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Widespread Detection of PER-1-Type Extended-Spectrum β-Lactamases among Nosocomial *Acinetobacter* and *Pseudomonas aeruginosa* Isolates in Turkey: a Nationwide Multicenter Study

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We studied the prevalence and molecular epidemiology of PER-1-type β-lactamases among *Acinetobacter*, *Klebsiella*, and *Pseudomonas aeruginosa* strains isolated over a 3-month period in eight university hospitals from distinct regions of Turkey. A total of 72, 92, and 367 *Acinetobacter*, *Klebsiella*, and *P. aeruginosa* isolates were studied, respectively. The presence of bla_{PER} was determined by the colony hybridization method and later confirmed by isoelectric focusing. We detected PER-1-type β-lactamases in 46% (33/72) of *Acinetobacter* strains and in 11% (40/367) of *P. aeruginosa* strains but not in *Klebsiella* strains. PER-1-type enzyme producers were highly resistant to ceftazidime and gentamicin, intermediately resistant to amikacin, and susceptible or moderately susceptible to imipenem and meropenem. Among PER-1-type-β-lactamase-positive isolates, five *Acinetobacter* isolates and six *P. aeruginosa* isolates from different hospitals were selected for ribosomal DNA fingerprinting with *Eco*RI and *Sal*I. The *Eco*RI-digested DNAs were later hybridized with a digoxigenin-labelled PER-1 probe. The ribotypes and the lengths of bla_{PER}-carrying fragments were identical in four *Acinetobacter* strains. A single isolate (Ac3) harbored a PER gene on a different fragment (approximately 4.2 kbp) than the others (approximately 3.4 kbp) and showed a clearly distinguishable ribotype. Ribotypes of *P. aeruginosa* strains obtained with *Eco*RI showed three patterns. Similarly, in *Pseudomonas* strains two different *Eco*RI fragments harbored bla_{PER} (approximately 4.2 kbp in five isolates and 3.4 kbp in one isolate). PER-1-type β-lactamases appear to be restricted to Turkey. However, their clonal diversity and high prevalence indicate a high spreading potential.

The majority of the extended-spectrum β-lactamases (ESBLs) arise by mutations that alter the hydrolytic activities of classical enzymes TEM-1, TEM-2, and SHV-1 (24). They are often encoded by plasmids and readily spread among the members of the *Enterobacteriaceae* (16). Surprisingly, until now, ESBLs have not been definitely observed in *Acinetobacter* species and only a small number of reports have appeared concerning their presence in *Pseudomonas* spp. Recently, a novel non-TEM-, non-SHV-derived class A ESBL (PER-1) was discovered in *Pseudomonas aeruginosa* isolates (23) and later in *Salmonella typhimurium* (33) isolates from Turkey. Most recently, we detected PER-1-type β-lactamases among nosocomial *Acinetobacter baumannii* isolates as well (unpublished data).

Acinetobacter species and *P. aeruginosa* are among the most important causes of nosocomial infections. Because of some unique features of the outer membrane porins (26, 27) and chromosomal β-lactamases (1, 17), these glucose nonfermenters are intrinsically less susceptible to several antibiotics, as well as to the newer expanded-spectrum β-lactams. By contrast, ceftazidime, piperacillin, and ticarcillin preserve their antipseudomonal antibacterial effect (3). Because ESBLs also confer resistance to these β-lactams, dissemination of them among *Acinetobacter* and *Pseudomonas* strains attracts considerable interest.

In order to understand the prevalence and molecular epidemiology of PER-1-type-β-lactamase-producing isolates, we conducted a multicenter study of nosocomial *Acinetobacter*, *Klebsiella*, and *P. aeruginosa* strains.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. We obtained nosocomial *Acinetobacter*, *Klebsiella*, and *P. aeruginosa* isolates collected over a period of 3 months from the hospitals of eight universities located in seven geographical regions of Turkey (Fig. 1). All bacteria were reidentified in our laboratory, initially by glucose and lactose fermentation and oxidation, citrate utilization, urea hydrolysis, indole and oxidase production, and motility tests. Selected isolates were further identified by the API 20NE test (bioMérieux, Lyon, France). *Acinetobacter* strains were tested for the ability to grow at 44°C in Trypticase soy broth (Oxoid, Unipath Ltd., Basingstoke, United Kingdom).

