FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 50, No. 1, 2012
pp. 46–51

ORIGINAL STUDY

VIA MEDICA

Susceptibility, phenotypes of resistance, and extended-spectrum β-lactamases in *Acinetobacter baumannii* strains

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Abstract: *Acinetobacter baumannii* plays an increasing role in the pathogenesis of infections in humans. The bacilli are frequently isolated from patients treated in intensive care units. A growing resistance to antibiotics is leading to the emergence of strains that are multidrug-resistant and resistant to all available agents. The objective of this study was to assess susceptibility to antibiotics and to determine the presence and current level of the extended-spectrum β-lactamases (ESBLs) and attempt to isolate the *Acinetobacter baumannii* strain carrying the bla_{PER} gene. A total of 51 strains of *A. baumannii* identified by phenotypic features were examined. That the strains belonged to the species was confirmed by the presence of the $bla_{OXA\text{-}51\text{-}like}$ gene. A broth microdilution method was used for antibacterial susceptibility testing. The occurrence of ESBLs was determined using phenotypic double-disk synergy tests. The PCR technique was used to confirm the presence of the $bla_{PER\text{-}1}$ gene encoding ESBL. The most active antibiotics were meropenem, cefepime and ampicillin/sulbactam, with susceptibility shown by 76.5%, 60.8% and 56.9% of the strains, respectively. The strains exhibited the highest resistance (> 75%) to piperacillin, tetracycline, ciprofloxacin and cefotaxime. Phenotypic tests revealed ESBL mechanism of resistance in approximately 20% of *Acinetobacter baumannii* isolates. However, the PCR technique did not confirm the presence of the $bla_{PER\text{-}1}$ gene in any of the *Acinetobacter baumannii* strains examined in our hospital. *Acinetobacter baumannii* strains demonstrate considerable resistance to many groups of antibiotics. Our findings indicate the involvement of enzymes belonging to families other than PER β-lactamase in resistance to β-lactams in *A. baumannii*. *(Folia Histochemica et Cytobiologica 2012; Vol. 50, No. 1, pp. 46–51)*

Key words: *Acinetobacter baumannii*, susceptibility to antibiotics, extended-spectrum β-lactamase

Introduction

In recent decades, *Acinetobacter baumannii* has become more prevalent as an opportunistic pathogen, especially as a multi-drug resistant agent (MDR) using different mechanisms of resistance [1–3]. β-lactamases have the essential participation in the resistance to β-lactams. *Acinetobacter baumannii* strains producing PER-1 β-lactamase have been described in Turkey, Korea and France [4–6].

The objective of this study was to isolate *Acinetobacter baumannii* strains producing the PER family extended spectrum β-lactamase in patients from the north eastern of Poland, and to assess their suscepti-

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bility to antibiotics, with the determination of resistance phenotypes. Until now, the occurrence of this enzyme in *Acinetobacter baumannii* strains in Poland has not been reported.

The PER-1 extended spectrum β-lactamase represents class A β-lactamases classified according to the scheme of Ambler. The enzyme is weakly related to other extended spectrum β-lactamases [7]. It conditions resistance to penicillin, cefotaxime, ceftazidime and aztreonam, and its activity is well inhibited by clavulanic acid, sulbactam and tazobactam [7, 8]. Currently, there are seven ESBL variants in the PER family [9]. PER-1 was first identified in 1993 in a *P. aeruginosa* strain isolated from a Turkish patient in France [7]. The strains have also been found in Belgium [10] and Italy [11].

Material and methods

Bacterial strains. The study material consisted of 51 *Acinetobacter baumannii* strains isolated from clinical samples obtained from patients treated in departments of the University Hospital in Bialystok. The bacteria were identified by the VITEK 2 GN card and the automatic system VITEK 2 (bioMerieux, Durham, NC, USA) according to the manufacturer's instructions. Additionally, advantage was taken of the ability of *Acinetobacter baumannii* to grow at a temperature of 44°C and of the presence of $bla_{\text{OXA-51-like}}$ carbapenemase genes that are naturally found in this species [12].

Detection of β-lactamases. The detection of extended spectrum β-lactamases was performed using a standard double-disk synergy test (DDST) [13], with disks containing ceftazidime, cefotaxime and additionally aztreonam and cefepime [14]. They were tested simultaneously on two media: (1) Mueller–Hinton agar (Oxoid Ltd, Basingstoke, UK) according to the classical double disk test [13] and (2) Mueller–Hinton agar (Oxoid) with the addition of cloxacillin (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 250 μg/ml [15]. *Klebsiella pneumoniae* ATCC 700603 strain was used as the ESBL producing control strain.

