VEB-1-Like Extended-Spectrum β-Lactamases in *Pseudomonas aeruginosa*, Kuwait

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Two clinical *Pseudomonas aeruginosa* isolates from patients in intensive care units in Kuwait were resistant to expanded-spectrum cephalosporins and showed a synergistic effect between ceftazidime and clavulanic acid. This is the first report of extended-spectrum enzymes from nosocomial isolates from the Middle East.

Pseudomonas aeruginosa has an inducible, naturally occurring cephalosporinase that confers low-level resistance to aminopenicillins and narrow-spectrum cephalosporins such as cephalothin and cefoxitin (1). Resistance to extended-spectrum cephalosporins may arise from overexpression of this cephalosporinase, acquired beta-lactamases, or both (1). The acquired beta-lactamases may be either clavulanic-acid inhibited (mostly Ambler class A enzymes) or clavulanic-acid resistant (class B and class D enzymes) (2). The class A extended-spectrum beta-lactamases (ESBLs) may derive from narrow-spectrum beta-lactamases of TEM and SHV types, as extensively reported for *Enterobacteriaceae* and rarely for *P. aeruginosa* (2). Other class A enzymes reported in *P. aeruginosa* include PER-1, which we first identified as chromosomally located and which is widespread in *P. aeruginosa* isolates in Turkey (11% of the hospital isolates) (3,4). Lately, another class A ESBL integron-located gene, $bla_{\text{VEB-1}}$, has been identified from *P. aeruginosa* and enterobacterial isolates from Southeast Asia (5-7).

We report on two novel VEB-1-like beta-lactamases from *P. aeruginosa* clinical isolates from Kuwait. This is the first report of extended-spectrum enzymes from nosocomial isolates from this part of the world.

The Study

P. aeruginosa KU-1 was isolated in January 1999 at Ibn Sina Hospital in Kuwait from endotracheal secretions of a 1-day-old infant with respiratory tract infection, hospitalized in the intensive care unit (ICU) unit because of severe enterocolitis. He was first treated with cefotaxime, amikacin, and metronidazole. These antibiotics were discontinued, and he was given imipenem. He improved and was discharged 7 days later. As it was a single isolate of a multidrug-resistant strain, isolation precautions were carried out only in the neonatal ICU. The infant's mother could not remember whether she had received antibiotic therapy during her pregnancy (which was uneventful). She had not traveled outside Kuwait.

P. aeruginosa KU-2 was also isolated in June 1999 from the urine of a 73-year-old man admitted to the ICU of another Kuwaiti hospital, Mubarak Al-Kabeer, with ischemic chest pain and bronchectiasis. On day 2 of his hospitalization, fever developed, but blood and urine cultures were negative. He was treated with ceftazidime for 12 days beginning on day 2. During his hospital stay, hematuria developed, followed by urine retention. On day 28, pus from a urinary catheter infection after transurethral prostatitic surgery grew *P. aeruginosa* KU-2 that was susceptible to norfloxacin. *P. aeruginosa* KU-2 was the only *P. aeruginosa* strain isolated from this patient's clinical specimens. He was treated with norfloxacin. Repeated urine cultures did not yield any organism, and he was discharged 15 days later. He had no history of travel outside Kuwait.

Strains from both patients were identified by using an API-20 NE system (Biomerieux, Marcy-l'Etoile, France). Preliminary antibiotic susceptibility testing by disc diffusion (5) revealed a slight synergy between ceftazidime- and clavulanic acid-containing discs for two clinical isolates, *P. aeruginosa* KU-1 and KU-2. Susceptibility testing of beta-lactams for *P. aeruginosa* KU-1 and KU-2 was then performed by a Mueller-Hinton agar dilution method (8). Both strains showed decreased susceptibility to all beta-lactams except imipenem and piperacillin/tazobactam (Table). MICs of

Table. MICs of beta-lactams for *Pseudomonas aeruginosa* KU-1 and KU-2 clinical isolates, *P. aeruginosa* PU21(pROT-1), and PU21 reference strain

	MICs (mg/L)			
Antibiotic[a]	*P. aeruginosa* KU-1	*P. aeruginosa* KU-2	*P. aeruginosa* PU21 (pROT-1)	*P. aeruginosa* PU21
Amoxicillin	>512	>512	>512	64
Ticarcillin	>512	512	512	16
Ticarcillin+CLA	64	16	16	16
Piperacillin	64	16	16	2
Piperacillin+TZB	32	8	8	1
Ceftazidime	512	512	512	0.25
Ceftazidime+CLA	16	8	8	0.25
Ceftazidime+TZB	8	4	8	0.50
Cefotaxime	512	128	128	4
Cefotaxime+CLA	64	16	16	2
Imipenem	2	2	2	0.12
Aztreonam	>512	>512	512	0.5
Aztreonam+CLA	64	8	8	0.5

[a]CLA: clavulanic acid at a fixed concentration of 2 mg/L; TZB: tazobactam at a fixed concentration of 4 mg/L.