Antibiotic susceptibility tests were performed on Mueller-Hinton (MH) agar (Oxoid). Antibiotic disks were obtained from Oxoid. Powder forms of the antibiotics were kindly supplied by the following companies: ceftazidime, Glaxo-Wellcome; ampicillin, cefoperazone, and sulbactam, Pfizer; ciprofloxacin, Bayer; imipenem, Merck Sharp and Dohme; meropenem, Zeneca; amikacin, Bristol-Myers-Squibb; gentamicin, Sigma (St. Louis, Mo.). MICs were determined by replicating approximately 10^4 CFU of bacteria per spot by the aid of a multipoint inoculator onto freshly prepared MH agar plates containing serial twofold dilutions of the related antibiotics. Agar plates were evaluated after 18 h of incubation at 37°C.

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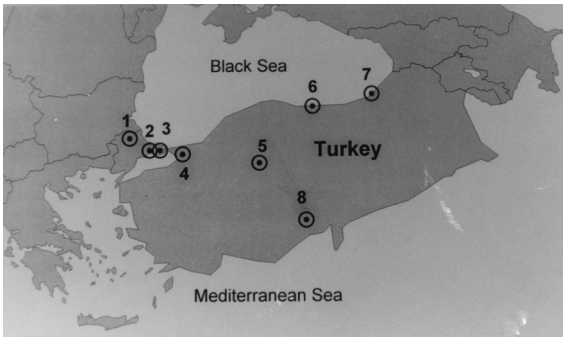


FIG. 1. Contributing centers are shown on the map of Turkey. For the locations corresponding to the numbers, see Table 1.

Colony hybridization and isoelectric focusing. For colony hybridizations, bacteria were spotted and grown overnight on positively charged nylon membranes (Boehringer GmbH, Mannheim, Germany) that were placed on MH agar plates. Later, the bacteria were lysed, denatured, and neutralized by laying these membranes on Whatman papers saturated, respectively, with 10% sodium dodecyl sulfate, denaturation solution (0.5 N NaOH, 1.5 M NaCl), and neutralization solution (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl) for 15 min each. The PER-1 probe was prepared by random labelling the 926-bp PCR product of bla_{PER-1} (33) with digoxigenin with the Digoxigenin Labelling and Detection Kit (Boehringer GmbH) as described by the manufacturer. The integron probe was a primer designed from the 3′ conserved region of the integron (5′-AAG CAG ACT TGA CCT GA-3′) (15), which was digoxigenin labelled with the 3′-End Labelling Kit according to the instructions of the manufacturer (Boehringer GmbH). All hybridizations were accomplished at 50°C. Prehybridization, hybridization, and detection experiments have been described in detail elsewhere (25).

β-Lactamases were released by freezing and thawing a dense suspension of bacteria in 0.1 M phosphate buffer (pH 7.0) 10 times. After centrifugation for 15 min at 12,000 × *g*, supernatants were subjected to an ampholine gel with a pH range of 3.5 to 10. Ampholine gels were prepared according to the formulation of Matthew et al. (18) but were supplemented with 10% sucrose. After focusing at 10 W for 90 to 120 min at 4°C on an isothermal-control electrophoresis apparatus (ISOLAB, Inc., Akron, Ohio; model CWS-2000), the enzymes were located with 1 mM nitrocefin in 0.1 M phosphate buffer (pH 7.0). Estimations of pI values were made by comparison with standards PER-1 (5.3), TEM-1 (5.4), TEM-2 (5.6), TEM-3 (6.3), and SHV-1 (7.6).

