TÜBİTAK

Original Article

Turk J Med Sci
2012; 42 (1): 149-156
© TÜBİTAK
E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1009-1095

Integron-associated resistance genes among multidrug-resistant *Pseudomonas aeruginosa* isolated from clinical specimens

Fatma BUDAK[1], Murat KASAP[2], Fetiye KOLAYLI[1], Aynur KARADENİZLİ[1], Mustafa Haluk VAHABOĞLU[3]

Aim: To characterize common beta-lactamase genes and the role of integrons in beta-lactam antibiotic resistance among multidrug-resistant (MDR) *P. aeruginosa* strains isolated from intensive care units in Turkey.

Materials and methods: After the examination of 67 *P. aeruginosa* isolates, 14 were found to be resistant to all of the tested beta-lactams and aminoglycosides. These 14 strains were studied to characterize the beta-lactamases and related integrons. Evaluation of minimum inhibitory concentrations, polymerase chain reaction (PCR) screening, isoelectric focusing, outer membrane isolation and analysis, and cloning and sequencing were performed according to published protocols.

Results: PCR screening for common beta-lactamases detected PER-1 and OXA-10 among the selected MDR *P. aeruginosa*. PCR screening results showed that 2 of the isolates had 5'-3' class I integrons. Sequence analysis of the integrons revealed the existence of 6'-N-acetyltransferase (aac(6)-Ib) and streptomycin 3'-adenyl transferase (aadA2) cassettes (GenBank accession no. EF368053). Coexistence of PER-1 and OXA-2 and of PER-1 and OXA-10 was demonstrated in 8 and 7 of the isolates, respectively, by PCR. Shotgun cloning and PCR screening with relevant primers revealed the existence of a class I integron-associated blaOXA-2 gene cassette in 2 of the isolates. Strains positive for OXA-2 were also positive for PER-1.

Conclusion: This study demonstrated the coexistence of PER-1 and OXA-2 in a single host. Coexistence of PER-1 with OXA-2 may increase the risk of the spreading of MDR *P. aeruginosa* in Turkish hospitals.

Key words: Integron, resistance genes, multidrug resistance, outer membrane protein, *Pseudomonas aeruginosa*

Klinik örneklerden soyutlanan, çoklu ilaç dirençli *Pseudomonas aeruginosa* suşlarında integronla ilişkili direnç genleri

Amaç: Bu çalışmada, Türkiye'de çoğul dirençli *P. aeruginosa* suşlarında beta laktam antibiyotik direncini kodlayan integronların tanımlanmasını amaçladık.

Yöntem ve gereç: Altmış yedi adet *P. aeruginosa* suşunun incelemesi sonrasında 14 tanesi tüm test edilen beta laktamazlara ve aminoglikozidlere dirençli bulunmuştur. Bu 14 suşun beta laktamazları çalışılmış ve integronla ilişkilendirilmiştir. Minimum inhibisyon konsantrasyonu, polimeraz zincir tepkimesi (PZT) taraması, izoelektrik odaklama, dış membran eldesi ve analizi, klonlama ve dizileme çalışmaları yayınlanmış protokollere göre yapılmıştır.

Bulgular: Seçilmiş çoklu ilaç dirençli *P. aeruginosa*'lar arasında beta laktamaz için PZT taramasında PER-1 ve OXA-10 tanımlanmıştır. Tüm suşlarda yapılan PZT ile iki suş 5'-3'sınıf I integron pozitif bulunmuştur. Bu integronların sekans analizi ile 6'-N-asetiltransferaz (aac(6)-Ib) ve streptomisin 3'-adenil transferaz (aadA2) kaseti (GenBank no. EF368053) varlığını göstermiştir. Sekiz suş PZT ile PER-1 ile OXA-2; 7 suş ise PER-1 ve OXA-10 pozitif bulunmuştur. Shotgun

Received: 03.09.2010 – Accepted: 10.01.2011

[1] Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Kocaeli University, Kocaeli - TURKEY
[2] Department of Medical Biology, Faculty of Medicine, Kocaeli University, Kocaeli - TURKEY
[3] Department of Infectious Diseases, Faculty of Medicine, Kocaeli University, Kocaeli - TURKEY
Correspondence: *Fatma BUDAK, Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Kocaeli University, 41380 Umuttepe, Kocaeli - TURKEY*
E-mail: fatma.budak@isbank.net.tr

klonlama ve uygun primerler ile PZT taraması sonucu 2 suşun sınıf I integronun varlığının blaOXA-2 gen kaseti ile ilişkili olduğu gösterilmiştir. OXA-2 pozitif suşların hepsi PER-1 için de pozitifdir.