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ceftazidime were decreased by addition of clavulanic acid and tazobactam, indicating the presence of a clavulanic-acid inhibited ESBL (Table). According to antibiotic susceptibility testing by Mueller-Hinton agar disc diffusion, *P. aeruginosa* KU-1 and KU-2 were also resistant to aminoglycosides (amikacin, gentamicin, netilmicin, tobramycin), chloramphenicol, tetracyclines, sulfonamides, and fluoroquinolones (except for KU-2).

Beta-lactamase extracts from cultures of *P. aeruginosa* KU-1 and KU-2 were obtained (8). Isoelectric focusing analysis (8) revealed beta-lactamases with isoelectric points of 7.4 and 8-8.4, the latter likely corresponding to *P. aeruginosa* AmpC cephalosporinases. Whole-cell DNAs of *P. aeruginosa* KU-1 and KU-2 were then obtained (3). Preliminary polymerase chain reaction (PCR) experiments were performed with DNAs of *P. aeruginosa* KU-1 and KU-2 as templates and primers specific for the following class A beta-lactamases: TEM, SHV, CARB (PSE-1), GES-1, PER-1, and VEB-1 (3,5,9-11). Only PCR using internal primers for $bla_{\text{VEB-1}}$ gave a positive result with an identical 642-bp fragment. External $bla_{\text{VEB-1}}$ specific primers gave 1,070-bp PCR fragments with DNAs of both *P. aeruginosa* strains as templates that were sequenced on both strands (6). The deduced amino acid sequences, obtained over the internet (8), identified VEB-1-like sequences that shared 99% amino acid identity with VEB-1 (Figure). Compared with VEB-1, the amino acid changes in VEB-1a and VEB-1b from *P. aeruginosa* KU-1 and KU-2, respectively, occurred in the putative leader peptide sequence (Figure). Thus, the hydrolytic activity of VEB-1a and VEB-1b should be identical to that reported for VEB-1 beta-lactamase (5).

The genetic background of $bla_{\text{VEB-1a}}$ and $bla_{\text{VEB-1b}}$ was further characterized. Plasmid extraction, conjugation, and electroporation experiments were performed (8). A plasmid (pROT-1) of ca. 70 kb carrying $\text{bla}_{\text{VEB-1a}}$ gene was identified according to hybridization results by using an internal PCR-obtained probe for $bla_{\text{VEB-1}}$ (5). Plasmid pROT-1 was self-conjugative from *P. aeruginosa* KU-1 to in vitro obtained rifampin-resistant *P. aeruginosa* PU21 reference strain after selection of transconjugants onto Mueller-Hinton agar plates each containing 150 µg/mL rifampin and 200 µg/mL ticarcillin. As assessed by antibiotic susceptibility testing by disc diffusion, plasmid pROT-1 conferred additional resistance to gentamicin, netilmicin, sulfonamides, and tobramycin. MICs of beta-lactams for *P. aeruginosa* PU21 (pROT-1) mirrored those obtained for *P. aeruginosa* KU-1 (Table). While the plasmid location of $bla_{\text{VEB-1}}$ gene is known only in Enterobacteriaceae (5-7), its report in *P. aeruginosa* may signal the evolution of its spread. The $bla_{\text{VEB-1b}}$ gene was not plasmid located, but a PCR-obtained 642-bp internal probe for $bla_{\text{VEB-1}}$ hybridized at chromosomal position of whole-cell DNA of *P. aeruginosa* KU-2.

