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Molecular Epidemiology of the Integron-Located VEB-1 Extended-Spectrum β-Lactamase in Nosocomial Enterobacterial Isolates in Bangkok, Thailand

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Over a 2½-month period in 1999, 37 ceftazidime-resistant nonrepetitive enterobacterial isolates were collected from 37 patients in a Bangkok hospital, Thailand. Eighty-one percent of these strains expressed a clavulanic acid-inhibited extended-cephalosporin resistance profile. An identical extended-spectrum β-lactamase (ESBL), VEB-1, was found in 16 unrelated enterobacterial isolates (*Escherichia coli*, $n = 10$; *Enterobacter cloacae*, $n = 2$; *Enterobacter sakazakii*, $n = 1$; and *Klebsiella pneumoniae*, $n = 3$) and in two clonally related *E. cloacae* isolates. The bla_{VEB-1} gene was located on mostly self-conjugative plasmids (ca. 24 to 200 kb) that conferred additional non-β-lactam antibiotic resistance patterns. Additionally, the bla_{VEB-1} gene cassette was part of class 1 integrons varying in size and structure. The bla_{VEB-1}-containing integrons were mostly associated with bla_{OXA-10}-like and *arr-2*-like gene cassettes, the latter conferring resistance to rifampin. These data indicated the spread of bla_{VEB-1} in Bangkok due to frequent transfer of different plasmids and class 1 integrons and rarely to clonally related strains. Plasmid- and integron-mediated resistance to rifampin was also found in enterobacterial isolates.

Plasmid-mediated extended-spectrum β-lactamases (ESBLs) were first identified in a *Klebsiella pneumoniae* isolate in Germany in 1983 (15). Since then, the infections caused by ESBL-producing members of the family *Enterobacteriaceae* have rapidly increased (20). These enzymes confer variable degrees of protection against expanded-spectrum cephalosporins such as cefotaxime, ceftazidime, and the monobactam aztreonam. Their activity is inhibited by clavulanic acid in vitro (13, 15). Most of the ESBLs that are disseminated worldwide are derivatives of narrow-spectrum TEM- and SHV-type β-lactamases, with one or more amino acid substitutions surrounding their active site, thus explaining the extension of their hydrolytic profile (20). In addition to these ESBLs, non-TEM, non-SHV derivatives with weak structural relationships have been detected with specific geographical distributions, such as CTX-M derivatives in Europe and South America, TOHO-1 and TOHO-2 in Japan, PER-1 in Turkey, and PER-2 in South America (2, 3, 7, 8, 11, 19, 25, 40, 41).

Strains expressing the ESBL VEB-1 (also named CEF-1) have rarely been identified, i.e., one *Escherichia coli* and one *K. pneumoniae* isolate from the same Vietnamese patient and two *Pseudomonas aeruginosa* isolates from two patients hospitalized in Thailand (23, 30, 38). Genetic analysis of bla_{VEB-1} revealed either its chromosome or its plasmid location and always its integration within class 1 integrons of variable structure. Integrons contain a site-specific recombination system able to capture and express genes as gene cassettes (4, 9). The essential components of class 1 integrons are the 5′ conserved segment (5′-CS) that includes an integrase gene, *intI,* which encodes a site-specific recombinase, an adjacent site, *attI,* that is recognized by the integrase and acts as a receptor for gene cassettes, and a common promoter region(s), P_{ant} (P_1) and/or P_2, from which integrated gene cassettes are expressed (5, 32). The 3′ conserved segment (3′-CS), located downstream of the integrated gene cassettes, usually contains a combination of the three genes *qacE1* (antiseptic resistance), *sulI* (resistance to sulfonamides), and an open reading frame (*orf5*) of unknown function (27). Each gene cassette is associated with a site-specific recombination site designated the 59-base element (59-be) and located downstream of the gene. Among the cassette-integrated β-lactamase genes, most of them encode β-lactamases of Ambler class D (oxacillin-hydrolyzing β-lactamases) and only rarely of class B (such as IMP-1, VIM-1, and VIM-2 carbapenem-hydrolyzing β-lactamases) or of class A (carbenicillin-hydrolyzing β-lactamases) (10, 22, 31, 32). The bla_{VEB-1} and bla_{GES-1} gene cassettes are the only class A ESBL gene cassettes so far known as part of class 1 integrons (29, 30).