Sonuç: Bu çalışma tek bir konakta, PER-1 ve OXA-2'nin birlikte varlığını göstermiştir. PER-1 ve OXA-2'nin birlikte varlığı, Türkiye'deki hastanelerde çoklu ilaç dirençli *P. aeruginosa* yayılma riskini arttırabilir.

Anahtar sözcükler: İntegron, direnç genleri, çoklu ilaç direnci, dış membran proteini, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a significant pathogen leading to severe infections in hospitals. Acquisition of multidrug resistance by *P. aeruginosa* is becoming a matter of concern in many hospitals worldwide (1,2). The increasing use of antibiotics and growing numbers of invasive procedures, together with the development of intrinsic and acquired resistance mechanisms of *P. aeruginosa*, cause the evolution of numerous multidrug-resistant (MDR) *P. aeruginosa* outbreaks in clinical settings.

Extended-spectrum beta-lactamases (ESBLs) that can confer resistance to cephalosporins are common in Enterobacteriaceae and have spread worldwide. Various class A ESBLs, such as TEM-, SHV-, VEB-, and PER-type ESBLs, and class D ESBLs such as OXA-type ESBLs have been identified in *P. aeruginosa* (3,4).

Integrons are genetic elements encoding the components of a site-specific recombination system that recognizes mobile gene cassettes, mostly resistance determinants (5). It is potentially a major agent in the dissemination of multidrug resistance among gram-negative bacteria and *Pseudomonas* (6,7). Class I integrons are predominant among integrons that carry resistance cassettes (8) and have a threatening potential for the development of antimicrobial resistance and the emergence of MDR profiles in clinical bacteria (9). Several resistance gene cassettes are associated with class I integrons in *P. aeruginosa* that confer resistance to antimicrobial agents (10). Typically, a class I integron is composed of a 5'-conserved segment (5'CS) including the integrase gene (*int*I), and a 3'-conserved segment (3'CS) encoding resistance to sulfonamides and disinfectants.

Previous studies of clinical isolates of *P. aeruginosa* reported that acquired resistance to carbapenems resulted from the complex interaction of several mechanisms, including loss of the OprD porin, overexpression of efflux systems (MexAB-OprM, MexEF-OprN), and production of carbapenemase activity (11). The most frequent causes of OprD mutational inactivation were frameshift mutations produced by insertion, deletions, and point mutations, leading to the creation of premature stop codons occurring in numerous isolates (12). These genetic events were located on the whole OprD gene.

In this study, we aimed to characterize common beta-lactamase genes and the role of integrons in beta-lactam antibiotic resistance among MDR *P. aeruginosa* strains isolated from intensive care units in Turkey.

Material and methods

Bacterial strains

A total of 67 *P. aeruginosa* specimens were isolated from 6 different university hospitals' surgery intensive care units during the years of 2000 and 2002. The hospitals were located in various regions of Turkey, including Kayseri Erciyes Medical Faculty, İzmir Dokuz Eylül Medical Faculty, Trabzon Karadeniz Technical University Medical Faculty, Samsun Ondokuz Mayıs University Medical Faculty, İstanbul Çapa Medical Faculty, and İstanbul Cerrahpaşa Medical Faculty. Bacteria were isolated from clinically important sites including 8 wounds, 23 urine samples, 5 tracheal aspirates, 13 sputum samples, and 18 catheters. All isolates were identified by conventional methods (13) and a single isolate per patient was retained in the study. Identifications were confirmed using a Vitek 2 GN card (bioMerieux, France). The 80% glycerol stocks of all isolates were stored at –70 °C until studied.

