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ORIGINAL ARTICLE

Efflux pump genes and antimicrobial resistance of *Pseudomonas aeruginosa* strains isolated from lower respiratory tract infections acquired in an intensive care unit

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The aim of this study was to determine the antimicrobial resistance rates and the resistance genes associated with efflux pumps of *Pseudomonas aeruginosa* strains isolated from the patients who acquired lower respiratory tract infection (LRTI) in intensive care unit (ICU). Fifty *P. aeruginosa* strains isolated from the lower respiratory tract specimens of the patients who acquired LRTIs in ICU were included in this study. *P. aeruginosa* strains were isolated from tracheal aspirate (27), bronchoalveolar lavage (14) and sputum (9). The susceptibilities of the isolates were investigated by the disk diffusion method. Multiplex PCR assay was carried out for the detection of 13 antibiotic-resistance genes. Antimicrobial resistance rates of the isolates were found high and the highest resistance rate of the isolates studied was determined against to mezlocillin (50%) followed by norfloxacin (48%), ciprofloxacin (46%), meropenem (40%). Fourty-three isolates (86%) were determined to carry one and more resistance genes. *NfxB* gene was most often determined in the genes that were investigated. The significant relation between the resistance to cefepime, piperacilline/tazobactam and the *mexC* gene, that between the resistance to mezlocillin, piperacilline/tazobactam, ceftazidime, cefepime and *ampC* genes, and that between the resistance to ciprofloxacin, norfloxacin and *oprJ, oprN* and *nfxB* genes was identified. Resistance caused by genes for carbapenemases, aminoglycoside-modifying enzymes and other mechanisms were not identified in this study. Understanding the prevalence and mechanism of antimicrobial resistance in *P. aeruginosa* may help to select empirical therapy for nosocomial LRTIs due to *P. aeruginosa* in our ICU.
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Keywords: intensive care unit; lower respiratory tract infection; *P. aeruginosa*; resistance genes

INTRODUCTION

Nosocomial lower respiratory tract infection (LRTI) is the most frequent hospital acquired infection. It is the most common cause of death among nosocomial infections and is the primary cause of death in intensive care units (ICUs).[1] *Pseudomonas aeruginosa* is an important pathogen of nosocomial LRTI especially in ICUs and is commonly resistant to many antibiotics.[2] Multidrug-resistant (MDR) *P. aeruginosa* (resistant to at least three of the following antimicrobials: ceftazidime, imipenem, gentamicin and ciprofloxacin) are often isolated from nosocomial infections in ICUs.[2] MDR is often related to the specific efflux pumps and porins in *P. aeruginosa* strains.[3,4] And four efflux pumps, all of the Resistance Nodule Cell Division Family (RND) type, have been described as *MexAB–OprM, MexCD–OprJ, MexEF–OprN* and *MexXY–OprM*, and an outer membrane porin (*OprD*). Three genes encoding these pumps are arranged as operons. The first gene encoding a membrane fusion protein that is associated with the cytoplasmic membrane (*MexA, MexC, MexE* and *MexX*). The second gene encodes the transporter (*MexB, MexD, MexF* and *MexY*) thought to export the substrate across the inner membrane. The third gene encodes an outer membrane protein (*OprM, OprJ* and *OprN*) that facilitates passage of the substrate across the outer membrane.[5] In many ICUs, MDR *P. aeruginosa* isolates represent a major therapeutic problem. Therefore, understanding the mechanisms of resistance and developing therapy alternatives for these isolates is very important.

The aim of this study was to determine the antimicrobial resistance rates and the resistance genes of *P. aeruginosa* strains isolated from the patients who had LRTI in ICU.

MATERIALS AND METHODS

The strains were collected during the period February 2007–April 2009 from the patients who acquired LRTIs in ICU of Mustafa Kemal University Hospital. This study was approved by the Local Ethical Committee and was carried out in Mustafa Kemal University, School of Medicine, Department of Medical Microbiology.