The aim of the present study was to evaluate the prevalence of nosocomial *Enterobacteriaceae* isolates that produced VEB-1 among nonrepetitive ceftazidime-resistant *Enterobacteriaceae* isolates over a 2½-month period in 1999 from patients hospitalized in a hospital in Bangkok, Thailand. To determine whether the enterobacterial isolates were epidemiologically related, they were compared for their β-lactamase content, their plasmid profile, and their genotype using arbitrary primer

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TABLE 1. Unpublished PCR primers used in this study

Gene	Primer[a]	Sequence 5′ to 3′	Accession no.[b]	Position of primer
bla_{VEB-1}	VEB-INV4F	ACGAAGAACAAATGCACAAGG	AF010416	660–681
bla_{VEB-1}	VEB-INV3B	GAACAGAATCAGTTCCTCCG	AF010416	610–591
bla_{OXA-10}	OXA-10promB	CGTTAATTCGACCTCAGAGGC	AF205943	7491–7471
bla_{OXA-10}	OXA-10casF	TTAGGCCTCGCCGAAGCG	AF205943	7331–7348
bla_{OXA-10}	OXA-10casB	CTTTGTTTTAGCCACCAATGATG	AF205943	8297–8319

[a] F, forward nucleotide sequence; B, backward nucleotide sequence.
[b] Accession numbers are from the published sequences in the GenBank and EMBL databases.

PCR analyses. Conjugation experiments (and/or electrotransformation) were performed to analyze the self-conjugative property of the plasmids. Finally, class 1 integrons were searched and analyzed in the VEB-1-positive isolates. This is, to our knowledge, the first work designed to study the distribution of an Ambler class A ESBL in this part of the world and the first study on the molecular epidemiology of an integron-located class A ESBL gene.

MATERIALS AND METHODS

Bacterial isolates. Thirty-seven nonrepetitive ceftazidime-resistant enterobacterial isolates were consecutively collected in the bacteriology laboratory in the Department for Microbiology at Siriraj Hospital, Bangkok (the biggest hospital facility in Thailand) from June to August 1999. The total number of enterobacterial isolates in the studied period was as follows: *E. coli* ($n = 266$), *K. pneumoniae* ($n = 62$), and *Enterobacter* sp. ($n = 46$). Isolates were identified by using the API20E system (bioMérieux SA, Marcy-l'Etoile, France). Electrocompetent *E. coli* DH10B (GIBCO BRL, Life Technologies, Cergy Pontoise, France) was used as a recipient strain in transformation experiments. Nalidixic acid-resistant *E. coli* JM109 was used as host in conjugation experiments (30). *E. coli* NCTC 50192, harboring 154-, 66-, 38-, and 7-kb plasmids, was used as a plasmid-containing reference strain (30). *E. coli* DH10B harboring recombinant plasmid pRLT1 that carries the bla_{VEB-1} gene was used as a VEB-1-producing reference strain, as described previously (30).

Susceptibility testing and screening for ESBL-producing strains. The antibiotic susceptibility of *Enterobacteriaceae* clinical isolates and their *E. coli* transformants was first determined by the disk diffusion method on Mueller-Hinton (MH) agar plates with β-lactam and non-β-lactam antibiotic-containing disks (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), according to the guidelines of the antibiogram committee of the French Society for Microbiology (30). Since in some cases transconjugants were obtained, plasmid DNAs of the transconjugants were electroporated into *E. coli* DH10B in order to make valid comparisons of the MICs for all transformants in an identical *E. coli* genetic background. The double-disk synergy test was performed with cefotaxime, ceftazidime, aztreonam, and amoxicillin-clavulanic acid disks on MH agar plates, and the results were interpreted as described previously (13).