DNA isolation and ribotyping. Genomic DNAs were isolated by the aid of the lytic effect of lysozyme and guanidine thiocyanate. Bacteria were collected from overnight plate cultures (9 cm in diameter) in 1 ml of 0.1 M phosphate buffer (pH 7.2), pelleted, suspended in 200 μl of lysozyme solution (20 mg of lysozyme per ml in TE buffer [10 mM Tris-HCl, 1 mM EDTA; pH 8.0]), and incubated for 30 min on ice. The cells were then lysed by adding 4 ml of lysis solution (5 M guanidine thiocyanate, 0.1 M EDTA [pH 8.0], 0.5% *N*-laurylsarcosine) and incubating at 60°C until the suspension was completely cleared. If lysis was not accomplished in 30 min, more lysis solution was added. After 7.5 M ice-cold ammonium acetate (0.50 volume) was added and the solution was vortexed vigorously, DNAs were extracted once with chloroform-isoamyl alcohol (24:1) and once with chloroform and later precipitated with isopropanol (0.50 volume) at room temperature for 10 min. Precipitated chromosomal DNAs were collected with a micropipette and transferred to a 1.5-ml Eppendorf tube, washed once with 90% ethanol and once with 70% ethanol, air dried, and resuspended in 100 μl of TE buffer. DNAs were digested with restriction enzyme *Eco*RI or *Sal*I (Boehringer GmbH) in 25-μl volumes for 4 h at 37°C, run on a 0.7% agarose gel at 20 V for 30 h, stained with ethidium bromide, and visualized with a UV light source. Southern transfers were achieved in 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) by a capillary transfer method described elsewhere (29). For ribotyping, a digoxigenin-labelled 16S-23S rRNA of *Escherichia coli* (Boehringer GmbH) was used. Labelling and hybridization procedures have been described previously (32). After the documentation of the *Eco*RI ribosomal DNA patterns, the nylon membrane was destained in *N*,*N*-dimethylformamide at 55°C by gentle agitation. This was followed by the stripping of the ribosomal DNA probe by incubating the membrane in probe-stripping solution (0.2 N NaOH, 0.1% sodium dodecyl sulfate) at 37°C for 15 min twice. This time, *Eco*RI-digested DNAs on the same membrane were hybridized with the PER-1 probe in order to determine the fragments harboring bla_{PER-1}.

Plasmid isolation and transconjugation experiments. Three methods, namely, that of Kado and Liu (13), the alkaline-lysis method (25), and the hot-Triton method (5), were applied for plasmid DNA isolation from 3-, 10-, and 50-ml overnight broth cultures, respectively. NCTC 50192, harboring four plasmids of known length (98, 42, 23.9, and 4.6 MDa), was the control strain for plasmid isolation experiments (30).

Transconjugations were studied at 37°C on agar plates. The recipients were *E. coli* J53-1 (*pro* Nal^r) and *E. coli* J53-2 (*met pro* Rif^r) (4). Briefly, overnight broth cultures of donor and recipient bacteria were mixed (8:2 [vol/vol]) and centrifuged for 10 min at 2,000 × *g*. Supernatants were discarded, and the pellets were incubated for 2 h at 37°C. Later, pellets were spread over antibiotic-free MH agar plates and further incubated at 37°C for 18 h. We attempted to select transconjugated J53-1 on ceftazidime (16 μg/ml)–nalidixic acid (100 μg/ml)-supplemented MH agar plates and transconjugated J53-2 on ceftazidime (16 μg/ml)–rifampin (100 μg/ml)-supplemented MH agar plates.

TABLE 1. PER-1-type-β-lactamase occurrence and numbers of isolates from contributing centers[a]

Center[b]	Presence of PER-1-type enzyme in:		No. of strains			
	Ac	*Ps*	*Ac*	*Klebs*	*Ps*	Total
1	N	N	10	10	10	30
2	P	P	5	5	15	25
3	P	P	9	5	18	32
4	N	N	5	5	9	19
5	P	P	7	15	30	52
6	P	P	18	15	47	80
7	N	P	5	5	150	160
8	P	P	13	32	88	133
Total			72	92	367	531

[a] *Ac*, *Acinetobacter*; *Klebs*, *Klebsiella*; *Ps*, *Pseudomonas*; N, negative; P, positive.
[b] 1, Edirne; 2, Istanbul-Çapa Tıp; 3, Istanbul-Cerrahpasa Tıp; 4, Kocaeli; 5, Samsun; 6, Trabzon; 7, Ankara; 8, Adana.

