Problematic clinical isolates of *Pseudomonas aeruginosa* from the university hospitals in Sofia, Bulgaria: current status of antimicrobial resistance and prevailing resistance mechanisms

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A total of 203 clinical isolates of *Pseudomonas aeruginosa* was collected during 2001–2006 from five university hospitals in Sofia, Bulgaria, to assess the current levels of antimicrobial susceptibility and to evaluate resistance mechanisms to antipseudomonal antimicrobial agents. The antibiotic resistance rates against the following antimicrobials were: carbenicillin 93.1 %, azlocillin 91.6 %, piperacillin 86.2 %, piperacillin/tazobactam 56.8 %, ceftazidime 45.8 %, cefepime 48.9 %, cefpirome 58.2 %, aztreonam 49.8 %, imipenem 42.3 %, meropenem 45.5 %, amikacin 59.1 %, gentamicin 79.7 %, tobramycin 89.6 %, netilmicin 69.6 % and ciprofloxacin 80.3 %. A total of 101 of the studied *P. aeruginosa* isolates (49.8 %) were multidrug resistant. Structural genes encoding class A and class D β -lactamases showed the following frequencies: *bla*_{VEB-1} 33.1 %, *bla*_{PSE-1} 22.5 %, *bla*_{PER-1} 0 %, *bla*_{OXA-groupI} 41.3 % and *bla*_{OXA-groupII} 8.8 %. IMP- and VIM-type carbapenemases were not detected. In conclusion, the studied clinical strains of *P. aeruginosa* were problematic nosocomial pathogens. VEB-1 extended-spectrum β -lactamases appear to have a significant presence among clinical *P. aeruginosa* isolates from Sofia. Carbapenem resistance was related to non-enzymic mechanisms such as a deficiency of OprD proteins and active efflux.

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INTRODUCTION

Pseudomonas aeruginosa is responsible for 10–15 % of nosocomial infections worldwide (Blanc *et al.*, 1998). The infections are frequently difficult to treat because of both the natural resistance of the species and its remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents. *P. aeruginosa* represents a phenomenon of antibiotic resistance, demonstrating practically all known enzymic and mutational mechanisms of bacterial resistance. These mechanisms are often present

simultaneously, conferring combined resistance to many strains (McGowan, 2006).

Multidrug-resistant strains of *P. aeruginosa* (resistant to at least three of the following antimicrobials: ceftazidime, imipenem, gentamicin and ciprofloxacin) are often isolated among patients suffering from nosocomial infections, particularly those receiving intensive care treatment (Tassios *et al.*, 1997). The increasing rate of *P. aeruginosa* strains in a wide spectrum of clinical settings determines them as emerging pathogens, especially in intensive care units (ICUs), and justifies the necessity for antimicrobial-resistance surveillance.

The aim of this study was to assess the current levels of antimicrobial susceptibility and to evaluate the resistance mechanisms to antipseudomonal antimicrobial agents among problematic clinical isolates of *P. aeruginosa* collected from five university hospitals in Sofia, Bulgaria.

Abbreviations: AAC, aminoglycoside acetyltransferase; ANT, aminoglycoside nucleotidyltransferase; ESBL, extended-spectrum β -lactamase; ICU, intensive care unit; LRTI, lower respiratory tract infection; MBL, metallo- β -lactamase; URTI, upper respiratory tract infection.

The GenBank/EMBL/DDBJ accession nos for the *P. aeruginosa* *bla*_{VEB-1} and *bla*_{PSE-1} gene sequences are DQ333895 and M69058, respectively.

METHODS

Bacterial isolates. A collection of 203 non-duplicate, problematic clinical isolates of *P. aeruginosa* (resistant to one or more of the following groups of antimicrobials: third- or fourth-generation cephalosporins, carbapenems, aminoglycosides and fluoroquinolones) were used in the present study. The strains were collected during the period 2001–2006 from in-patients of different types of ward in five university hospitals in Sofia: surgical, orthopaedic, internal, paediatric, neurological and ICUs. The isolates were obtained from urine (79), tracheal aspirates (27), sputum (14), bronchial lavage (12), pleural fluid (2), surgical wounds or abscesses (30), drainages (9), blood (9), nose (9), throat (7), ear (1), rectal swabs (2) and bile (2). Bacterial identification was performed using a BBL Enteric/Nonfermenter ID system (Becton Dickinson).