MICs were determined for selected β-lactams by an agar dilution technique on MH agar with an inoculum of 10^4 CFU per spot, as described previously (28). MICs of some β-lactams were determined alone or in combination with a fixed concentration of either clavulanic acid (2 μg/ml) or tazobactam (4 μg/ml). MIC results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (24).

Conjugation, electroporation, and plasmid DNA content analysis. Conjugation experiments were performed between clinical isolates and *E. coli* JM109 in solid and liquid media at 37°C as reported previously (28). Transconjugants were selected on Trypticase soy (TS) agar plates containing 100 μg of nalidixic acid per ml and 2 μg of ceftazidime per ml. Non-self-conjugative plasmid DNAs of the enterobacterial isolates and plasmid DNAs of the transconjugants were extracted as described previously (28). They were electroporated into *E. coli* DH10B, and recombinant strains were selected on ceftazidime-containing (2 μg/ml) TS agar plates. Plasmid DNAs of these transformants were analyzed as described previously (28).

IEF analysis. Cultures of the *E. coli* electroporants were grown overnight at 37°C in 10 ml of TS broth containing 2 μg of ceftazidime per ml. Then, 1 ml of each overnight culture was further grown for 3 h at 37°C in 10 ml of TS broth with ceftazidime. Analytical isoelectric focusing (IEF) was performed using an ampholine polyacrylamide gel as described previously (28).

PCR amplification for detection of ESBL genes, analysis of class 1 integrons, and sequencing. Under standard PCR conditions (33), a series of primers was designed for detection of Ambler class A β-lactamase genes. Detection was performed for genes encoding TEM (TEM-A and TEM-B [29, 35]), SHV (SHV-F and SHV-B [21]), PER-1/PER-2 (PER-A and PER-B [25, 29]), VEB-1 (VEB-1A and VEB-1B [23]), CTX-M (CTXM-2A and CTXM2B [8]), TOHO-1/TOHO-2 (TOHO-A and TOHO-B [11, 29]), and GES-1 (GES-1A and GES-1B [29]) (Table 1). For each reaction, 0.5 μg of whole-cell DNA of the ESBL-possessing *Enterobacteriaceae* isolates or 0.5 μg of plasmid DNA from *E. coli* DH10B electroporants was used. Primers for the detection of class 1 integrons were located in the 5′-CS and in the 3′-CS regions (primers 5′-CS and 3′-CS [16]). A combination of 5′-CS or 3′-CS and bla_{VEB-1} primers was also used for the determination of the genetic content of the class 1 integrons (primers 5′-CS and VEB-B, or 3′-CS and VEB-F [16, 23]). Additionally, since *oxa-10* and *arr-2* genes had been found to be associated with the bla_{VEB-1} gene (23, 38), their detections by PCR amplification were performed (primers OXA-10casF and -casB [Table 1] and ARR-2-F and ARR-2-B [37]). Their position relative to the bla_{VEB-1} gene was determined by PCR using primers for bla_{VEB-1} (primers VEB-1A and VEB-1B [23] and VEB-INV4F and VEB-INFV3B [Table 1]), for bla_{OXA-10} (primers OXA-10promB [Table 1] and OPR-1 and OPR-2 [42]), and for *arr-2* (primers ARR-2-F and ARR-2-B).

For direct DNA sequencing, PCR products using external primers for the bla_{VEB-1} gene cassette (primers VEBcas-F and VEBcas-B [23]) were purified with PCR purification columns (Qiagen). Sequencing reactions were performed using the same bla_{VEB-1} specific primers and an automated sequencer (ABI 377; Applied Biosystems, Foster City, Calif.). The nucleotide and deduced protein sequences were analyzed with software available over the Internet (29).

RAPD fingerprinting. Random amplified polymorphism DNA (RAPD) analysis was performed and interpreted as described by Williams et al. (43) with some modifications as reported previously (28). The RAPD primers were ERIC-2 and 628 (28) and AP1 and AP4 (1).