Antimicrobial susceptibility test

In order to easily determine the minimum inhibitory concentration (MIC) values of different test agents

for a large number of bacteria, the agar dilution method was used with Mueller-Hinton agar (Oxoid, Basingstoke, UK) and an inoculum of 10^4 colony-forming units per spot as recommended by the Clinical and Laboratory Standards Institute (formerly NCCLS) (14). Endpoints were read after 18 h of incubation at 37 °C. *P. aeruginosa* ATCC 25983 was used as the control strain.

Antimicrobial agents whose sources and ranges were tested were amikacin (Eczacıbaşı; 0.125-512 µg/L), ceftazidime (GlaxoSmithKline; 0.25-1024 µg/L), cefepime (Bristol-Myers Squibb; 0.12-512 µg/L), ciprofloxacin (Bayer; 0.12-512 µg/L), imipenem (Merck; 0.064-128 µg/L), meropenem (Astra Zeneca; 0.064-128 µg/L), piperacillin (Wyeth; 0.25-512 µg/L), piperacillin/tazobactam (0.25-128 µg/L), and cefoperazone/sulbactam (1-256 µg/L). Experiments were repeated 3 times and the results were evaluated together.

Polymerase chain reaction experiments

A loopful of bacteria was suspended in 100 µL of double distilled water. The bacterial extract containing the DNA was obtained by incubating bacterial suspensions at 95 °C for 10 min and sedimenting the debris for 10 min at 12,000 × *g*. The supernatant was stored at –20 °C until use. Polymerase chain reaction (PCR) screenings were accomplished in a final volume of 50 µL with 5 µL of DNA extract. The master mixture was composed of 1× buffer supplied with DNA Taq polymerase (Fermentas, Lithuania), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of each primer, and 1.5 U of Taq polymerase. Amplification was accomplished after 5 min of initial denaturation at 94 °C with 40 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at an appropriate annealing temperature (9,15-17), and 90 s of elongation at 72 °C. The annealing temperature for each primer set is given in Table 1. PCR products were examined on a 1.5% agarose gel at a constant 12 V/cm and visualized using an image analysis system (SciGene, UK). The sequence analyses were accomplished after product purification using the High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland). The sequencing was performed at İontek, Inc. (İstanbul, Turkey).

Sequence data were read and edited with either ChromasPro (http://www.technelysium.com.au) or BioEdit version 7.0.5.2.

Isoelectric focusing

For isoelectric focusing (IEF), crude cell extracts were prepared by sonication. IEF was performed on 5% polyacrylamide gels containing ampholytes (pH range of 3-10; Fluka, Switzerland) with a Model 111 Mini IEF Cell (Bio-Rad Laboratories, USA) (18). Enzymes were focused at a constant 1 W for 45 min and detected by overlaying the gel with 1 mM nitrocefin solution. Known enzymes SHV-1 (pI 7.6), OXA-14 (pI 6.2), and TEM-1 (pI 5.4) were used as standards (19).

Isolation of outer membrane proteins

Outer membrane proteins were isolated according to a published procedure (20). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Table 1. Primers used for PCR screening.

Primers	5'-3' nucleotide sequence	Annealing temp. (°C)	References
Integron I	5'CS-GGC ATC CAA GCA GCA AG 3'CS-AAG CAG ACT TGA CCT GA	58.5	9
PER-1	F(5-'AAT TTG GGC TTA GGG CAG AA-3') R(5-' ATG AAT GTC ATT ATA AAA GC-3')	52	15
OXA-10	F (5'-TCT TTC GAG TAC GGC ATT AGC-3') R(5'-CCA ATG ATG CCC TCA CTT TCC-3')	51	16
OXA-2	F(5-'GCC AAA GGC ACG ATA GTT GT-3') R(5-'GCG TCC GAG TTG ACT GCC GG-3')	51.5	17

(SDS-PAGE) was performed using the procedure of Laemmli (21) and the gels (12%) were stained with Coomassie blue before analysis. OprD-positive (PT5) and OprD-negative (PT149) standard strains, kindly provided by T. Kohler in 1997, were used.