Antimicrobial-susceptibility testing. The susceptibility of the investigated *P. aeruginosa* isolates to 17 antimicrobial agents was determined by the disc diffusion method on Mueller–Hinton II agar plates (Becton Dickinson) using antibiotic-containing discs provided by Becton Dickinson, Mast Diagnostics and Bul Bio, and was interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) (now the Clinical and Laboratory Standards Institute) 2004 recommendations (NCCLS, 2004). Control strains included *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

Phenotypic methods for detection of resistance mechanisms to antimicrobial agents

Detection of group 1 inducible β -lactamases. The prevalence of inducible AmpC β -lactamase (molecular class C, functional group 1) (Bush *et al.*, 1995) in the studied strains of *P. aeruginosa* was investigated using a disc approximation test method (Sanders & Sanders, 1992). A ceftazidime (30 μ g) disc was placed at a distance of 20 mm (centre to centre) from an imipenem (10 μ g) disc on a Mueller–Hinton II agar plate inoculated with a suspension of the test organism, adjusted to a McFarland no. 0.5 tube. After overnight incubation, distinct flattening of the inhibitory zone around the ceftazidime-containing disc on the side nearest to the imipenem disc was taken to indicate the presence of inducible AmpC β -lactamase.

Screening for extended-spectrum β -lactamases (ESBLs). The presence of ESBLs was investigated by the double disc synergy test (Jarlier *et al.*, 1988). Ceftazidime (30 μ g), cefepime (30 μ g), ceftipime (30 μ g) and aztreonam (30 μ g) discs were placed next to an amoxicillin/clavulanic acid (20/10 μ g)-containing disc at a distance of 20 mm (centre to centre) on a Mueller–Hinton II agar plate inoculated with the test organism. After overnight incubation at 37 °C, an enhancement of the inhibition zone around at least one of these discs toward the clavulanate-containing disc indicated the presence of ESBLs. All studied strains were tested additionally by a disc diffusion method with imipenem (10 μ g) and ceftazidime (30 μ g) discs for the presence of synergism (Weldhagen *et al.*, 2003).

Screening for metallo- β -lactamases (MBLs). The presence of Ambler class B MBLs (Bush *et al.*, 1995) was studied using the modified Hodge test (Lee *et al.*, 2001).

Detection of presumptive aminoglycoside-modifying enzymes. This test was performed according to the substrate profile, as described by the Aminoglycoside Resistance Study Groups (1994).

PCR amplification and sequencing of β -lactamase genes. Total DNA from *P. aeruginosa* isolates was extracted by boiling. The detection of *bla*_{VEB-1}, *bla*_{PER-1}, *bla*_{PSE-1}, *bla*_{OXA-group I}, *bla*_{OXA-group II}, *bla*_{IMP-like} and *bla*_{VIM-like} genes in the investigated strains was performed by PCR with the specific primers (Alpha DNA) listed in Table 1. PCR was carried out with 2 μ l template DNA, 0.25 μ M each primer, 0.2 mM deoxyribonucleoside triphosphates, 1 $\times$ reaction buffer, 2 mM MgCl₂ and 1.5 U Prime *Taq* DNA polymerase (GENET BIO) in a total volume of 25 μ l. The DNA was amplified in a Techgen PCR thermocycler (Techne) using the following protocol: initial denaturation (94 °C for 5 min), followed by 30 cycles of denaturation (94 °C for 45 s), annealing (50–64 °C, from 45 s to 1 min) and extension (72 °C, from 45 s to 1 min), with a single final extension of 7 min at 72 °C. PCR products were separated in 1% agarose gel for 50 min at 150 V, stained with ethidium bromide (0.5 μ g ml⁻¹) and detected by a UV transillumination (wavelength 312 nm). The amplified genes were identified on the basis of fragment size (shown in Table 1). Selected VEB-1 and PSE-1 PCR products were purified with ExoSAP-IT reagent (Amersham Biosciences). Sequencing

