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Clinical importance of extended-spectrum β-lactamase (PER-1-type)-producing *Acinetobacter* spp. and *Pseudomonas aeruginosa* strains

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Recently, an extended-spectrum β-lactamase (PER-1) was found to be disseminated among *Acinetobacter* spp. and *Pseudomonas aeruginosa* isolates in Turkey. A population-based cohort study was conducted to elucidate predictive mortality factors in patients with nosocomial infections caused by *Acinetobacter* spp. and *P. aeruginosa*, with particular reference to PER-1-type extended-spectrum β-lactamase (ESBL) production. The study group comprised 16 and 21 non-survivors and 82 and 126 survivors in cohorts infected with *Acinetobacter* and *P. aeruginosa*, respectively. In the *Acinetobacter*-infected cohort, nosocomial pneumonia, hypotension and infection with a PER-positive isolate were independent predictors of mortality. In the *P. aeruginosa*-infected cohort, impaired consciousness, a PER-positive isolate, male sex and (with a negative relative risk) urinary tract infection were independent predictors of death. This study demonstrated the relationship of PER-1-type ESBL-producing *Acinetobacter* spp. and *P. aeruginosa* with poor clinical outcome.

Introduction

Acinetobacter spp. and *Pseudomonas aeruginosa* are important nosocomial pathogens with high mortality rates [1, 2]. Both have intrinsic resistance to the extended-spectrum cephalosporins, have an outer membrane with selective permeability to β-lactams [3, 4] and, by modification of outer-membrane porins, diminish permeability to other antibiotics [5]. Also, they have chromosomal β-lactamases. All of these intrinsic mechanisms cause resistance to the extended-spectrum β-lactam antibiotics [6].

Conjugative plasmid-encoded β-lactamases with extended-spectrum activity (ESBLs) have been described in *Klebsiella* and *Escherichia coli* [7], but are uncommon among *Acinetobacter* spp. and *P. aeruginosa* [8]. However, an ESBL, PER-1, was found recently to be highly prevalent in *Acinetobacter* spp. and *P. aeruginosa* isolates in Turkish hospitals [9].

Risk factors and clinical impact of intrinsically multi-resistant *Acinetobacter* spp. and *P. aeruginosa* have been studied previously [10–12]. ESBL-associated resistance among *Acinetobacter* spp. and *P. aeruginosa* is a new phenomenon and the clinical impact of this resistance genotype is unknown. A population-based cohort study was conducted and the influence of various risk factors on outcome was compared, with particular reference to PER-1-type ESBL-producing *Acinetobacter* spp. and *P. aeruginosa*.

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Materials and methods

Isolates and patient data

Ten university hospitals located in distinct geographical

regions of Turkey participated in this study. Between September 1997 and May 1998, clinical data and the isolates were collected from consecutive patients nosocomially infected with *Acinetobacter* spp. and *P. aeruginosa*. Only one isolate from each patient was included in the study. Isolates stored at −20°C in a preservation medium such as skimmed milk, and passages on agar slants were submitted together with patient data. Bacteria were re-identified in the laboratory at Kocaeli University by Sceptor System Pseudomonas Resistant MIC/ID panel (cat. no. 4480429; Becton Dickinson Diagnostic Instrument Systems, USA) and by classical methods such as oxidase test, motility, urease test (on Christensen urea agar) and oxidation of glucose. *Acinetobacter* spp. were further tested for growth at 44°C. The genus *Acinetobacter* is composed of 19 genomospecies (by DNA–DNA hybridisation). However, phenotypic tests, when coupled with the ability to grow at 44°C, could identify *A. baumannii* [13]. Therefore, isolates growing at 44°C and identified as *A. baumannii* by the phenotypic tests were referred to as *A. baumannii*.

The contributors applied the nosocomial infection definitions of the Centers for Disease Control [14] to distinguish true infection from colonisation. Colonised patients were not included in the study.

Identification of β-lactamases

Cefrazidime MICs of the isolates were determined by an agar dilution method on Mueller-Hinton Agar (Oxoid, Hemakin, Istanbul, Turkey). Isolates were screened for PER-1-type β-lactamases by membrane hybridisation. For isolates with a positive or suspected hybridisation signal, the PER-1 β-lactamase gene (bla_{PER-1}) was detected by PCR and the enzyme was demonstrated by iso-electric focusing (IEF) as described previously [9]. Extended-spectrum activity of β-lactamases was determined by an antibiotic-agar overlay method as follows. IEF gels were loaded and run in duplicate. After focusing was completed, nitrocefin solution was spread over one of the gels to stain the β-lactamase bands; the other gel was left untreated. A Mueller-Hinton agar and broth mixture of 2:1 (v:v) was prepared and was supplemented with ceftazidime 0.5 mg/L at 55°C in a water bath. The unstained gel was overlaid with this agar-antibiotic mixture to provide a covering layer of medium 3–4 mm thick (control of thickness is extremely important). After incubation at 37°C for 2 h, a suspension of *Escherichia coli* ATCC 25922 (10^8 cfu/ml) was spread over the gel-antibiotic-agar with a cotton swab and incubated at 37°C overnight. Visible growth of *E. coli* on the gel indicated the presence of a ceftazidime-hydrolysing β-lactamase.