Shotgun cloning

Shotgun cloning was used to hunt and clone beta-lactamases and related integrons for genetic characterization from genomic DNA. For shotgun cloning, genomic DNA of isolate 13 was partially digested with MboI and ligated to the dephosphorylated BamHI arms of pZerO (Stratagene, USA) overnight at 16 °C, then transformed to electrocompetent *E. coli* DH10B cells (Invitrogen, USA) by electroporation at 1700 V in a 50-µL volume. Positive clones were selected on agar plates supplemented with ampicillin (50 µg/mL) and Zeocin (50 µg/mL) (22). The insert's sequence was determined by the dye-terminator cycle sequencing method using universal T3 and T7 primers (İontek, Inc.). When necessary, new sequencing primers were synthesized by İontek and used for DNA walking.

Cloning of PCR products

The PCR products were cloned into a pCR2.1-TOPO vector according to the instructions of the manufacturer of the TOPO TA Cloning Kit (Invitrogen).

Results

Antimicrobial susceptibility test

Among the 67 isolates, 53 isolates were susceptible to all antimicrobial agents tested. It was found that 14 of them isolated from 6 different intensive care units locations were resistant to all of the tested beta-lactams and aminoglycosides (Table 2).

PCR experiments

Integron screening of these 14 isolates by PCR revealed 2 integron-positive strains. PCR products from the positive strains were sequenced and revealed a single sequence identity (GenBank accession no. EF368053). In addition, PER-1 and OXA-10 PCR screening of 14 isolates revealed the presence of 8 bla_{PER-1}-positive and 8 bla_{OXA-10}-positive strains. Furthermore, by PCR, 8 isolates had PER-1 and OXA-2, and 7 isolates had PER-1 and OXA-10 (Table 3).

Isoelectric focusing

Isoelectric focusing of the 14 isolates indicated the presence of 2 bands in some experiments at around pI 8.6 and in the others at 7.6, in addition to PER-1 and OXA-10 bands (Figure 1). Cloning and sequencing efforts allowed us to identify these bands as OXA-2, which was expected to give a single band of pI 6.8.

Table 2. MIC (µg/mL) values of 14 MDR isolates.

No.	IMP	MEM	CAZ	CEF	CIP	AK	PIP/TAZO	PIP	CEF/SUL
1	32	32	256	128	1	16	64	32	64
2	32	16	512	128	1	32	32	32	64
3	16	32	256	64	64	16	64	64	64
4	64	32	16	16	32	8	64	64	64
5	32	16	512	128	4	32	64	64	64
6	32	16	512	64	0.5	32	32	64	32
7	64	64	256	128	>64	32	128	128	128
8	16	16	128	64	64	8	32	32	64
9	32	32	512	64	32	512	128	128	128
10	32	16	512	64	2	32	32	16	64
11	32	16	512	128	8	16	128	256	128
12	64	32	512	512	16	32	64	256	128
13	32	32	512	16	0.5	16	32	128	32
14	32	32	512	512	2	32	64	128	128

IMP, imipenem; MEM, meropenem; CAZ, ceftazidime; CEF, cefepime; CIP, ciprofloxacin; AK, amikacin; PIP/TAZO, piperacillin/tazobactam; PIP, piperacillin; CEF/SUL, cefoperazone/sulbactam.

Table 3. Integron PCR and resistance genes of 14 MDR isolates.

No.	Int/PCR	PER	OXA-10	OprD	OXA-2
1		Pos	Pos		Pos
2	Pos		Pos	Neg	
3		Pos	Pos	Neg	Pos
4					
5		Pos		Neg	Pos
6	Pos	Pos	Pos	Neg	Pos
7		Pos	Pos		Pos
8		Pos	Pos	Neg	Pos
9				Neg	
10				Neg	
11					
12				Neg	
13		Pos	Pos		Pos
14		Pos	Pos	Neg	Pos

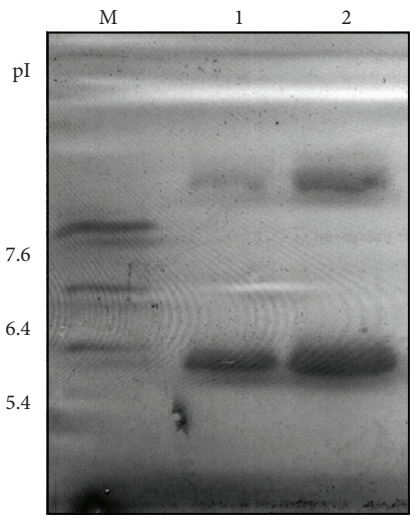

Figure 1. IEF bands of isolates positive for PER-1 (pI 5.3) and OXA-2 (pI 8.6).