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Bacterial strains

Fifty *P. aeruginosa* strains isolated from the lower respiratory tract specimens of the patients who acquired LRTIs in ICU were included in this study. *P. aeruginosa* strains (fifty) were isolated from tracheal aspirate (27), bronchoalveolar lavage (14) and sputum (9). Isolates were identified as *P. aeruginosa* based on colony morphology, odor, Gram staining, production of blue-green pigment on Mueller Hinton agar, reactions (k/k) on triple sugar iron agar slants, positive oxidase reaction.[5] The species identification was confirmed with the Vitek 2 compact system (bioMérieux, Marcy l'Etoile, France) as required.

Antibiotic susceptibility testing

The isolates were evaluated for their susceptibilities to mezlocillin (75 μg), piperacillin/tazobactam (100/10 μg), ceftazidime (30 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), amikacin (30 μg), tobramicin (10 μg), ciprofloxacin (5 μg), and norfloxacin (10 μg; Oxoid, Basingstoke, UK) by the disk diffusion method, and evaluated according to Clinical and Laboratory Standards Institute.[6]

Determination of the resistance genes by PCR

Bacterial DNA was extracted from the strains using the method of Chen and Kuo[7] with some modifications. The primers of resistance genes were selected from a research article of Dumas *et al*.,[8] shown in Table 1. Multiplex PCR assay was carried out for the detection of antibiotic-resistance genes in a thermal cycler (Bioder/Thermal Blocks xp cycler, Tokyo Japan). The primers were selected according to their base pair. There were four primer groups including; *mexE* (114 bp), *mexR* (150 bp), *mexT* (216 bp), *mexA* (316 bp) as the first, *oprD* (156 bp), *oprD* (232 bp) and *oprJ* (305 bp) as the second, *mexC* (164 bp), *mexC* (344 bp) and *ampC* (218 bp) as the third, and *nfxB* (164 bp), *oprN* (235 bp) and *mexX* (326 bp) as the fourth group.

The PCR amplification was carried out in a total volume of 25 μl reaction mixture. PCR amplification was performed as follows: The reaction mixture consisted of 2.5 ml of 10× reaction buffer without $MgCl_2$ (Promega, Madison, WI, USA); 200 μM of each deoxynucleoside triphosphate (ABgene, Epsom, UK), 2 mM $MgCl_2$; 0.4 μM of primers and ~10 ng of template DNA, and brought up to a 25 μl final volume with distilled water. Reactions were started at 94 °C for 4 min and placed on ice, and 1 U of Taq polymerase (Fermentas, Hanover, MD, USA) was added. The amplification process was started with an initial denaturation step (94 °C, 1 min). Each cycle consists of three steps (denaturation, annealing and extension). PCR reaction consisted of 35 cycles of amplification for only *mexA, mexT, mexE* and *mexR* genes. The other PCR reaction consisted of 30 cycles of amplification. Amplification consisted of denaturation at 94 °C for 1 min, annealing at 57 °C for 45 s and DNA chain extension at 72 °C for 45 s. And a final extension cycle was performed at 72 °C for 10 min. After the amplification of antibiotic-resistance genes, 10 μl volumes of PCR samples were mixed with 3 μL of loading buffer (10% (w/v), ficoll 400; 10 mmol l^{-1} Tris-HCl, pH 7.5; 50 mmol l^{-1} EDTA; 0.25% bromophenol blue). The PCR products were analyzed in a 2% (w/v) agarose gel in 1×TAE buffer (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA). Ethidium bromide (0.5 μg ml^{-1} TAE)-stained DNA amplicons were visualized using a gel imaging system (Wealtec, Dolphin-View, NV, USA). To determine the expected bp lengths, DNA marker with defined molecular weights in the range 100–2000 were used.

Statistical methods

Analysis was performed using Statistical Package for Social Sciences version 13.0 (SSPS Inc, Chicago, IL, USA). Comparison for categorical variables was calculated using χ^2 test. A *P*-value <0.05 was considered statistically significant.