Assessment of drug-susceptibility. A broth microdilution method was used to assess susceptibility to antibiotics [16]. The minimum inhibitory concentrations (MICs) were determined within the range of 0.032–512 μg/ml for the following antibiotics: ampicillin/sulbactam (SAM), piperacillin (PIP), piperacillin/tazobactam (TZP), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP), imipenem (IMP), meropenem (MEM), tetracycline (TE), gentamicin (GM), netilmicin (NET), amikacin (AN), and ciprofloxacin (CIP). *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 35218 were used as reference strains. Following the incubation period, the MIC values of the antibiotics being studied were read for the *Acinetobacter baumannii* strains and susceptibility was interpreted according to the criteria established by the Clinical and Laboratory Standards Institute [17]. Drug-resistance phenotypes of the respective strains were determined.

Genetic methods. *Acinetobacter baumannii* genomic DNA was isolated using a Genomic Mini kit (A&A Biotechnology, Poland). Genes were detected by the PCR technique. The PCR reaction was conducted in the final volume of 25 μl assay mixture, containing 12.5 μl of 2 × PCR RED (DNA-Gdansk, Poland), 3 μl of extracted DNA, and 10 pmol of each of the starters (PERf 5'-AGTCAGCGGCTTAGATA-3'; PERr 5'-CGTATGAAAAGGACAATC-3' [18], in a Cyclone 96 (PEQLAB Biotechnology GmbH, Germany) following the procedure: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 1 min, at 57°C for 1 min, at 72°C for 1 min; 1 cycle at 72°C for 10 min [19]. PCR products were analyzed using horizontal agarose gel electrophoresis (1.5%). The resultant electrophoretic distributions — the expected amplification product of 978 bp — were assessed in ultraviolet radiation (UV) and then archived using the gel documentation system ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). *Acinetobacter baumannii* LMG 1025 strain was used in the study as a control.

Results

A total of 51 *Acinetobacter baumannii* strains were identified and examined. Most (78.0%) of these strains were obtained from patients treated in the intensive care unit. Approximately 75% of these strains were isolated from samples collected from the respiratory tract, mostly from bronchial secretion (52.9%).

The ESBL type β-lactamases were determined based on phenotypic tests. The minimum and difficult to interpret effects of inhibition zone extension around the cephalosporin disks towards the inhibitor were observed only in the test performed on Mueller–Hinton agar with cloxacillin, which inhibits chromosomal cephalosporinases that mask simultaneous occurrence of ESBL. Despite an ambiguous ESBL test pattern, approximately 20% of the strains can be treated as positive.

However, molecular biology techniques (PCR), which confirmed the presence of β-lactamase, failed to show the $bla_{\text{PER-1}}$ β-lactamase gene in any of the *Acinetobacter baumannii* strains studied.

Figure 1 presents the overall susceptibility of *Acinetobacter baumannii* strains to the antibiotics studied. Most strains were susceptible to meropenem (76.5%), cefepime (60.8%) and ampicillin combined with sulbactam (56.9%). At the same time, the lowest levels of resistance were found with meropenem and ampicillin with sulbactam (13.7%) and piperacillin combined with tazobactam (17.6%). About 50% of the strains were susceptible to netilmicin, which

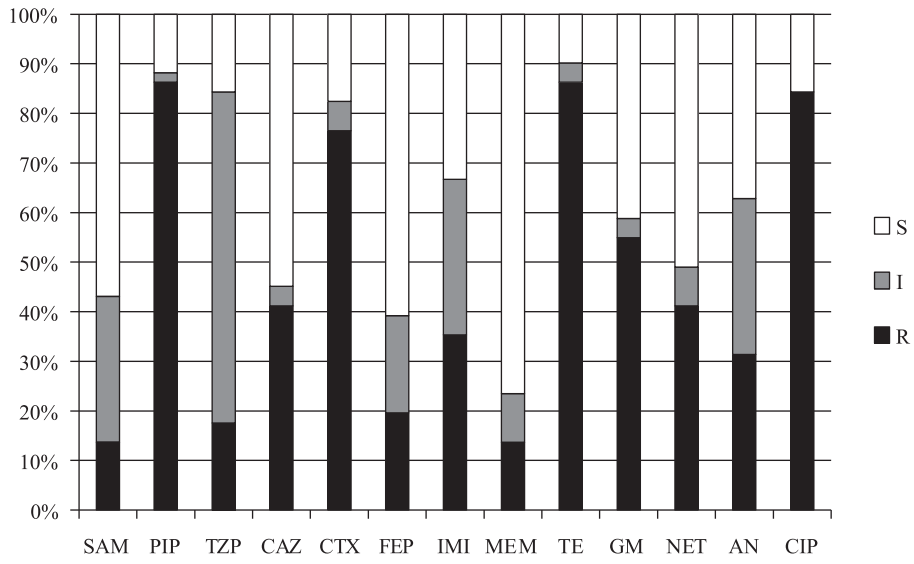

Figure 1. *In vitro* susceptibility of *Acinetobacter baumannii* strains (n = 51). SAM — ampicillin/sulbactam; PIP — piperacillin; TZP — piperacillin/tazobactam; CAZ — ceftazidime; CTX — cefotaxime; FEP — cefepime; IMI — imipenem; MEM — meropenem; TE — tetracycline; GM — gentamicin; NET — netilmicin; AN — amikacin; CIP — ciprofloxacin; R — resistant; I — intermediate; S — susceptible

was the most active antibiotic among the aminoglycosides examined. A considerable percentage of the strains showed intermediate susceptibility to piperacillin with tazobactam (66.7%). The highest level of resistance was noted to piperacillin, tetracycline, ciprofloxacin and cefotaxime.