Isolation of outer membrane proteins

Since OprD expression is related to the acquired antibiotic resistance, we intended to look for the expression of OprD in the 14 isolates mentioned above. Detailed analysis of SDS-PAGE results with Image J indicated that, except for isolate 12, all other studied strains lacked OprD expression (Figure 2). Outer membrane protein isolation and examination revealed the presence of D2 porin, while 13 of the 14 isolates lacked D2 porin protein and were MDR.

Shotgun cloning

Among the isolates with a pI value of 8.6, isolate 13 was randomly selected for shotgun cloning. A 4402-bp fragment carrying the gene for an enzyme of pI 8.6 was cloned. The sequence analysis revealed the presence of the bla_{OXA-2} gene in a type I integron. The antibiotic resistance profile of this clone was then compared with isolate 13 (Table 4). The clone displayed a similar profile to that of isolate 13.

Discussion

The emergence of ESBL-producing gram-negative clinical pathogens poses a severe challenge to antimicrobial chemotherapy, with an increasing number of reports worldwide (23,24). Integrons and transposons have been detected repeatedly in MDR *P. aeruginosa* isolates. In this study, we aimed to determine the rate of multidrug resistance to several antimicrobial agents in *P. aeruginosa* isolated from 6 different intensive care units, with special emphasis on ESBLs.

According to this study, 14 isolates out of 67 that were isolated from 6 different intensive care units displayed a resistance against beta-lactams and aminoglycosides. PCR screening for common beta-lactamases detected PER-1 and OXA-10 among these 14 MDR *P. aeruginosa*, and 8 of 14 MDR *P. aeruginosa* strains were found to be positive for PER-1. This indicated that the PER-1 promoter is active in *P. aeruginosa*, as previously observed (25,26). In our study, 7 of 14 beta-lactamase- and aminoglycoside-resistant *P. aeruginosa* strains were found to be both OXA-2- and OXA-10-positive, in addition to PER-1. OXA-10, OXA-11, and OXA-14, which have extended spectra, were previously identified in *P. aeruginosa* isolated from a hospital in Turkey (27,28). In this study, we showed that 8 isolates had narrow-spectrum OXA-10 activity.

It was seen that 2 isolates gave a PCR product with a 5'-3' class I integron PCR. Sequence analysis of these integrons revealed the existence of 6'-N-acetyltransferase (aac(6)-Ib) and streptomycin 3'-adenyl transferase (aadA2) cassettes (GenBank accession no. EF368053). Characterization of resistance gene cassettes associated with class I integrons in MDR clinical isolates is of considerable

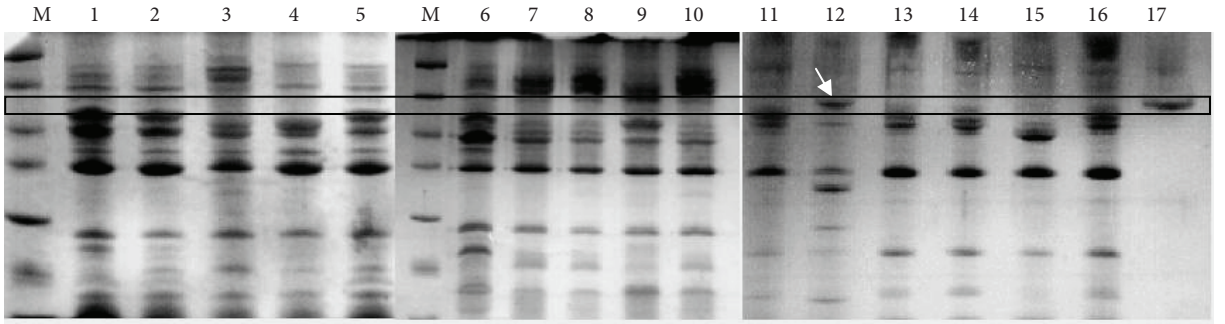

Figure 2. Outer membrane proteins. M: protein markers; 1-3: standard strains (1: OprD-positive, 2 and 3: OprD-negative); 4-16: study strains; 17: pure OprD protein.