RESULTS

The highest resistance rate was found against to mezlocillin (50%), followed by norfloxacin (48%), ciprofloxacin (46%), meropenem (40%; Table 2). We measured gene expression of seven mex efflux pumps, the chromosomal ampC β-lactamase, the porin *oprD, oprJ, oprN* and *nfxB* in clinical isolates. Expression of *mexA, mexE, mexR, mexT* genes in group one, *oprD* and *oprJ* genes in group two, *ampC* and *mexC* genes in group three and *nfxB, oprN* and *mexX* genes in group four is shown in Figures 1–4, respectively.

Table 1 Primer sequences used in the study

Primer	*5′-sequence-3′*	*Length (bp)*	*Product length (bp)*
mexR1	CGCGAGCTGGAGGGAAGAAACC	22	150
mexR2	CGGGGCAAACAACTCGTCATGC	22	
mexA1	CGACCAGGCCGTGAGCAAGCAGC	23	316
mexA2	GGAGACCTTCGCCGCGTTGTCGC	23	
nfxB1	CGCCTGATCAAGGAACACCTCACC	24	164
mfxB2	CGAAACACGCCTTTCTGCTGTCC	23	
mexC1	ATCCGGCACCGCTGAAGGCTGCG	23	344
mexC2	CGGATCGAGCTGCTGGATGCGCG	23	
mexC3	GTACCGGCGTCATGCAGGGTTC	22	164
mexC4	TTACTGTTGCGGCGCAGGTGACT	23	
oprJ1	GTTCCGGGCCTGAATGCCGCTGC	23	305
oprJ2	TCGCGGCTGACCAGGGTCTGACG	23	
mexX1	TGAAGGCGGCCCTGGACATCAGC	23	326
mexX2	GATCTGCTCGACGCGGGTCAGCG	23	
mexT1	CAGCACCGCGGTGTTCCGCATCG	23	216
mexT2	ACGGTCTTGCGCTTGGCGTTGGC	23	
mexE4	CCAGGACCAGCACGAACTTCTTGC	24	114
mexE5	CGACAACGCCAAGGGCGAGTTCACC	25	
oprN1	CAACCGGGAGTGACCGAGGACCG	23	235
oprN2	TGCTCAGGGCAATCTTCTCGCGC	23	
ampC1	CGGCTCGGTGAGCAAGACCTTC	22	218
ampC2	AGTCGCGGATCTGTGCCTGGTC	22	
oprD1	ATCTACCGCACAAACGATGAAGG	23	156
oprD2	GCCGAAGCCGATATAATCAAACG	23	
oprD3	CTCGACGGCACCTCCGACAAGAC	23	232
oprD4	AGCCCTTCGAATTCGCTGCTCTG	23	

Table 2 The resistance rates of strains by the disk diffusion method

Antibiotics	*Number (%)*
Mezlocillin	25 (50)
Norfloxacin	24 (48)
Ciprofloxacin	23 (46)
Meropenem	20 (40)
Gentamicin	19 (38)
Tobramicin	18 (36)
Imipenem	18 (36)
Ceftazidime	15 (30)
Piperacilline/tazobactam	12 (24)
Cefepime	9 (18)
Amikacin	8 (16)

Seven of 50 *P. aeruginosa* strains had none of these resistance genes. Fourty-three isolates (86%) were determined to be positive for one and more resistance genes. Only four isolates were found to be positive for one resistance gene. The presence of the resistance genes by multiplex PCR is shown in Table 3.

Number of the resistant isolates to cefepime and piperacilline/tazobactam carrying *mexC* gene were found to be 12 (24%; *P*=0.048) and 15 (30%; *P*=0.025), respectively; and the number of the resistant isolates to mezlocillin, piperacilline/tazobactam, ceftazidime and cefepime-carrying *ampC* gene were found to be 16 (32%; *P*=0.002), 15 (30%; *P*=0.002), 12 (24%; *P*=0.035) and 12 (24%; *P*=0.008), respectively. The isolates carrying *nfxB, oprN* and *oprJ* genes