Study design and definitions

For this prospective surveillance of nosocomial infections caused by *Acinetobacter* spp. and *P. aeruginosa*, staff members of Infectious Diseases Departments visited the patients immediately after the identification of *Acinetobacter* spp. or *P. aeruginosa* from clinical specimens and these patients were evaluated to determine the presence or absence of nosocomial infection. Patients diagnosed as having a 'true infection' were included in the study and a computer-assisted protocol was completed for each patient. Patients infected with *Acinetobacter* spp. or *P. aeruginosa* were followed prospectively until in-hospital death or discharge.

The following defined variables were recorded: time from admission (days between admission to hospital and diagnosis of the nosocomial infection); the presence of a central venous catheter (CVC) of any kind; PER-positive isolate (a nosocomial infection caused by a PER-1-type ESBL-producing *Acinetobacter* spp. or *P. aeruginosa*); hypotension (systolic pressure $\leqslant$90 mm Hg or a decrease of $\geqslant$ 40 mm Hg from baseline value); ICU acquisition (nosocomial infection acquired in an intensive care unit); impaired consciousness (levels of unconsciousness ranging from somnolence to deep coma); bacteraemia (a positive blood culture isolate of *Acinetobacter* spp. or *P. aeruginosa*); urinary tract infection; prior surgery; nosocomial pneumonia (pneumonia occurring after the first 48 h of hospitalisation including ventilator-associated pneumonia); prior antibiotic usage (any antibiotic for any reason between admission to the hospital and diagnosis of the infection); co-morbidity (including diabetes, renal failure or cancer); multiple resistance (infection with a strain resistant to three or more antibiotics of different classes, e.g., cephalosporins, carbapenems, quinolones and aminoglycosides).

Isolates were sent to a central laboratory and examined as above. Isolates that had both a positive PCR amplification for bla_{PER-1} plus an enzyme co-focusing with the PER-1 control in IEF were considered to be PER-1-type β-lactamase producers. Agar-antibiotic overlay experiments confirmed the extended-spectrum activity of these PER-1-type enzymes. PER-negative isolates with ceftazidime MICs $\geqslant$ 32 mg/L were also subjected to IEF and agar-antibiotic overlay experiments.

Statistical analysis

Statistical analyses were performed with the SPSS software (version 7.5, SPSS, Chicago, IL, USA). Categorical variables were compared by Fisher's exact test and continuous variables were compared by 'independent variables (Students) *t* test'. Relative risk values were determined in the univariate analysis. All p values and confidence intervals (CI) were two-sided. Variables with a p value of $\leqslant$0.1 were entered into a non-conditional logistic regression analysis. This multivariate analysis made it possible to evaluate the variables simultaneously and select those that were

independent predictors. Logistic regression analyses were executed separately for *Acinetobacter* spp. and *P. aeruginosa* subsets of the study group.

Results

In total, 288 patients with *Acinetobacter* spp. or *P. aeruginosa* infections were enrolled. Among these, 43 patients died due to other complications not directly attributable to a nosocomial infection; 37 deaths were directly related to nosocomial infections and 208 patients survived. Sixteen patients who died and 82 survivors were infected with *Acinetobacter* spp.; *A. baumannii* was isolated from 15 fatal cases and 76 survivors.

Univariate comparison of the variables between non-survivors and survivors is shown in Table 1. Nine variables were included in the multivariate analysis for the *Acinetobacter* subset. Among these nosocomial pneumonia (wald, 3.939; p, 0.047), hypotension (wald, 10.420; p, 0.001) and PER-positive isolate (wald, 4.983; p, 0.026) were found to be significant in multivariate analysis, while mortality due to *A. baumannii* compared with other *Acinetobacter* spp. was not significant (Table 2).

In the *P. aeruginosa* subset, nine variables were included in the multivariate analysis. Among these, impaired consciousness (wald, 10.351; p, 0.001), PER-positive isolate (wald, 9.375; p, 0.002), male sex (wald, 5.468; p, 0.019) and urinary tract infection (wald, 4.121; p, 0.042) were found to be significant. The relative risk of urinary tract infection for fatal outcome was negative. While not an unexpected result, inclusion of this variable allowed the elimination of the possible confounding effect of this prevalent and self-limiting infection.