RESULTS

A total of 531 strains isolated from nosocomial infections were submitted to our laboratory. Of these 72, 92, and 367 were identified as *Acinetobacter* spp., *Klebsiella* spp. and *P. aeruginosa*, respectively (Table 1). Ceftazidime resistance was initially determined by disk diffusion tests. All isolates were examined for the presence of bla_{PER} by colony hybridization. The isolates giving positive signals were further tested for PER-1-type-enzyme production by isoelectric focusing. Ceftazidime-resistant and hybridization-positive isolates for which enzyme cofocusing with the PER-1 control (isolate B [33]) at pI 5.3 was observed were accepted as PER-1-type-β-lactamase-positive isolates. For further studies, one negative and five PER-1-type-enzyme-positive *Acinetobacter* isolates from different hospitals were randomly selected and designated Ac1 to Ac6. Selected *Acinetobacter* strains were identified as *A. baumannii* with API 20NE, and all were able to grow at 44°C, another typical characteristic of *A. baumannii*. Similarly, one PER-1-type-β-lactamase-negative, ceftazidime-susceptible isolate, one PER-1-type-negative, ceftazidime-resistant isolate, and six PER-1-type-β-lactamase-positive *P. aeruginosa* isolates from different regions were selected and designated Ps1 to Ps8. The details of ceftazidime resistance and PER-1-type-β-lactamase prevalence are presented in Table 2, and the properties of the isolates and the MICs of various antibiotics for the isolates are presented in Table 3.

Ceftazidime resistance was found in 76% (55/72) of *Acinetobacter* isolates, in 39% (36/92) of *Klebsiella* isolates, and in 28% (104/367) of *Pseudomonas* isolates. PER-1-type β-lactamases were responsible for 60% (33/55) and 38% (40/104) of the ceftazidime-resistant *Acinetobacter* and *Pseudomonas* strains, respectively (Table 2). Besides β-lactam resistance, PER-1-type ESBL producers had a high level of resistance to gentamicin and a low level of resistance to amikacin but were susceptible or moderately susceptible to imipenem and meropenem. On the other hand, for two isolates, Ac4 and Ps3, the

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TABLE 2. Prevalence of ceftazidime resistance and PER-1-type β-lactamase

Bacterial genus or species[a]	PER-1-type-β-lactamase prevalence (no. of isolates [% of total])		
	Positive	Negative	Total
Acinetobacter			
Ctaz resistant	33 (46)	22 (30)	55 (76)
Ctaz susceptible	0	17 (24)	17 (24)
Total	33 (46)	39 (54)	72 (100)
Klebsiella			
Ctaz resistant	0	36 (39)	36 (39)
Ctaz susceptible	0	56 (61)	56 (61)
Total	0	92 (100)	92 (100)
P. aeruginosa			
Ctaz resistant	40 (11)	64 (17)	104 (28)
Ctaz susceptible	0	263 (72)	263 (72)
Total	40 (11)	327 (89)	367 (100)

[a] Ctaz, ceftazidime. Ctaz resistant, MIC ≥ 32 mg/liter; Ctaz susceptible, MIC ≤ 8 mg/liter.

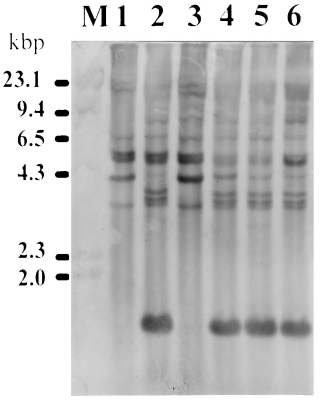


FIG. 2. Ribotypes of selected *Acinetobacter* strains obtained with the *Sal*I restriction enzyme. Lane M, *Hin*dIII-digested lambda-DNA marker; lanes 1 to 6, Ac1 to Ac6, respectively.

MICs of meropenem were more than twofold higher than those of imipenem (Table 3).

We detected PER-1-producing *Acinetobacter* strains in five hospitals and PER-1-producing *Pseudomonas* strains in six hospitals. Two patterns in the ribotypes of PER-1-type-enzyme-positive *Acinetobacter* isolates were observed. The ribotype of Ac3, obtained either with *Sal*I or *Eco*RI, was distinguishable from the rest (Fig. 2 and 3). Furthermore, the *Eco*RI fragment bearing the bla_{PER} of Ac3 (approximately 4.2 kbp) was different in length from the fragments of other *Acinetobacter* isolates (Ac2 and Ac4 to Ac6: approximately 3.4 kbp) (Fig. 4). Taken together, these data suggested that Ac3 might belong to a different clone. On the other hand, the fact that Ac2 and Ac4 to Ac6 had identical ribotypes and similar-length fragments with bla_{PER} suggested that these isolates were clonally related.