Hybridizations. DNA-DNA hybridizations were performed as described by Sambrook et al. with a Southern transfer of an agarose gel containing plasmid DNA from *E. coli* DH10B electroporants as a template (33). The probe consisted of a 650-bp PCR fragment generated from recombinant plasmid pRLT-1 and internal to bla_{VEB-1} (30). Labeling of the probe and signal detection were carried out using a nonradioactive labeling and detection kit according to the manufacturer's instructions (Amersham Pharmacia Biotech).

RESULTS

Epidemiology and preliminary PCR detection of β-lactamase genes. A total of 37 nonrepetitive ceftazidime-resistant enterobacterial isolates were collected from hospitalized patients in a 2,000-bed hospital in Bangkok from June to August 1999. Ceftazidime resistance accounted for 4.5% (12 of 266) of *E. coli*, 41% (19 of 46) of *Enterobacter* sp., and 9.6% (6 of 62) of *K. pneumoniae* isolates. Preliminary antibiotic susceptibility testing by disk diffusion showed synergy between ceftazidime- and clavulanate-containing disks for 30 of these 37 isolates. The seven isolates that did not show synergy image were *Enterobacter cloacae*. They likely had a derepressed expression of their cephalosporinase since the diameter around the ceftazidime disk increased when antibiograms were performed on oxacillin-containing MH plates (200 μg/ml) (data not shown).

Most of the synergy-positive isolates were not *K. pneumoniae*

TABLE 2. Clinical features of the ceftazidime-resistant bla_{VEB-1}-positive enterobacterial isolates[a]

Isolate	Date of isolation[b]	Dates of hospitalization[b]	Hospitalization unit	Source	Underlying disease	Treatment	Clinical outcome
E. coli 1	06-15-99	07-09-95 to 10-01-00	Medicine	Urine	UTI	None	Improved
E. coli 2	06-16-99	05-21-99 to 06-25-99	Medicine	Urine	Diabetes mellitus, septicemia with UTI	Imipenem	Dead
E. coli 3	07-09-99	05-09-99 to 08-10-99	Medicine	Urine	Septicemia with UTI	Sulbactam-cefoperazone	Cured
E. coli 4	07-18-99	06-16-99 to 07-24-99	Medicine	Urine	Diabetes mellitus, pneumonia, UTI	Amoxicillin-clavulanic acid	Cured
E. coli 5	07-20-99	06-26-99 to 08-03-99	Medicine	Urine	Psychiatric disorder, UTI	None	Cured
E. coli 6	07-23-99	07-22-99 to 07-26-99	Pediatrics	Urine	Neuroblastoma, UTI	None	Cured
E. coli 7	08-03-99	08-01-99 to 08-07-99	Surgery	Urine	Chronic aortic dissection, UTI	None	Cured
E. coli 8	08-07-99	05-26-99 to 09-17-99	Medicine	Urine	Diabetes mellitus, UTI	Ceftazidime	Cured
E. coli 9	08-09-99	07-30-99 to 08-06-99	Pediatrics	Urine	UTI	Ceftazidime	Cured
E. coli 10	08-26-99	07-08-99 to 09-15-99	Pediatrics	Pus	Scalp abscess	Gentamicin	Cured
E. cloacae 11	06-26-99	06-21-99 to 06-28-99	Surgery ICU	Pus	Septicemia, congenital diaphragmatic hernia	Imipenem	Dead
E. cloacae 12	07-13-99	06-28-99 to 08-19-99	Surgery	Pus	Neonatal intestinal obstruction	Cefotaxime-netilmicin	Cured
E. cloacae 13	07-31-99	06-17-99 to 10-03-99	Surgery	Urine	UTI	Unknown	Unknown
E. cloacae 14	08-03-99	07-24-99 to 11-07-99	Medicine ICU	Urine	Pneumonia, UTI	Imipenem, netilmicin	Cured
E. sakazakii 15	08-16-99	08-02-99 to 10-07-99	Medicine	Pus	Triple vessel disease, diabetes mellitus	Ceftazidime, cefoperazone-sulbactam	Cured
K. pneumoniae 16	08-03-99	07-08-99 to 09-15-99	Neurology ICU	Urine	Cerebral tumor, pneumonia, UTI	Imipenem	Dead
K. pneumoniae 17	08-17-99	06-16-99 to 08-28-99	Surgery	Bile	Infected cholangiocarcinoma	Unknown	Dead
K. pneumoniae 18	08-20-99	08-06-99 to 09-16-99	Surgery	Urine	Coronary artery disease, UTI	Ofloxacin	Cured