Table 4. MIC values of antimicrobials for isolate 13 and clone 13, with and without clavulanic acid.

	Clavulanate neg/pos			
	Isolate 13		Clone 13	
Ceftazidime	256	16	32	1
Cefepime	64	8	1	1
Pip/Tazo	16	<1	16	<1
Cefotaxime	256	64	0.5	0.5
Imipenem	32	32	1	1
Meropenem	32	16	0.0125	0.0125

significance (29). Class I integrons, inducing resistance to antibiotics, play an important role in the development of antimicrobial resistance and emergence of MDR strains for 3 reasons. First, the integron-mediated circular gene cassette capturing system inserts resistance gene cassettes at the specific attI site downstream of class I integrons with the help of the integrase enzyme (30). Second, gene cassette arrays have only one promoter Pc, which strongly expresses antibiotic resistance genes, causing cross-resistance (9). Third, integrons associated with transferable plasmids and transposons widely diffuse among bacteria and develop antibiotic resistance (31).

Another reason for carbapenem resistance is the absence of the D2 porin protein (32,33). The main mechanism of imipenem resistance in *P. aeruginosa* is the repression or inactivation of the OprD gene encoding the OprD porin. *P. aeruginosa* mutants with high MICs for imipenem are easily produced in vitro or in vivo and are often found in patients during treatment with imipenem (34). Although OprD could be regulated by multiple systems, the downregulation of the porin gene alone is sufficient to induce high-level imipenem resistance, as shown for 13 isolates.

Shotgun cloning from 1 isolate and PCR screening with OXA-2 primers revealed the existence of a class I integron-associated bla_{OXA-2} gene cassette in 2 isolates. OXA-2-positive isolates were also positive for PER-1. A multicenter study reported ceftazidime resistance in 28% of *P. aeruginosa* isolates, with PER-1-type beta-lactamases being found in 38% of the ceftazidime-resistant isolates (35). The results of the present study show that isolates of *P. aeruginosa* producing PER-1 and OXA-10 beta-lactamases are encountered frequently in Turkish hospitals, and that their clonal diversity and high prevalence indicate a considerable potential for spread among patients (36).

The pattern determined in the IEF of isolate 13 appeared to indicate the presence of 2 enzymes in some experiments with pI values of 8.6 and others of 7.6. However, the pattern seems to be a misleading one, as the recombinant clone that possesses a class I integron cassette harboring the bla_{OXA-2} gene displayed a similar pattern. A similar phenomenon was also reported by Holland et al. (37) and interpreted as satellite bands.

Different from other OXA enzymes, OXA-2 hydrolyzes ceftazidime and cefotaxime and was

inhibited by clavulanate (37). The OXA-2 of this study also hydrolyzed imipenem, but weakly. In another study, similar hydrolyzing properties were reported for OXA-2, which provided resistant to ceftazidime and cefotaxime and was influenced by imipenem weakly (38).

This is the first study in Turkey that reports the isolation of OXA-2 from 8 of 14 strains (57% frequency). Although there was a weak imipenem hydrolysis by OXA-2, we believe that this hydrolysis did not contribute much to imipenem resistance. There must be another mechanism responsible for carbapenem resistance, as well. In this study and in a study in Poland in 2007, PER-1 and OXA-2 enzymes coexisted in the same isolates (39).

Integrons are natural expression vectors that permit the insertion of antibiotic resistance genes by a site-specific recombinational mechanism. By using molecular techniques, we have determined the content and order of the antibiotic resistance gene inserted between the conserved segments in the integrons of resistant clinical *P. aeruginosa*. PCR mapping of genes inserted as cassettes into integrons will provide valuable information for studies of gene expression as it relates to the position of these genes within the integrons. It is possible that the 59-base element, a potential stem-loop-forming structure found at the downstream end of each inserted gene cassette, may act as an inefficient terminator of transcription, resulting in diminished expression of a gene inserted in the second, third, and further positions in an antibiotic resistance operon. The present study demonstrated the coexistence of PER-1 and OXA-2 in a single host. Coexistence of PER-1 with OXA-2 may increase the risk of the spreading of MDR *P. aeruginosa* in Turkish hospitals.

