. Antibiotic resistance patterns of bacterial isolates from blood in San Francisco County, California, 1996–1999. *Emerg. Infect. Dis.* 8(2): 195–201.

Jesudason M.V., A. Kandathil and V. Balaji. 2005. Comparison of two methods to detect carbapenemase and metallo-beta-lactamase production in clinical isolates. *Indian J. Med. Res.* 121(6): 780–783.

Jones R.N., H.S. Sader and M.L. Beach. 2003. Contemporary *in vitro* spectrum of activity summary for antimicrobial agents tested against 18 569 strains non-fermentative Gram-negative bacilli isolated in the SENTRY Antimicrobial Surveillance Program (1997–2001). *Int. J. Antimicrob. Agents.* 22(6): 551–556.

Kouda S., M. Ohara, M. Onodera, Y. Fujiue, M. Sasaki, T. Kohara, S. Kashiyama, S. Hayashida, T. Harino and T. Tsuji. 2009. Increased prevalence and clonal dissemination of multidrug-resistant *Pseudomonas aeruginosa* with the $bla_{IMP\text{-}1}$ gene cassette in Hiroshima. *J. Antimicrob. Chemother.* 64(1): 46–51.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259): 680–685.

Landman D., S. Bratu, M. Alam and J. Quale. 2005. Citywide emergence of *Pseudomonas aeruginosa* strains with reduced susceptibility to polymyxin B. *J. Antimicrob. Chemother.* 55(6): 954–957.

Landman D., C. Georgescu, D.A. Martin and J. Quale. 2008. Polymyxins revisited. *Clin. Microbiol. Rev.* 21(3): 449–465.

Liu Q., X. Li, W. Li, X. Du, J.-Q. He, C. Tao and Y. Feng. 2015. Influence of carbapenem resistance on mortality of patients with *Pseudomonas aeruginosa* infection: a meta-analysis. *Sci. Rep.* 5: 11715.

Livermore D.M. 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clin. Infect. Dis.* 34(5): 634.

Mavroidi A., E. Tzelepi, A. Tsakris, V. Miriagou, D. Sofianou and L. Tzouvelekis. 2001. An integron-associated β-lactamase (IBC-2) from *Pseudomonas aeruginosa* is a variant of the extended-spectrum β-lactamase IBC-1. *J. Antimicrob. Chemother.* 48(5): 627–630.

Nishino K. and A. Yamaguchi. 2004. Role of histone-like protein H-NS in multidrug resistance of *Escherichia coli*. *J. Bacteriol.* 186(5): 1423–1429.

Nordmann P. 2010. Gram-negative bacteriae with resistance to carbapenems (in French). *Med. Sci.* (*Paris*) 26(11): 950–959.

Osman K., M. Alabady, N. Ata, N. Ezzeldin and M. Aly. 2010. Genotypic Characterization of *Pseudomonas aeruginosa* Isolated from Human and Animal Sources in Egypt. *Zoonoses and Public Health.* 57(5): 329–338.

Poirel L., T. Naas, D. Nicolas, L. Collet, S. Bellais, J.D. Cavallo and P. Nordmann. 2000. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrob. Agents Chemother.* 44(4): 891–897.

Poirel L., D. Girlich, T. Naas and P. Nordmann. 2001. OXA-28, an extended-spectrum variant of OXA-10 β-lactamase from *Pseudomonas aeruginosa* and its plasmid-and integron-located gene. *Antimicrob. Agents Chemother.* 45(2): 447–453.

Quale J., S. Bratu, J. Gupta and D. Landman. 2006. Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* 50(5): 1633–1641.

Queenan A.M. and K. Bush. 2007. Carbapenemases: the versatile β-lactamases. *Clin. Microbiol. Rev.* 20(3): 440–458.

Rodriguez-Martinez J.M., L. Poirel and P. Nordmann. 2009. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53(11): 4783–4788.

Shehabi A.A., A.A. Haider and M.K. Fayyad. 2011. Frequency of antimicrobial resistance markers among *Pseudomonas aeruginosa* and *Escherichia coli* isolates from municipal sewage effluent water and patients in Jordan. *The International Arabic Journal of Antimicrobial Agents.* 1(1); doi: 10: 3823/700.

Song J.H. 2008. What's new on the antimicrobial horizon? *Int. J. Antimicrob. Agents.* 32(suppl. 4): S207–213.

Strateva T., V. Ouzounova-Raykova, B. Markova, A. Todorova, Y. Marteva-Proevska and I. Mitov. 2007. Problematic clinical isolates of *Pseudomonas aeruginosa* from the university hospitals in Sofia, Bulgaria: current status of antimicrobial resistance and prevailing resistance mechanisms. *J. Med. Microbiol.* 56(7): 956–963.

Strateva T. and D. Yordanov. 2009. *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance. *J. Med. Microbiol.* 58(9): 1133–1148.

Suárez C., C. Peṇa, L. Gavaldā, F. Tubau, A. Manzur, M. Dominguez, M. Pujol, F. Gudiol and J. Ariza. 2010. Influence of carbapenem resistance on mortality and the dynamics of mortality in *Pseudomonas aeruginosa* bloodstream infection. *Int. J. Infect. Dis.* 14: e73–e78.

Tawfik A.F., A.M. Shibl, M.A. Aljohi, M.A. Altammami and M.H. Al-Agamy. 2012. Distribution of Ambler class A, B and D β-lactamases among *Pseudomonas aeruginosa* isolates. *Burns* 38(6): 855–60.

Van Delden C. and B. H. Iglewski. 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg. Infect. Dis.* 4(4): 551.

Walsh T.R. 2010. Emerging carbapenemases: a global perspective. *Int. J. Antimicrob. Agents.* 36(suppl. 3): S8–14.

Wolter D.J., N. Khalaf, I.E. Robledo, G.J. Vázquez, M.I. Santé, E.E. Aquino, R.V. Goering and N.D. Hanson. 2009. Surveillance of carbapenem-resistant *Pseudomonas aeruginosa* isolates from Puerto Rican Medical Center Hospitals: dissemination of KPC and IMP-18 β-lactamases. *J. Antimicrob. Chemother.* 53(4): 1660–1664.

Woodford N., J. Zhang, M.E. Kaufmann, S. Yarde, M. del Mar Tomas, C. Faris, M.S. Vardhan, S. Dawson, S.L. Cotterill and D.M. Livermore. 2008. Detection of *Pseudomonas aeruginosa* isolates producing VEB-type extended-spectrum β-lactamases in the United Kingdom. *J. Antimicrob. Chemother.* 62(6): 1265–1268.