Ribotypes of PER-1-type-β-lactamase-positive *P. aeruginosa* showed three clearly distinct patterns (pattern 1, Ps3 and Ps4; pattern 2, Ps5; pattern 3, Ps6 to Ps8), indicating that at least some of these isolates belong to different clones (Fig. 3). The *Eco*RI fragments harboring bla_{PER}s of the selected *Pseudomonas* strains were similar in length (approximately 4.2 kbp), except the fragment of Ps5 (approximately 3.4 kbp) (Fig. 4).

Transconjugation and plasmid isolation experiments failed to determine a plasmid harboring bla_{PER}, and none of the isolates hybridized with the integron probe.

DISCUSSION

ESBLs spread on plasmids among the members of the *Enterobacteriaceae*. *Acinetobacter* strains are ubiquitous in the hospital environment and probably have frequent contacts with ESBL genes, but these plasmidic enzymes, except in a single, incompletely defined report (2), have not been documented in this genus. We found PER-1-type β-lactamase in *Acinetobacter* strains from five of the eight hospitals, in 46% (33 of 72) of the total isolates, in two clones, and on two different *Eco*RI fragments. *P. aeruginosa* is another species in which ESBLs have rarely been detected. TEM-42 (21), OXA-10 derivates with extended-spectrum activity (7–9), and PER-1 are the only ex-

TABLE 3. MICs for selected *A. baumannii* and *P. aeruginosa* strains

Isolate	PER-1[b] status	MIC (μg/ml) of[a]:									
		Ctaz	Am/Sulb	Cfp	Cfp/Sulb	Cip	Pip	Inem	Mnem	Ak	Gm
Ac1	Negative	2	1	<1	<1	<1	<1	<1	<1	1	0.5
Ac2	Positive	≥256	32	≥256	32	<1	16	<1	<1	32	≥256
Ac3	Positive	128	256	128	64	1	32	<1	<1	16	64
Ac4	Positive	≥256	128	≥256	32	1	256	1	8	32	128
Ac5	Positive	≥256	64	≥256	32	1	32	<1	<1	32	≥256
Ac6	Positive	≥256	64	≥256	32	>128	256	<1	1	32	≥256
Ps1	Negative	4	64	<1	<1	<1	≥256	<1	<1	1	<1
Ps2	Negative	64	32	64	64	<1	16	<1	<1	32	≥256
Ps3	Positive	≥256	≥256	≥256	64	1	64	<1	8	32	≥256
Ps4	Positive	≥256	≥256	64	64	32	32	8	8	32	128
Ps5	Positive	128	≥256	16	4	<1	4	<1	1	32	≥256
Ps6	Positive	64	128	64	32	<1	8	<1	<1	16	128
Ps7	Positive	≥256	≥256	64	64	16	32	8	16	32	≥256
Ps8	Positive	128	64	32	8	<1	32	<1	1	64	≥256
Ps-ATCC[c]	Negative	2	64	4	8	<1	2	1	1	4	1

[a] Abbreviations of antibiotics: Ak, amikacin; Am/Sulb, ampicillin-sulbactam; Cfp, cefoperazone; Cfp/Sulb, cefoperazone-sulbactam; Ctaz, ceftazidime; Cip, ciprofloxacin; Gm, gentamicin; Inem, imipenem; Mnem, meropenem; Pip, piperacillin.
[b] PER-1, PER-1-type β-lactamase.
[c] Ps-ATCC, *P. aeruginosa* ATCC 27853.

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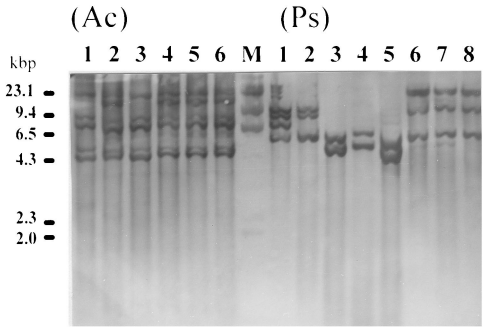


FIG. 3. Ribotypes of *Acinetobacter* (Ac) and *Pseudomonas* (Ps) strains obtained with *Eco*RI. Lanes: (Ac) 1 to 6, Ac1 to Ac6, respectively; M, *Hin*dIII-digested lambda-DNA marker; (Ps) 1 to 8, Ps1 to Ps8, respectively.

tended-spectrum enzymes reported to date in *Pseudomonas* strains. Recently, PER-1 was found in most of the *P. aeruginosa* isolates obtained from a Turkish hospital (6). However, the prevalence in Turkey and the molecular epidemiology of PER-1 producers have not been studied. Here, we found PER-1-type ESBLs in the isolates from six of the eight hospitals and in different clones of *P. aeruginosa*. More interestingly, like *Acinetobacter* isolates, these strains harbored bla_{PER} on two different *Eco*RI fragments. The results of this study show that PER-1-type β-lactamases are highly prevalent in Turkey and have already spread among different species and clones.