Conclusion

The ability of multigene antibiotic-resistant mutants of *P. aeruginosa* to cause extensive outbreaks is associated with significant morbidity and mortality. Our findings showed the need to differentiate polyclonal emergence of MDR *P. aeruginosa* from clonal outbreaks, which require targeted infection control measures.

Acknowledgments

We are grateful to T. Kohler for the generous provision of the type strains. This project was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK), project number 104S337.

References

1. Goossens H. Susceptibility of multi-drug-resistant *Pseudomonas aeruginosa* in intensive care units: results from the European MYSTIC Study Group. Clin Microbiol Infect 2003; 9: 980-3.
2. Obritsch MD, Fish DN, 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 2004; 48: 4606-10.
3. Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin Infect Dis 2002; 34: 634-40.
4. Nordmann P, Guibert M. Extended-spectrum β-lactamases in *Pseudomonas aeruginosa*. J Antimicrob Chemother 1998; 42: 128-31.
5. Recchia GD, Hall RM. Gene cassettes: a new class of mobile element. Microbiology 1995; 141: 3015-27.
6. Bunny KL, Hall M, Stokes HW. New mobile gene cassettes containing an aminoglycoside resistance gene, aacA7, and a chloramphenicol resistance gene, catB3, in an integron in pBWH301. Antimicrob Agents Chemother 1995; 39: 686-93.
7. Lee MD, Sanchez S, Zimmer M, Idris U, Berrang ME , McDermott PF. Class 1 integron-associated tobramycin-gentamicin resistance in *Campylobacter jejuni* isolated from the broiler chicken house environment. Antimicrob Agents Chemother 2002; 46: 3660-6.
8. Recchia GD, Hall R. Origins of the mobile gene cassettes found in integron. Trends Microbiol 1997; 5: 389-94.
9. Levesque C, Brassard S, Lapointe J, Roy PH. Diversity and relative strength of tandem promoters for the antibiotic resistance genes of several integrons. Gene 1994; 142: 49-54.
10. Naas T, Poirel L, Karim A, Nordmann P. Molecular characterization on In50, a class 1 integron encoding the gene for the extended-spectrum beta-lactamase VEB-1 in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 1999; 176: 411-9.