Table 1. Oligonucleotides used as primers for amplification and sequencing

Primer*	Target	Sequence (5'→3')†	Product size (bp)	Annealing temperature (°C)	Reference
VEB-F	VEB-1 ESBL	CGACTTCCATTTCCCGATGC	643	58	Naas <i>et al.</i> (2000)
VEB-B		GGACTCTGCAACAAATACGC			
PER-F	PER-1 ESBL	AATTTGGGCTTAGGGCAGAA	925	50	Claeys <i>et al.</i> (2000)
PER-B		ATGAATGTCATTATAAAAGC			
PSE-F	PSE-1	AATGGCAATCAGCGCTTC	699	54	De Champs <i>et al.</i> (2002)
PSE-B		GCGCGACTGTGATGTATA			
OXA-10-F	OXA group I	TCTTTCGAGTACGGCATTAGC	759	58	Naas <i>et al.</i> (2000)
OXA-10-B		CCAATGATGCCCTCACTTTCC			
OXA-2-F	OXA group II	GCCAAAGGCACGATAGTTGT	701	64	De Champs <i>et al.</i> (2002)
OXA-2-B		GCGTCCGAGTTGACTGCCGG			
IMP-F	IMP-type MBLs	GAAGGYGTTTATGTTTCATAC	587	56	Nordmann & Poirel (2002)
IMP-B		GTAMGTTTCAAGAGTGATGC			
VIM-F	VIM-type MBLs	ATGGTGTTTGGTCGCATATC	510	60	Nordmann & Poirel (2002)
VIM-B		TGGGCCATTACGCCAGATC			

*F, Forward; B, backward.

†Y=C or T; M=A or C.

reactions were performed using the same *bla*_{VEB-1}- and *bla*_{PSE-1}-specific primers and a BigDye terminator v3.1 kit (Applied) in an automated sequencer (ABI 310 sequence genetic analyser; Applied Biosystems). The nucleotide and deduced amino acid sequences were analysed with software available from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

Statistical analysis. Student's *t*-test was used to assess differences in resistance rates. A *P* value below 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

The antimicrobial-resistance testing results are presented in Table 2. The established antimicrobial resistances, in increasing order, were to: polymyxin B < imipenem < meropenem < ceftazidime < cefepime < aztreonam < piperacillin/tazobactam. Polymyxin B remained active against all isolates. The antimicrobial resistance to antibiotics of the investigated problematic strains of *P. aeruginosa* was higher than the mean *P. aeruginosa* resistance found in Bulgaria in 2003, according to data from the national program BulSTAR: 45.8 vs 24.5 % to ceftazidime, 42.3 vs 8.3 % to imipenem, 59.1 vs 24.9 % to amikacin, 79.7 vs 38.7 % to gentamicin and 80.3 vs 30.7 % to ciprofloxacin (Petrov *et al.*, 2005). Approximately half of our isolates (49.8 %) were multidrug resistant.

The comparative temporary resistance rates among the studied *P. aeruginosa* isolates are shown in Fig. 1(a, b). The strains isolated during 2004–2006 were significantly more resistant ($P < 0.001$) than those obtained during 2001–2003

to the following antibiotics: ceftazidime (79.5 vs 20.0 %), cefepime (75.0 vs 26.9 %), ceftiofime (81.6 vs 7.6 %), aztreonam (73.9 vs 31.0 %), imipenem (59.1 vs 31.3 %), meropenem (62.5 vs 27.8 %) and amikacin (72.7 vs 47.8 %). Thus, these results demonstrated increasing resistance rates to extended-spectrum antipseudomonal cephalosporins, carbapenems, monobactams and amikacin in *P. aeruginosa* isolates from the university hospitals in Sofia during the last 3 years.

The strains of *P. aeruginosa* from ICUs were more resistant to antibiotics than the overall studied strains, except to ceftiofime and aztreonam (Table 2). The ICU isolates were significantly more resistant to meropenem (61.4 %) than all investigated isolates as a whole (45.5 %, $P < 0.05$), which is related to the widespread use of meropenem for the treatment of life-threatening infections in ICUs.