The distribution of antibiotic MICs for *Acinetobacter baumannii* strains is presented in Table 1. Among the most active antibiotics (meropenem, cefepime, ampicillin/sulbactam), the commonest MIC values were as follows: meropenem MIC = 4 μg/ml in 41.3%, cefepime MIC = 4 μg/ml in 23.5%, and ampicillin/sulbactam MIC = 16 μg/ml in 29.4% of the strains. In the group of the least active antibiotics, the commonest MIC values were: piperacillin MIC ≥ 256 μg/ml in 62.7%, tetracycline MIC = 64 μg/ml in 21.6%, ciprofloxacin MIC = 16 μg/ml in 49.0%, and cefotaxime MIC = 64 μg/ml in 29.4% of the strains.

Based on the resistant strain category, phenotypes of resistance were determined, of which the commonest among *Acinetobacter baumannii* strains are shown in Table 2. Most strains were resistant to six or seven antibiotics (nine strains in each group), which in both groups presented various resistance phenotypes. The commonest profile of resistance was: PIP^R, CAZ^R, CTX^R, TE^R, CIP^R, which was observed in six strains. No strain was found to be resistant to all the antibiotics (13). Two *A. baumannii* isolates showed resistance to the largest number of antibiotics (11), whereas another two strains exhibited resistance only to tetracycline and amikacin.

Discussion

The phenotypic tests used to detect extended spectrum β-lactamases in 51 *Acinetobacter baumannii* strains indicate their potential occurrence in approximately 20% of isolates. When compared to literature data, this percentage seems reasonable, and the relatively high level may in most cases result from the plasmid nature of the enzymes and a selective pressure due to excessive use of antibiotics. Irrespective of the family, extended spectrum β-lactamases have been found in approximately 6.5–28% of isolates [20, 21]. Routine detection of *Acinetobacter* spp. strains producing ESBL using phenotypic methods may be difficult, because the effects of synergy between cephalosporins and clavulanic acid, typically observed in ESBL-positive rods of *Enterobacteriaceae,* can be minimal [21].

The $bla_{\text{PER-1}}$ gene is widespread in the strains of *Acinetobacter* spp., *P. aeruginosa*, *Salmonella enterica* serowar Typhimurium and *Providencia rettgeri* in Turkey [9, 22], in the region where PER-1 enzyme is present even in 32% of *Acinetobacter* spp. and 55% of *P. aeruginosa* [22]. A high prevalence of PER-1 positive *Acinetobacter* spp. strains has been observed in Korea (54.6%) [11]. Although microorganisms with PER-1 expression have been found to predominate in Turkey, outbreaks induced by *P. aeruginosa* strains have been reported from Italy [5], where PER-1 has also been detected in *P. mirabilis* and *Alcaligenes faecalis* [23, 24]. Likewise, the presence of this enzyme has also been noted in France and Spain [25, 26]. PER-1

Table 1. Minimum concentrations of antibiotic (MICs) inhibiting the growth of *Acinetobacter baumannii* strains

Antibiotic	Number of strains with a definite MIC															MIC range	MIC_{50}	MIC_{90}
	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	> 256			
SAM[a]						6	7	6	10	15	7					1–32	8	32
PIP								2	4			1		12	32	4– ≥ 256	≥ 256	≥ 256
TZP[b]								4	3	1	18	16	8	1		4–256	32	128
CAZ						4	4	3	17	2	8	13				1–64	8	64
CTX								5	4	1	2	15	8	13	3	4– ≥ 256	64	256
FEP					2	6	1	12	10	10	4	6				0.5–64	8	64
IMI			2	2	3	2	3	5	16	7	8	3				0.125–64	8	32
MEM		6	2	1	4	4		22	5	6	1					0.064–32	4	16
TE							1	4	2	6	10	11	4	6	7	2– ≥ 256	64	≥ 256
GM			4	3	6	2		6	2	3	3	13			9	0.125– ≥ 256	16	≥ 256
NET				1	6	3	3	9	4	4	12	5		1	3	0.25 – ≥ 256	8	64
AN						1	2	1	3	12	16	9	5	1	1	1– ≥ 256	32	128
CIP	3	2	2	1					7	25	6	5				0.032–64	16	32