11. Livermore DM. Of *Pseudomonas*, porins, pumps and carbapenems. J Antimicrob Chemother 2001; 47: 247-50.
12. Lepelletier D, Cady A, Caroff N, Maraillac J, Reynoud A, Lucet JC et al. Imipenem-resistant *Pseudomonas aeruginosa* gastrointestinal carriage among hospitalized patients: risk factors and resistance mechanisms. Diagn Microbiol Infect Dis 2010; 66: 1-6.
13. Forbes BA, Sahm DF, Weissfeld AS, eds. Bailey & Scott's diagnostic microbiology. 11th ed. St. Louis (MO): Mosby, Inc; 2002. p.358-98.
14. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. CLSI document M7-A7. Wayne (PA): CLSI; 2006.
15. Danel F, Hall LM, Gur D, Akalin HE, Livermore DM. Transferable production of PER-1 beta-lactamase in *Pseudomonas aeruginosa*. J Antimicrob Chemother 1995; 35: 281-94.
16. De Champs C, Poirel L, Bonnet R, Sirot D, Chanal C, Sirot J et al. Prospective survey of beta-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolated in a French hospital in 2000. Antimicrob Agents Chemother 2002; 46: 3031-4.
17. Naas T, Benaoudia F, Massuard S, Nordmann P. Integron-located VEB-1 extended-spectrum beta-lactamase gene in a *Proteus mirabilis* clinical isolate from Vietnam. J Antimicrob Chemother 2000; 46: 703-11.
18. Vahaboglu H, Dodanli S, Eroglu C, Ozturk R, Soyletir G, Yildirim I et al. Characterization of multiple-antibiotic-resistant *Salmonella typhimurium* stains: molecular epidemiology of PER-1-producing isolates and evidence for nosocomial plasmid exchange by a clone. J Clin Microbiol 1996; 34: 2942-6.
19. Gacar GG, Midilli K, Kolayli F, Ergen K, Gundes S, Hosoglu S et al. Genetic and enzymatic properties of metallo-b-lactamase VIM-5 from a clinical isolate of *Enterobacter cloacae*. Antimicrob Agents Chemother 2005; 49: 4400-3.
20. Ojanen T, Helander IM, Haahtela K, Korhonen TK, Laakso T. Outer membrane proteins and lipopolysaccharides in pathovars of *Xanthomonas campestris*. Appl Environ Microbiol 1993; 59: 4143-51.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 1970; 227: 680-5.
22. Sambrook J, Russell DW: Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2001.
23. Weldhagen GF, Poirel L, Nordmann P. Ambler class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. Antimicrob Agents Chemother 2003; 47: 2385-92.
24. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 2005; 18: 657-8.
25. Nordmann P, Naas T. Sequence analysis of PER-1 extended-spectrum beta-lactamase from *Pseudomonas aeruginosa* and comparison with class A beta-lactamases. Antimicrob Agents Chemother 1994; 38: 104-14.
26. Kolayli F, Gacar G, Karadenizli A, Sanic A, Vahaboglu H, Study Group. PER-1 is still widespread in Turkish hospitals among *Pseudomonas aeruginosa* and *Acinetobacter* spp. FEMS Microbiol Lett 2005; 249: 241-5.
27. Danel F, Hall LM, Gur D, Livermore DM. OXA-14, another extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1995; 1881-4.
28. Hall LM, Livermore DM, Gur D, Akova M, Akalin HE. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1993; 37: 1637-44.
29. Norrby SR. Integrons: adding another threat to the use of antibiotic therapy. Clin Infect Dis 2005; 41: 10-11.
30. Mazel D. Integrons and the origin of antibiotic resistance gene cassettes. ASM News 2004; 70: 520-5.
31. Leverstein-van Hall MA, Box ATA, Blok HEM, Paauw A, Fluit AC, Verhoef J. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant Enterobacteriaceae in a clinical setting. J Infect Dis 2002; 186: 49-56.
32. Satake S, Yoshihara E, Nakae T. Diffusion of beta-lactam antibiotics through liposome membranes reconstituted from purified porins of the outer membrane of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1990; 34: 685-90.
33. Quinn JP, Studemeister AE, DiVincenzo CA, Lerner SA. Resistance to imipenem in *Pseudomonas aeruginosa*: clinical experience and biochemical mechanisms. Rev Infect Dis 1988; 10: 892-8.
34. Quinn JP, Dudek EJ, DiVincenzo CA, Lucks DA, Lerner SA. Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. J Infect Dis 1986; 154: 289-94.
35. Vahaboglu H, Oztürk R, Aygün G, Coşkunkan F, Yaman A, Kaygusuz A et al. Widespread detection of PER-1-type extended-spectrum beta-lactamases among nosocomial *Acinetobacter* and *Pseudomonas aeruginosa* isolates in Turkey: a nationwide multicenter study. Antimicrob Agents Chemother 1997; 41: 2265-9.
36. Aktaş Z, Poirel L, Şalcıoğlu M, Özcan PE, Midilli K, Bal Ç et al. PER-1- and OXA-10-like b-lactamases in ceftazidime-resistant *Pseudomonas aeruginosa* isolates from intensive care unit patients in Istanbul, Turkey. Clin Microbiol Infect 2005; 11: 193-8.
37. Holland S, Dale JW. Improved purification and characterization of the OXA-2 beta-lactamase. Biochem 1984; 224: 1009-13.
38. Danel F, Hall LM, Gur D, Livermore DM. OXA-15, an extended-spectrum variant of OXA-2 beta-lactamase, isolated from a *Pseudomonas aeruginosa* strain. Antimicrob Agents Chemother 1997; 41: 785-90.
39. Empel J, Filczak K, Mrówka A, Hryniewicz W, Livermore DM, Gniadkowski M et al. Outbreak of *Pseudomonas aeruginosa* infections with PER-1 extended-spectrum beta-lactamase in Warsaw, Poland: further evidence for an international clonal complex. J Clin Microbiol 2007; 45: 2829-34.