Resistance genes, once they appear, often disseminate rapidly all around the world. The dissemination of a particular clone is one of the proposed mechanisms (19, 20). However, the exact mode of dissemination of a clone between hospitals remained unclear. In a recent study, we showed that PER-1-producing *S. typhimurium* isolates spread between hospitals by the transfer of patients colonized by *S. typhimurium* strains (32). The clonal relationship between some of the *Acinetobacter* and *Pseudomonas* isolates of this study from different cities could be explained by a similar dissemination mode. Nevertheless, in this study we have not obtained any patient data supporting this hypothesis.

Resistance genes are encoded either on the bacterial chromosome or on a transferable genetic element, such as an integron (15), a transposon (11), or a plasmid (12). Formerly, because conjugative transfer had failed, PER-1 was proposed by Nordmann et al. to be located on the chromosome of *P. aeruginosa* RNL-1 (23). In a later study, it was successfully transferred from one *P. aeruginosa* strain to another *P. aeruginosa* strain (pU21) and was shown to be encoded on a plasmid larger than 154 kbp (6). Here, we could not demonstrate an integron by hybridization or a plasmid by transconjugation in these isolates. In those unsuccessful former experiments, as here, *E. coli* was the recipient. Hedges and Matthew showed that some plasmids confined to the genus *Pseudomonas* could not replicate and so could not be maintained in *E. coli* (10). Moreover, the sequence of the promoter of $bla_{\mathrm{PER\text{-}1}}$ was shown to be quite distinct from the usual promoter consensus sequences of *E. coli* (22), which meant that *E. coli*, even if it acquired bla_{PER}, might fail to express the enzyme. Hence, the failure of the conjugative transfer of PER-encoding genes does not necessarily indicate a chromosomal location. In other words, PER-1-type enzymes may be encoded on a plasmid incompatible with *E. coli*. However, despite the application of the recommended methods several times, we could not demonstrate a plasmid.

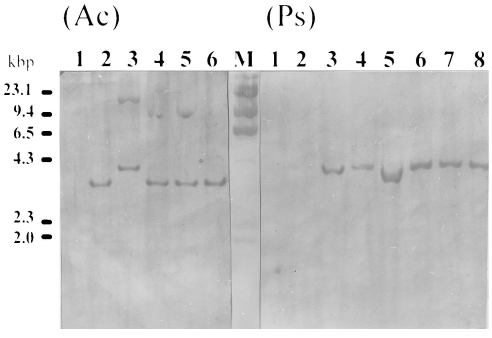


FIG. 4. Hybridization with the PER-1 probe of *Eco*RI-digested total chromosomes after destaining and probe stripping the membrane of Fig. 3. Lanes: (Ac) 1 to 6, Ac1 to Ac6, respectively; M, *Hin*dIII-digested lambda-DNA marker; (Ps) 1 to 8, Ps1 to Ps8, respectively.

Acinetobacter strains, particularly *A. baumannii*, have emerged as multiply resistant nosocomial isolates (28). Multiple resistance was not the consequence of ESBL production, and resistant *Acinetobacter* strains remained susceptible in variable degrees to some β-lactams and β-lactam–sulbactam combinations (14). Sulbactam, per se, has an antibacterial effect on *Acinetobacter* strains, and previously sulbactam–β-lactam combinations were, in vitro (1) and in vivo (31), found to be highly effective against the genus. It is noteworthy that PER-1 production not only was related to increased MICs of expanded-spectrum cephalosporins but also gave rise to ampicillin-sulbactam resistance and could increase the MICs of cefoperazone-sulbactam to a resistant level for the *Acinetobacter* isolates. Since PER-1-type-ESBL-producing *Acinetobacter* and *P. aeruginosa* strains are highly resistant to β-lactams, their emergence and dissemination deserve great attention.

In conclusion, the clonal diversity, association with different *Eco*RI fragments, and high prevalence of PER-1-type ESBLs imply that they may not long remain restricted to Turkish hospitals.

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