[a] — for ampicillin/sulbactam, the value of sulbactam, which constitutes 1/2 of ampicillin, was not given; [b] — for piperacillin/tazobactam, the value of tazobactam, which constitutes 1/8 of piperacillin, was not given; MIC — minimum concentration of antibiotic inhibiting the growth of microorganisms; MIC_{50} — minimum concentration of antibiotic inhibiting the growth of 50% of strains examined; MIC_{90} — minimum concentration of antibiotic inhibiting the growth of 90% of strains examined; SAM — ampicillin/sulbactam, PIP — piperacillin, TZP — piperacillin/tazobactam, CAZ — ceftazidime, CTX — cefotaxime, FEP — cefepime, IMI — imipenem, MEM — meropenem, TE — tetracycline, GM — gentamicin, NET — netylmicin, AN — amikacin, CIP — ciprofloxacin; □ range of MIC values in the criterion of susceptible strains; ▒ range of MIC values in the criterion of intermediate susceptible strains; ■ range of MIC values in the criterion of resistant strains

Folia Histochem Cytobiol. 2012
10.5603/FHC.2012.0006

Table 2. Commonest phenotypes of resistance to antibiotics among the strains of *Acinetobacter baumannii*

Resistance to a definite number of antibiotics	Phenotypes of resistance to antibiotics												Number of strains
11	PIP^R	TZP^R	CAZ^R	CTX^R	FEP^R	IMI^R	MEM^R	TE^R	GM^R	NET^R		CIP^R	2
8	PIP^R			CTX^R		IMI^R		TE^R	GM^R	NET^R	AN^R	CIP^R	2
5	PIP^R		CAZ^R	CTX^R				TE^R				CIP^R	6
4	PIP^R			CTX^R				TE^R				CIP^R	3
3	PIP^R			CTX^R								CIP^R	2
2								TE^R			AN^R		2

PIP — piperacillin; TZP — piperacillin/tazobactam; CAZ — ceftazidime; CTX — cefotaxime; FEP — cefepime; IMI — imipenem; MEM — meropenem; TE — tetracycline; GM — gentamicin; NET — netylmicin; AN — amikacin; CIP — ciprofloxacin; ^{R} — resistant

producing *Acinetobacter baumannii* has been reported from France, Belgium, Romania, Russia and Hungary [10, 19, 27–29]. Subsequent variants have been described in the PER family in various bacteria (also in *A. baumannii*), among which PER-2 is found to predominate in some South American countries [30, 31].

Most strains of *A. baumannii* with the bla_{PER-1} gene, investigated in various countries, do not exhibit a consanguineous relationship to one another, something that is suggested by a clonal model of spread of this mechanism of resistance [9, 11]. The analysis of the bla_{PER-1} gene in various Gram-negative bacteria revealed not only (in most cases) their chromosomal, but also plasmid, location [32, 33]. These genes were identified within transposon Tn*1213* limited by two insertion sequences IS*Pa12* and IS*Pa13*, when they had chromosomal location in *P. aeruginosa*, *P. stuartii* and *A. baumannii*. Contrary to that, the PER-1 gene carried on plasmids was not part of the above-mentioned transposon — only the IS*Pa12* sequence above the bla_{PER-1} gene was identified. These plasmids have been isolated from *Salmonella enterica* serowar Typhimurium and *A. baumannii*. The relation between bla_{PER-2} genes also with the IS*Pa12* sequence seems to suggest a similar mechanism of gene mobilization on separate continents (bla_{PER-2} in South America vs. bla_{PER-1} in Europe and Asia). In both cases, the expression of the bla_{PER-1} gene was controlled by the promoter sequence located in IS*Pa12*. It is interesting that the bla_{PER-3} gene detected in the *Aeromonas punctata* strain was combined with the IS*CR1* element situated within class 1 integron In39. This finding may indicate a different mode of bla_{PER} gene spread.

In Poland, ESBLs are seldom detected in other groups of bacteria (non-fermentative bacilli), apart from the *Enterobacteriaceae* family. Initial reports on PER-1 refer to *P. aeruginosa* strains [34], which, compared to our findings, indicates a low involvement of these enzymes in resistance to β-lactams in non-fermentative bacilli. The appearance of PER β-lactamases in new species of bacteria in various regions suggests the continuous spread of resistance determinants.

Conclusions

The application of phenotypic tests used to detect ESBL showed a likely presence of this mechanism of resistance in approximately 20% of *Acinetobacter baumannii* strains. Our strains seem not to use ESBL PER-1 as a determinant of resistance to β-lactam antibiotics. The most active antibiotics against the strains examined included meropenem, cefepim and ampicillin with sulbactam.

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Submitted: 25 August, 2011
Accepted after reviews: 23 November, 2011