The antimicrobial resistance in *P. aeruginosa* varied among different clinical specimens (Table 2). The *P. aeruginosa* isolates from in-patients with lower respiratory tract infections (LRTIs) were more resistant to piperacillin than the isolates obtained from wounds and drainages ($P < 0.05$). The strains of *P. aeruginosa* isolated from in-patients with LRTIs and upper respiratory tract infections (URTIs) were more resistant to piperacillin/tazobactam than those from urine and wounds ($P < 0.01$ and $P < 0.001$, respectively). The observed resistance rate to ceftazidime in *P. aeruginosa* from wounds and drainages was lower than that in URTI isolates ($P < 0.05$). URTI strains of *P. aeruginosa* were more resistant to ceftiofime than LRTI isolates ($P < 0.05$). The antibiotic resistance of *P. aeruginosa*

Table 2. Antimicrobial resistance as percentage of isolates from different sources among 203 isolates of *P. aeruginosa*

Antimicrobial agent	Urine (n=79)	LRTI (n=55)	Wounds and drainages (n=39)	URTI (n=17)	Blood culture (n=9)	ICU (n=68)	Total* (n=203)
Carbenicillin	89.9	96.4	94.9	2.3	100.0	97.2	93.1
Azlocillin	89.3	96.2	91.7	87.5	87.5	95.7	91.6
Piperacillin	84.1	97.5	77.3	84.6	75.0	94.5	86.2
Piperacillin + tazobactam	47.9	75.0	34.5	81.2	37.5	70.5	56.8
Ceftazidime	46.8	40.0	35.9	64.7	77.8	49.3	45.8
Cefoperazone	85.3	94.0	77.1	75.0	87.5	95.6	86.2
Cefepime	45.6	51.0	47.4	52.9	66.7	53.1	48.9
Ceftiofime	55.3	51.8	59.5	76.5	77.8	55.7	58.2
Aztreonam	51.9	41.5	41.0	64.7	77.8	43.5	49.8
Imipenem	32.9	43.6	43.6	64.7	77.8	52.1	42.3
Meropenem	31.6	53.7	43.6	64.7	77.8	61.4	45.5
Amikacin	59.5	58.2	74.4	17.6	77.8	66.2	59.1
Gentamicin	86.1	79.6	92.3	29.4	88.9	82.8	79.7
Tobramycin	93.7	96.3	97.4	35.3	88.9	95.7	89.6
Netilmicin	70.5	79.1	74.4	35.3	44.4	75.0	69.6
Ciprofloxacin	92.4	78.2	87.2	29.4	66.7	80.3	80.3
Polymyxin B	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Antimicrobial resistance of all strains of *P. aeruginosa*, including two rectal swab and two bile isolates.

Fig. 1. Comparative temporary antimicrobial resistance in the studied *P. aeruginosa* isolates. $\blacktriangle$, 2001–2003 ($n=115$); $\square$, 2004–2006 ($n=88$). (a) Resistance to β -lactams. CAR, Carbenicillin; AZL, azlocillin; PIP, piperacillin; TZP, piperacillin + tazobactam; CAZ, ceftazidime; CPZ, cefoperazone; FEP, cefepime; CPO, cefpirome; ATM, aztreonam; IMP, imipenem; MEM, meropenem. (b) Resistance to aminoglycosides and fluoroquinolones. AMK, Amikacin; GEN, gentamicin; TOB, tobramycin; NET, netilmicin; CIP, ciprofloxacin.

from urine samples towards imipenem and meropenem was lower than that among URTI strains ($P<0.02$ and $P<0.01$, respectively). URTI isolates were significantly more susceptible than the *P. aeruginosa* isolates from urine, LRTIs, and wounds and drainages to the following antimicrobials: amikacin ($P<0.001$), gentamicin ($P<0.001$), tobramycin ($P<0.001$), netilmicin ($P<0.01$, $P<0.001$ and $P<0.01$, respectively) and ciprofloxacin ($P<0.001$).

A total of 104 of the 203 investigated problematic *P. aeruginosa* isolates (51.2%) showed a 'penicillinase production phenotype' (resistance to carboxypenicillins and ureidopenicillins, and susceptibility to ceftazidime) (Bert *et al.*, 2003). These strains were presumptive producers of narrow-spectrum β -lactamases. Additionally, they expressed an inducible AmpC β -lactamase (cephalosporinase).

The ceftazidime resistance rate was 45.8%. A total of 57 of the strains of the 203 studied *P. aeruginosa* isolates (28.1%) were resistant to extended-spectrum cephalosporins, including ceftazidime, and were characterized as presumptive producers of ESBLs according to the double disc

synergy test. These strains also displayed *in vitro* synergism between imipenem and ceftazidime, typical of the producers of clavulanic acid- and tazobactam-inhibited ESBLs from molecular class A, such as VEB-, PER- or GES-type (Weldhagen *et al.*, 2003).