Discussion

This study demonstrated that nosocomial infection with *Acinetobacter* spp. and *P. aeruginosa* strains that are PER-1-type ESBL producers was significantly related to an increased incidence of fatal outcome in Turkish patients. It is possible that the clonal dissemination of $bla_{\mathrm{PER-1}}$ and the high virulence of the clone carrying $bla_{\mathrm{PER-1}}$ could be responsible for this mortality. If PER-positive isolates belonged to a single clone that was highly virulent, the high mortality associated with PER production might be related to the virulence of that clone, *per se*. This argument was not directly answered in the present study, but a previous study by this group with typing by restriction fragment-length polymorphism of 16S–23S ribosomal DNA (ribotyping) showed that $bla_{\mathrm{PER-1}}$ was disseminated among different clones and species in Turkey [9]. Therefore, PER-1 producers in this study should also be expected to be diverse in clonal nature.

Table 1. Significance of the variables in this study population in univariate analysis

	Acinetobacter spp. (n = NS16/S82)			*P. aeruginosa* (n = NS21/S126)		
Variable	NS/S	RR (95% CI)	p value	NS/S	RR (95% CIs)	p value
Prior antibiotic usage	11/69	0.49 (0.19–1.24)	0.16	17/102	1.00 (0.36–2.74)	1.00
Nosocomial pneumonia	7/11	3.45 (1.48–8.04)	< 0.01	5/23	1.32 (0.53–3.31)	0.55
Co-morbidity	1/17	0.29 (0.04–2.10)	0.29	5/21	1.45 (0.58–3.61)	0.53
Male sex	10/57	0.77 (0.30–1.93)	0.57	16/70	2.27 (0.87–5.86)	0.09
Urinary tract infection	0/13	nc	0.11	1/29	0.16 (0.02–1.30)	0.07
Bacteraemia	6/11	2.85 (1.20–6.80)	0.03	5/11	2.55 (1.08–6.04)	0.05
Multiple resistance	11/43	1.79 (0.67–4.77)	0.28	11/36	2.34 (1.07–5.12)	0.04
CVC	13/44	3.11 (0.94–10.24)	0.05	17/41	6.52 (2.31–18.41)	< 0.01
Acquired in ICU	11/26	3.62 (1.36–9.61)	0.01	13/36	3.25 (1.44–7.31)	< 0.01
Hypotension	8/3	7.90 (3.72–16.79)	< 0.01	6/6	4.50 (2.14–9.43)	< 0.01
Impaired consciousness	11/9	8.58 (3.36–21.87)	< 0.01	14/13	8.88 (3.97–19.89)	< 0.01
PER-positive isolate	11/26	3.62 (1.36–9.61)	0.01	8/10	4.41 (2.12–9.14)	< 0.01
Prior surgery	3/48	0.21 (0.06–0.70)	< 0.01	9/62	0.80 (0.36–1.78)	0.64
Mean (SD) age (years)	41.0 (28.8)/43.5 (25.9)		0.72	38.4 (29.0)/34.8 (27.2)		0.58
Mean (SD) time from admission (days)	12.8 (10.0)/26.0 (28.0)		0.06	21.6 (22.5)/25.6 (34.7)		0.61

NS, non-survivors; S, survivors; RR, relative risk; 95% CIs, 95% confidence intervals (lower–upper); nc, not calculated due to the '0' cell value. Categorical variables were compared with Fisher's exact test and continuous variables were compared by Student's *t* test.

Table 2. Comparison of mortality rates between *A. baumannii* and other *Acinetobacter* species

Organism	Non-survivor	Survivor	RR (95% CIs)	p value*
A. baumannii	15	76	1.15 (0.17–7.50)	1.00
Acinetobacter spp.	1	6	…	…

*Fisher's exact test.

ESBLs are mostly derivates of the narrow-spectrum classical enzymes TEM-1, TEM-2 or SHV-1. ESBLs belonging to TEM or SHV superfamilies of resistance genes are mostly encoded and preserved on certain plasmids [15], and *Acinetobacter* spp. and *P. aeruginosa* have frequent exposure to these genes. However, these TEM- or SHV-derived genes have not been disseminated among *Acinetobacter* spp. and *P. aeruginosa*, perhaps because of intrinsic incompatibility [16].

Adaptation of exogenous resistance genes to bacteria is facilitated by the selective pressure of antibiotics. *Acinetobacter* spp. and *P. aeruginosa* are under continuous selective pressure of antibiotic exposure in hospital environments and so ESBLs should have been widely disseminated among these species. However, *Acinetobacter* spp. and *P. aeruginosa* may bear some type of intrinsic resistance mechanisms to deter gene transfer. Several possible explanations include a negative influence by exposure to the selective pressure of oxyimino-cephalosporins or other extended-spectrum β-lactams or that *Acinetobacter* spp. and *P. aeruginosa* have no need to acquire extrinsic resistance genes to survive under the pressure of oxyimino-cephalosporins. Therefore, it is extremely interesting to find an ESBL in high frequency in *Acinetobacter* spp. and *P. aeruginosa* and the significant impact of this ESBL in relationship to clinical outcome.

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