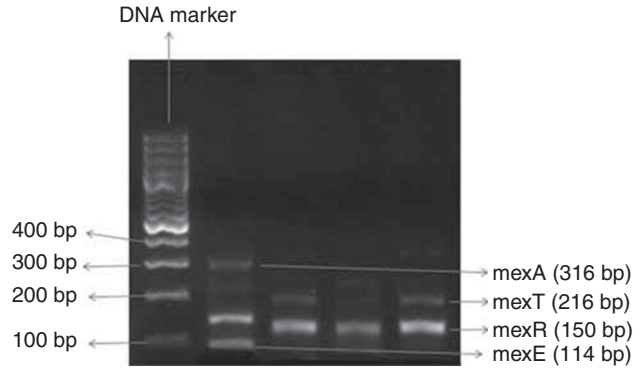

Figure 1 Multiplex PCR amplification products showing expression of the *mexA*, *mexT*, *mexR* and *mexE* genes of *P. aeruginosa*. A 100-bp DNA size ladder is shown; 100 bp DNA size ladder includes fragments of 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp.

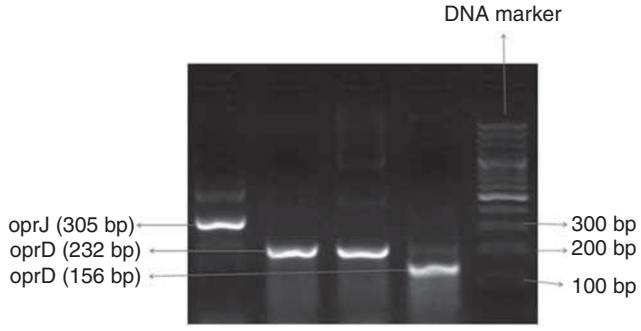

Figure 2 Multiplex PCR amplification products showing expression of the *oprJ* and *oprD* genes of *P. aeruginosa*. A 100-bp DNA size ladder is shown.

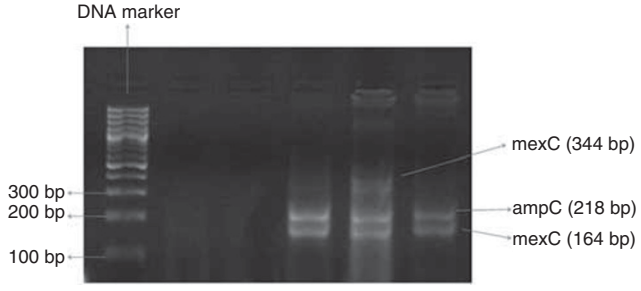

Figure 3 Multiplex PCR amplification products showing expression of the *mexC* and *ampC* genes of *P. aeruginosa*. A 100-bp DNA size ladder is shown.

Figure 4 Multiplex PCR amplification products showing expression of the *mexX*, *oprN*, *nfxB* genes of *P. aeruginosa*. A 100-bp DNA size ladder is shown.

Table 3 The presence of antibiotic resistance genes in *Pseudomonas aeruginosa* by multiplex PCR method

Genes (bp)	*Isolate number (%)*
nfxB (164)	32 (64)
oprN (235)	26 (52)
mexE (114)	26 (52)
mexC (164)	24 (48)
ampC (218)	21 (42)
oprD (232)	21 (42)
mexR (150)	17 (34)
mexC (344)	12 (24)
oprJ (305)	11 (22)
oprD (156)	9 (18)
mexA (316)	7 (14)
mexT (216)	5 (10)
mexX (326)	2 (4)

were found to be more resistant to norfloxacin and ciprofloxacin ($P<0.05$). The relationship between antibiotic resistance and the presence of the resistance genes is shown in Table 4.