Twelve strains of *P. aeruginosa* (5.9%) were resistant to all β -lactams except carbapenems, and showed a negative result in the double disc synergy test. In these isolates, resistance to extended-spectrum cephalosporins was related mainly to the overproduction of a chromosomal AmpC cephalosporinase from molecular class C.

Of the 203 *P. aeruginosa* isolates, 12 (5.9%) possessed a OprD⁻ mutant phenotype (resistant only to imipenem and meropenem, but susceptible to other β -lactams). Carbapenem resistance is mostly due to OprD deficiency and is independent of susceptibility towards other β -lactam agents (Livermore, 2001). This resistance mechanism demands continued expression of the chromosomal AmpC β -lactamase (Livermore, 1992).

Overproduction of active efflux systems with wide substrate profiles was the prevailing resistance mechanism in eight *P. aeruginosa* isolates (3.9%). In our study, the presumptive efflux systems were: MexA–MexB–OprM associated with decreased susceptibility or resistance to all β -lactams, except imipenem, and with decreased susceptibility or resistance to quinolones (*nalB* or *nalC* mutants) (Llanes *et al.*, 2004), and MexC–MexD–OprJ conferring resistance to fourth-generation cephalosporins (cefepime and cefpirome) and resulting from mutation in *nfxB* (Poole *et al.*, 1996).

Sixty strains of *P. aeruginosa* (29.6%) were resistant to all β -lactams, including carbapenems, and thus could be related to a phenotype of Ambler class B MBL-producing strains (Nordmann & Poirel, 2002). All carbapenem-resistant strains of *P. aeruginosa* showed a negative Hodge test and therefore were not producers of MBLs. Most probably, the resistance to β -lactams resulted from a combination of different mechanisms: OprD deficiency, derepression of chromosomal AmpC cephalosporinase, ESBL production and overexpression of active efflux systems.

One hundred and twenty isolates out of the studied strains of *P. aeruginosa* (59.1%) were resistant to amikacin and 69.6–89.6% to the other aminoglycosides (netilmicin, gentamicin and tobramycin). The most widespread mechanism of resistance to these antimicrobials involves enzymic modification by aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) or aminoglycoside phosphotransferases (Poole, 2005). The prevailing phenotypes of aminoglycoside resistance in our strains were: (i) amikacin + gentamicin + tobramycin + netilmicin (49.9%), associated with AAC (6')-I $\pm$ ANT (2''); (ii) gentamicin + tobramycin + netilmicin (14.1%), associated with AAC (3)-V or ANT (2'') + AAC (3)-Ia; and (iii) amikacin + gentamicin + tobramycin (14.1%), related

to aminoglycoside phosphotransferase (3')-VI+ANT (2'') (Aminoglycoside Resistance Study Group, 1994).

One hundred and sixty three (80.3 %) of the isolates were resistant to ciprofloxacin. The most important mechanisms of quinolone resistance are structural alterations of the primary or secondary targets because of chromosomal point mutations in *gyrA/gyrB* or *parC/parE* genes, respectively, followed by an active efflux of these antimicrobial agents (Hooper, 2001).

A molecular genetic study was carried out for the presence of β -lactamases belonging to different molecular classes. A total of 160 isolates were investigated, and 53 (33.1 %) were found to be VEB-1 producers. The sequence of *bla*_{VEB-1} amplified from different selected isolates was identical for all isolates and 100 % identical to the known *veb-1* sequence (GenBank accession no. DQ333895). The frequency of VEB-1 ESBLs among the ceftazidime-resistant *P. aeruginosa* was 57.0 % (53/93). A total of 36 of the 160 isolates (22.5 %) produced PSE-1 enzyme. Selected PSE-1 PCR products showed 100 % identity to *bla*_{PSE-1} (GenBank accession no. M69058). The frequency of OXA group I and OXA group II β -lactamases was 41.3 % (66/160) and 8.8 % (14/160), respectively.

The distribution of the Ambler class A and D β -lactamases among the investigated strains of *P. aeruginosa* is presented in Table 3. As shown, the relative proportion of β -lactamase-producing strains of *P. aeruginosa* (66.8 %) was higher than the proportion of β -lactamase-non-producing strains (33.1 %). An analogous study carried out recently in Korea established that β -lactamase-non-producing strains of *P. aeruginosa* were more widespread than producers (74.6 vs 25.4 %; Lee *et al.*, 2005).