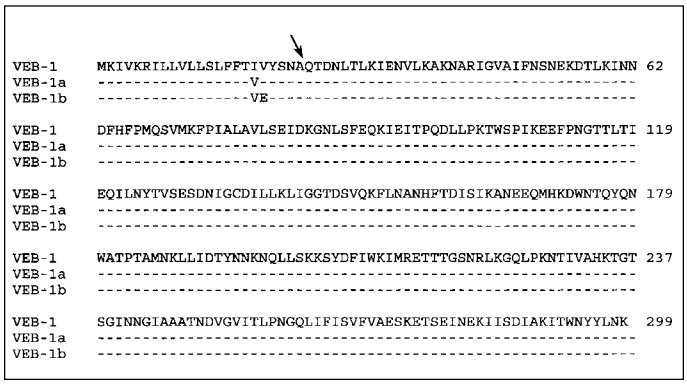

Figure. Comparison of the amino acid sequence of VEB-1, VEB-1a, and VEB-1b beta-lactamases from *Pseudomonas aeruginosa* JES from Thailand and KU-1 and KU-2 from Kuwait. Dashed lines indicate identical amino acids. Arrow indicates position of the putative cleavage site of the peptide leader. Numbering is according to Ambler designation (12).

The $bla_{\text{VEB-1}}$ gene is located on different structures of class 1 integrons (5-7). Integrons comprise two conserved regions (5'-CS and 3'-CS) flanking an internal variable region usually containing several gene cassettes (13). Integrons are in fact expression vectors for antibiotic resistance genes that are included as gene cassettes and are neighbored (13). By using primers located either in the 5'-CS sequence and the 5' end of $bla_{\text{VEB-1}}$ or in the 3' end of $bla_{\text{VEB-1}}$ and the 3'-CS sequence (5,14), PCR amplification experiments were performed with whole-cell DNAs of *P. aeruginosa* KU-1 and KU-2 as templates. In one case (strain KU-1), a PCR fragment was obtained by using $bla_{\text{VEB-1}}$ and 5'-CS primers, indicating that $bla_{\text{VEB-1a}}$ was located downstream of a class 1 integrase gene. In this case, the 4-kb PCR fragment differed from those of known $bla_{\text{VEB-1}}$ containing integrons identified in *Escherichia coli* and *P. aeruginosa* isolates (5-7). Amplimers of 1 kb were obtained for both strains using $bla_{\text{VEB-1}}$ and 3'-CS primers, showing that the 3'-CS end was present in both cases and that the $bla_{\text{VEB-1}}$-like sequences were located next to the 3'-CS end within class 1 integrons. The *attC (59-be)* recombination sites (15) located downstream of gene cassettes were identical for $bla_{\text{VEB-1a}}$ and $bla_{\text{VEB-1b}}$ to those described for $bla_{\text{VEB-1}}$ in *P. aeruginosa* and enterobacterial isolates identified so far from Southeast Asia (5-7). Therefore, an identical $\text{bla}_{\text{VEB-1}}$-like gene cassette may be located on different class 1 integrons. Using 5'-CS and 3'-CS primers, two additional PCR fragments were obtained for each *P. aeruginosa* strain, showing that both strains contained another bla_{VEB}-negative class 1 integron. For *P. aeruginosa* KU-1, a 950-bp PCR fragment for an *aadA1a* gene coding for an aminoglycoside modifying enzyme was found to be plasmid- and integron-located in *Salmonella enterica* serotype Typhimurium (16). For *P. aeruginosa* KU-2, a 500-bp PCR fragment encoding a putative 95 amino acid protein of unknown function was PCR amplified. It shared 71% amino acid identity with an amino acid sequence from a gene that was Tn*1696* transposon-located and In4 integron-located in *P. aeruginosa* (17).

Finally, *P. aeruginosa* KU-1 and KU-2 isolates containing VEB-1-like beta-lactamases were compared with VEB-1 positive *P. aerugionosa* strain JES from Thailand by using random amplified polymorphic DNA technique (10,18). The isolates were not clonally related (data not shown). Although the patients had not traveled outside Kuwait, introduction of *P. aeruginosa* into Kuwaiti hospitals by travelers or patients from Southeast Asia cannot be ruled out.

Conclusions

The presence of clavulanic-acid inhibited ESBLs in *P. aeruginosa* isolates may account for part of the 50% resistance to ceftazidime of *P. aeruginosa* strains isolates from ICUs in Kuwait (19). ESBLs in *P. aeruginosa* in Kuwait

and other Middle Eastern hospitals may be underestimated because routine detection with a double disc synergy test may be difficult. Identification of ESBLs is of interest since they confer resistance to all extended-spectrum cephalosporins and aztreonam, whatever their MICs. This has been confirmed by experimental data using a model of pneumonia in rats with the Ambler class A ESBL, PER-1 (20).

This work underscores that very similar ESBLs may be identified in different parts of the world. It is the first report of ESBL genes characterized from *P. aeruginosa* isolates from the Middle East.

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