DISCUSSION

P. aeruginosa is an important pathogen associated with serious nosocomial infections. In 2003, *P. aeruginosa* was reported to be the most commonly isolated Gram-negative bacteria (18.1%) for nosocomial pneumonia in the United States.[9] Inside and outside ICUs, MDR-*P. aeruginosa* strains has becoming an increasingly reported problem.[10] The ICU isolates gained significant resistance to the antibiotics used for the treatment of the life-threatening infections in ICUs.[10] Increasing resistance rates to the antibiotics in *P. aeruginosa* strains were reported by several studies during the last years.[10–13]

In this study, 50 *P. aeruginosa* isolates from the patients with LRTIs in ICU were investigated for 13 genes, mostly for efflux proteins leading to antimicrobial resistance. To our knowledge, although there are studies investigating the resistance genes from Turkey,[14,15] there aren't any studies investigating a large number of resistance genes in *P. aeruginosa* strains isolated from nosocomial LRTIs. The results of the study have shown antimicrobial resistance rates of the isolates were found high, and 86% of them were determined to carry at least one resistance gene. *P. aeruginosa* exhibited the highest rates of resistance to mezlocillin, with resistance to norfloxacin and ciprofloxacin ranging from 46 to 50%. In the National Surveillance Program in USA, it was reported that antimicrobial resistance was highest for the beta-lactams and ciprofloxacin.[13] Our findings support the results of that study. In our study, the resistance rates of *P. aeruginosa* were higher than the resistance rates in multicenter study in Spain[12] and lower than the resistance rates in the study from Bulgaria.[11]

Beta lactams in combination with aminoglycosides are commonly used as antipseudomonal agents because they may exhibit synergy with aminoglycosides.[16] In the current study, the highest resistance rate of the isolates were determined against mezlocillin (50%). The resistance rates against other beta lactams; ceftazidime, piperacilline/tazobactam and cefepime were determined to be 30, 24, 18 and 16%, respectively. These resistance rates were lower than the study performed in an another university hospital in Turkey.[17] Antipseudomonal beta lactam antibiotics (piperaciline, cefepime and meropenem) are among *mexCD-OprJ*'s substrates.[18] We found that the

Table 4 Relationship between antibiotic resistance and the presence of the resistance genes

	mexA		*mexC*		*mexE*		*mexX*		*mexR*		*mexT*		*oprD*		*oprJ*		*oprN*		*nfxB*		*ampC*	
Resistance to antibiotics	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*	*Pos. (n)*	*Neg. (n)*
Piperacilline/tazobactam	3	20	15*	8			1	22			1	22									15*	8
Mezlocillin																					16*	9
Cefepim	3	15	12*	6			1	17	7	11	1	17			6	12	12	6			12*	6
Ceftazidime	4	16					1	19	8	12	1	19									12*	8
Meropenem	3	17	11	9			0	20	8	12	1	19	7	7	6	14	13	7				
Imipenem							0	18			1	17	7	7	5	13	12	6				
Norfloxacin	3	21			13	11	1	23	8	16	2	22			9*	15	16*	8	24*	0		
Ciprofloxacin	3	21			13	11	1	23	8	16	2	22			9*	15	16*	8	24*	0		
Amikacin							1	13														
Gentamicin							1	20														
Tobramicin							1	18														

Abbreviations: neg., negative; pos., positive.
*$P<0.05$.

isolates carrying *mexC* gene were more resistant to cefepime and piperacilline/tazobactam.

Beta lactam antibiotics (piperacilline, ceftazidime, cefepime aztreonam and meropenem) are substrats of *mexAB-OprM*.[19] Also, no significant relation was determined between *mexA* gene and ceftazidime, cefepime and piperacilline/tazobactam resistance. Piperacilline, cefepime, ceftazidime, meropenem, imipenem are substrats of *mexXY-OprM* efflux system.[18] Furthermore, no significant relation between *mexX* gene and resistance against these antibiotics was found.

MexR negatively regulates *mexAB-oprM* efflux system.[20] However, no relation was found between *mexR* gene and resistance against ceftazidime, cefepime and meropenem in our study.