In our investigation, the β -lactamase producers were mostly present as VEB-1 + OXA group I (20.0 %), followed by PSE-1 (13.1 %) and OXA group I (12.5 %). The frequency of the Ambler class A β -lactamases (55.6 %) was approximately equal to the frequency of class D β -lactamases (50.1 %). In comparison, class D OXA-type

enzymes were detected more frequently than class A in *P. aeruginosa* from Korea (21.0 vs 6.3 %; Lee *et al.*, 2005), in contrast to the data from Europe (31.3 vs 64.9 %; Bert *et al.*, 2002).

A high frequency of distribution of ESBLs in the ceftazidime-resistant isolates of *P. aeruginosa* was established in our study. In all the university hospitals monitored in Sofia, widespread dissemination of *bla*_{VEB-1} in clinical isolates of *P. aeruginosa* was found. Recently, Bachvarova *et al.* (2005) reported a significantly lower ($P < 0.01$) rate of prevalence of VEB-1-type β -lactamases among ceftazidime-resistant strains of *P. aeruginosa* obtained from distinct regions of Bulgaria during 1998–2003 than that determined in our study (36.8 vs 57.0 %). Thus, the observed trend towards an increasing rate of VEB-1-producing *P. aeruginosa* strains in Bulgaria relates to the last 2 years. VEB-1 and VEB-1-like enzymes are widespread in Asia (Thailand, Kuwait, India and China) (Weldhagen *et al.*, 2003; Girlich *et al.*, 2002; Poirel *et al.*, 2001), but in European countries have been detected only in France (Naas *et al.*, 1999).

A total of 42 (26.3 %) of the 160 isolates studied possessed both VEB-1 and OXA group I enzymes. It is likely that the strains produced the narrow-spectrum OXA-10 from OXA group I (Sanschagrin *et al.*, 1995), encoded by a gene located on class 1 integron *In50*, as well as *bla*_{VEB-1} (Girlich *et al.*, 2002). Lee *et al.* (2005) reported that OXA-10 was the most prevalent enzyme (13.5 %) in Korea in 2002.

PSE-1 β -lactamases belonging to Ambler class A and functional group 2c (Bush *et al.*, 1995) were detected in 22.5 % of the investigated strains. In 2002, Nordmann (2002) reported 11 % CARB-producing strains of *P. aeruginosa* in France, 90 % of which were PSE-1.

Our study did not reveal *bla*_{PER-1}, in contrast to the widespread detection of these genes in Europe. Epidemics caused by PER-1-producing *P. aeruginosa* have been reported previously in Turkey and Italy (Vahaboglu *et al.*, 1997; Luzzaro *et al.*, 2001). Isolates of *P. aeruginosa* with

Table 3. Prevalence of Ambler class A and D β -lactamases in 160 *P. aeruginosa* isolates

Class	Type of β -lactamase	No. (%) of isolates
Class A	PSE-1	21 (13.1)
	VEB-1	8 (5.0)
	VEB-1 and PSE-1	2 (1.3)
Class D	OXA group II	20 (12.5)
	OXA group I	10 (6.3)
Combined	VEB-1 and OXA group I	32 (20.0)
	VEB-1, PSE-1 and OXA group I	8 (5.0)
	VEB-1, PSE-1, OXA group I and OXA group II	2 (1.3)
	PSE-1 and OXA group I	2 (1.3)
	PSE-1 and OXA group II	1 (0.6)
	VEB-1, OXA group I and OXA group II	1 (0.6)
	β -lactamase-non-producing	53 (33.1)
None		

PER-1 enzymes have also been observed in France, Belgium and Poland (De Champs *et al.*, 2002; Claeys *et al.*, 2000; Empel *et al.*, 2005).

The established frequency of OXA group II β -lactamases comprising OXA-2, -3, -15 and -20 (Sanschagrin *et al.*, 1995) was the lowest in our research. It is likely that the oxacillinases from group II were predominantly narrow spectrum, such as OXA-2 or -3, as these enzymes were detected mainly in ceftazidime-susceptible strains of *P. aeruginosa*. In comparison, OXA group II enzymes were disseminated among 2.3 % of the studied *P. aeruginosa* isolates in Korea and all strains were determined as OXA-2 producers (Lee *et al.*, 2005). The frequency of *bla*_{OXA-groupII} in our strains of *P. aeruginosa* (8.8 %) was similar to the dissemination rate of OXA group II β -lactamases in France during 1994–1999 (9.9 %) (Bert *et al.*, 2002).