AmpC gene causes production of chromosomal beta lactamase. The overproduction of *AmpC* beta lactamases can result in resistance to nearly all beta-lactam antibiotics except the carbapenems.[21] We found that isolates carrying *ampC* gene were more resistant to mezlocillin, piperacilline/tazobactam, ceftazidime and cefepime.

mexT negatively regulates *mexAB-oprM* efflux system and *oprD*.[22] We didn't find a significant relation between *mexT* gene and ceftazidime, cefepime, piperacilline/tazobactam, imipenem and meropenem (the substrates of *mexAB-oprM* efflux system and *oprD*).

Carbapenems are one of the most active groups of beta lactam antibiotics against *P. aeruginosa*. The outer membrane protein *OprD* allows entry of carbapenems, and its reduced expression is frequently noted in carbapenem-resistant isolates.[23] In this study, no relation was found between the persistence of the *oprD* gene and susceptibility of carbapenems. Outer membrane proteins; *oprJ* and *oprN* are related to multidrug resistance.[23] No relation between the antibiotics (cefepime, imipenem and meropenem) that were investigated in the other studies and these genes (*oprJ* and *oprN*) was determined in this study.

Carbapenem remains as an important agent for the therapy of serious infections secondary to MDR *P. aeruginosa*. The development of carbapenem resistance severely compromises effective therapeutic options. In the absence of carbapenem-hydrolyzing enzymes, the mechanism leading to carbapenem resistance is usually multifactorial. We determined that the isolates that were resistant to carbapenems were also resistant to other beta lactam antibiotics in this study. Only one isolate was resistant to carbapenems and didn't show cross-resistance to other beta lactams so it can be imipenem-resistant *P. aeruginosa* mutant.[24]

We investigated the relation between *mexA*, *mexC*, *mexX*, *mexE* genes and quinolones because they are substrates of four efflux system but we didn't find. We also found no relation between *mexR* gene that negatively regulates *mexAB-oprM*, and *mexT* gene that positively regulates *mexCD-oprJ* and the resistance against the antibiotics.

The isolates carrying *oprJ* and *oprN*, which cause multidrug resistance, and *nfxB* gene were determined to be more resistant to ciprofloxacin and norfloxacin.

Aminoglycosides are frequently used in pseudomonal infections.[16] In our study, the aminoglycoside resistance rates in *P. aeruginosa* were lower than that in the study from Korea.[25] Aminoglycoside resistance arises more frequently via enzymatic modification of the aminoglycosides, and less frequently via *mexXY-oprM* efflux systems.[16] So no relation was found between the presence of *mexX* gene and aminoglycosides (amikacin, gentamicin and tobramycin).

CONCLUSION

These data showed that antimicrobial resistance rates of the isolates were high and the highest resistance was against mezlocillin. Most of the isolates were determined to carry one and more resistance genes. *NfxB* gene was most often seen in the genes that were investigated.

There were strains that were susceptible to most of the antibiotics although they contained large number of antibiotic resistance genes. These strains have very high chance of developing resistance during treatment. And also it should be remembered that the mechanism leading to antimicrobial resistance is usually multifactorial. For this reason, rather than investigating the susceptibility to antimicrobials by phenotypic methods, investigating genotypically the antimicrobial-resistance genes is more meaningful. Understanding the prevalence and mechanism of antimicrobial resistance may help to select empirical therapy for nosocomial LRTIs due to *P. aeruginosa* in our ICU.