Carbapenem-hydrolysing IMP- and VIM-type metalloenzymes belonging to Ambler class B were not detected in this study. The investigated carbapenem-resistant strains of *P. aeruginosa* from Sofia did not harbour *bla*_{VIM-like} genes, although the detection of these genes is widespread, especially in neighbouring countries such as Greece and Turkey (Tsakris *et al.*, 2000; Mavroidi *et al.*, 2000; Pournaras *et al.*, 2002; Bahar *et al.*, 2004). The carbapenem resistance was related to non-enzymic mechanisms such as OprD deficiency and active efflux.

The comparative antimicrobial resistances of β -lactamase-producing and β -lactamase-non-producing *P. aeruginosa* are summarized in Table 4. The β -lactamase producers were significantly more resistant than non-producers to ceftazidime, cefepime, ceftiofime, aztreonam, amikacin, tobramycin and ciprofloxacin ($P < 0.001$) and to gentamicin

($P < 0.01$). The susceptibilities to extended-spectrum cephalosporins, aztreonam and carbapenems among β -lactamase-producing strains of *P. aeruginosa* were lower than those in non- β -lactamase producers and were similar to the susceptibilities in analogous *P. aeruginosa* isolates from Korea in 2002 (Lee *et al.*, 2005). Moreover, in our study and the Korean study, the cross-class resistance to aminoglycosides and ciprofloxacin was significantly higher in class A and D β -lactamase-producing *P. aeruginosa*. As described previously, VEB-1 was the first class A enzyme found to be encoded by an integron-located gene cassette (Poirel *et al.*, 1999). In the *bla*_{VEB-1}-containing integrons of *P. aeruginosa*, the *veb-1* cassette is often associated with aminoglycoside resistance gene cassettes (Girlich *et al.*, 2002).

In conclusion, the studied clinical strains of *P. aeruginosa* were problematic nosocomial pathogens and half were found to be multidrug resistant. From 2001 to 2006, the rates of resistance to third- or fourth-generation cephalosporins, monobactams, carbapenems and amikacin showed significant increases among the investigated *P. aeruginosa* strains from the monitored hospitals. The interpretation of the phenotypic patterns of antimicrobial susceptibility showed a variety of resistance mechanisms, from which the prevalent were expression of an inducible AmpC cephalosporinase, production of ESBLs and a combination of mechanisms conferring resistance to multiple groups of antimicrobials. The carbapenem resistance was not related to enzymic hydrolysis by Ambler class B MBLs. The clavulanic acid-inhibited VEB-1-type ESBLs appear to have a significant presence among *P. aeruginosa* isolates in the university hospitals in Sofia and cause serious impediments in antimicrobial treatment and difficulties in limiting their dissemination in Bulgaria.

Table 4. Comparison of antimicrobial resistance (%) between the class A and/or class D β -lactamase-producing and -non-producing *P. aeruginosa* isolates

Antimicrobial agent	β -Lactamase producing (<i>n</i> =107)	β -Lactamase non-producing (<i>n</i> =53)	<i>P</i> value
Carbenicillin	100.0	98.1	$P > 0.1$
Azlocillin	99.1	98.1	$P > 0.1$
Piperacillin	90.5	90.9	$P > 0.1$
Piperacillin + tazobactam	48.4	60.5	$P > 0.1$
Ceftazidime	69.2	28.3	$P < 0.001$
Cefoperazone	95.2	90.2	$P > 0.1$
Cefepime	72.5	35.3	$P < 0.001$
Ceftiofime	79.4	49.1	$P < 0.001$
Aztreonam	67.6	35.8	$P < 0.001$
Imipenem	53.3	41.5	$P > 0.1$
Meropenem	52.3	47.2	$P > 0.1$
Amikacin	77.6	35.8	$P < 0.001$
Gentamicin	88.8	67.9	$P < 0.01$
Tobramycin	99.1	75.5	$P < 0.001$
Netilmicin	65.0	73.1	$P > 0.1$
Ciprofloxacin	91.6	67.9	$P < 0.001$

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