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1 Hernández, G., Rico, P., Díaz, E. & Rello, J. Nosocomial lung infections in adult intensive care units. *Microbes Infect.* **6,** 1004–1014 (2004).
2 Vanhems, P. *et al.* Nosocomial pulmonary infection by antimicrobial-resistant bacteria of patients hospitalized in intensive care units: risk factors and survival. *J. Hosp. Infect.* **45,** 98–106 (2000).
3 Poole, K. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. *Antimicrob. Agents Chemother.* **44,** 2233–2241 (2000).
4 Poole, K. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J. Mol. Microbiol. Biotechnol.* **3,** 255–264 (2001).
5 Kiska, D. L. & Gilligan, P. H. In: *Man. Clin. Microbio.* 8th edn (eds Muray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH) (ASM Press, Washington, 2003).
6 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing-Nineteenth Informational Supplement. CLSI document M100-S21* (Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA, 2011).
7 Chen, W. & Kuo, T. A simple and rapid method for the preparation of gram negative bacterial genomic DNA. *Nucleic Acids Res.* **21,** 2260 (1993).
8 Dumas, J. L., van Delden, C., Peron, K. & Köhler, T. Analysis of antibiotic resistance gene expression in Pseudomonas aeruginosa by quantitative real-time-PCR. *FEMS Microbiol. Lett.* **254,** 217–225 (2006).
9 Gaynes, R. & Edwards, J. R. Overview of nosocomial infections caused by gram-negative bacilli. *Clin. Infect. Dis.* **41,** 848–854 (2005).
10 Karlowsky, J. A., Draghi, D. C., Jones, M. E., Thornsberry, C., Friedland, I. R. & Sahm, D. F. Surveillance for antimicrobial susceptibility among clinical isalates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from hospitalized patients in the United States, 1998 to 2002. *Antimicrob. Agents. Chemother.* **47,** 1681–1688 (2003).
11 Strateva, T., Ouzounova Raykova, V., Markova, B., Todorova, A., Marteva Proevska, Y. & Mitov, I. 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,** 956–963 (2007).
12 Sánchez-Romero, I. *et al.* Evolution of the antimicrobial resistance of *Pseudomonas aeruginosa* in Spain: Second National Study (2003). *Rev. Esp. Quimioterap.* **20,** 222–229 (2007).
13 Obritsch, M. D., Fish, D. N., MacLaren, R. & Jung, R. National surveillance of antimicrobial resistance in *Pseudomonas aeruginosa* isolates obtained from intensive care unit patients from 1993 to 2002. *Antimicrob. Agents Chemother.* **48,** 4606–4610 (2004).
14 Savli, H. *et al.* Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J. Med. Microbiol.* **52,** 1–6 (2003).
15 Kolayli, F. *et al.* Effect of carbapenems on the transcriptional expression of the oprD, OprM, OprN genes in *Pseudomona aeruginosa*. *J. Med. Microbiol.* **53,** 915–920 (2004).
16 Poole, K. Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **49,** 479–487 2005.
17 Ozer, B., Tatman-Otkun, M., Memis, D. & Otkun, M. Characteristics of *Pseudomonas aeruginosa* isolates from intensive care unit. *Cent. Eur. J. Med.* **4,** 156–163 (2009).
18 Masuda, N. *et al.* Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM, efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **44,** 3322–3327 (2000).
19 Maseda, H. *et al.* Enhancement of the mexAB-oprM efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the mexEF-oprN efflux pump operon in Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **48,** 1320–1328 (2004).
20 Adewoye, L., Sutherland, A., Srikumar, R. & Poole, K. The MexR repressor of mexAB-oprM multidrug efflux operon in *Pseudomonas aeruginosa*: characterization of mutations compromising activity. *J. Bacteriol.* **184,** 4308–4312 (2002).
21 Quale, J., Bratu, S., Gupta, J. & Landman, D. Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of Pseudomonas aeruginosa clinical isolates. *Antimicrob. Agents Chemother.* **50,** 1633–1641 (2006).
22 Kohler, T., Epp, S. F., Curty, L. K. & Pechere, J. C. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J. Bacteriol.* **181,** 6300–6305 (1999).
23 Livermore, D M. Interplay of impermeability and chromosomal beta lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **36,** 2046–2048 (1992).
24 Sakyo, S., Tomita, H., Tanimoto, K., Fujimoto, S. & Ike, Y. Potency of carbapenems for the prevention of carbapenem-resistant mutants of Pseudomonas aeruginosa. *J. Antibiot.* **59,** 220–228 (2006).
25 Kim, J. Y. *et al.* Occurrence and mechanisms of amikacin resistance and its association with beta-lactamases in *Pseudomonas aeruginosa*: a Korean nationwide study. *J. Antimicrob. Chemother.* **62,** 479–483 (2008).