[a] ICU, intensive care unit; UTI, urinary tract infection.
[b] Dates are given as month-day-year.

but *Enterobacter* sp. and *E. coli* (24 of 30 isolates). PCR experiments using primers specific for bla_{VEB-1}, bla_{TEM}, bla_{SHV}, bla_{GES-1}, bla_{PER-1}, and $bla_{CTX-M-1}$ gave bla_{VEB-1}-positive results for 18 of these 30 strains (Table 2). Among the 12 bla_{VEB-1}-negative strains, all *E. cloacae* isolates ($n = 6$) were bla_{SHV} positive and 4 of these strains were also bla_{TEM} positive, the *Enterobacter sakazakii* isolates were bla_{SHV} positive and one of them was additionally bla_{TEM} positive, the *E. coli* isolate was bla_{TEM} and bla_{SHV} positive, and the naturally bla_{SHV}-positive *K. pneumoniae* strain was also bla_{TEM} positive (data not shown). Thus, the 12 bla_{VEB-1}-negative isolates that showed a positive synergy test possessed either a bla_{TEM}- and/or a bla_{SHV}-derivative gene that may explain their ESBL phenotype.

The study was then focused on the 18 bla_{VEB-1} PCR-positive isolates. Random PCR showed that these isolates were not clonally related within a given enterobacterial species except *E. cloacae* 11 and 12 from two patients hospitalized in the same surgery department at different times (Table 2).

MICs of β-lactam antibiotics and IEF analysis. In all cases, the MICs of ceftazidime and aztreonam were higher than those of cefotaxime, which is consistent with the presence of VEB-1 β-lactamase (Table 3) (30). The addition of clavulanic acid consistently decreased the MICs of ceftazidime, cefotaxime, and aztreonam (Table 3). The MICs of β-lactams for transformants were very similar to those found for clinical isolates (Table 3). IEF analysis showed that the transformants expressed a β-lactamase pI value of 7.4 that corresponded to VEB-1 in all cases (Table 4). An additional β-lactamase with a pI value of 6.3 was found in all cases except for three transformants (*E. cloacae* strains 11, 12, and 14) (Table 4). In one case (transformant *E. cloacae* 14) a β-lactamase with a pI value of 6.5 instead of 6.3 was found. Additionally, a β-lactamase with a pI value of 5.4 that may correspond to a TEM derivative was found in four transformants (*E. coli* 1, *E. cloacae* 13, *E. cloacae* 15, and *K. pneumoniae* 18), all of them being bla_{TEM} PCR positive (Table 4).

Transfer of resistance and plasmid analysis. The transmissibility of the ceftazidime resistance marker was tested by conjugation. The transfer of resistance was obtained for 8 of 18 strains at a high frequency (10^{-3} to 10^{-4}). For the remaining 10 strains, transfer of the ceftazidime resistance marker was obtained by electroporation using plasmid extracts from the clinical isolates. Plasmid DNAs of the transconjugants were extracted and retransformed into *E. coli* DH10B in order to make a valid comparison between transconjugants and transformants. All transformants were checked to be bla_{VEB-1} positive by hybridization (data not shown). The plasmid sizes of the transformants ranged from ca. 24 to 210 kb (Table 4). A correlation was established between plasmids of similar size and non-β-lactam antibiotic resistance markers of the transformants (Table 4). This was the case for transformants of *E. coli* strains 2, 3, 4, 6, 7, and 9 on the one hand and transformants of *E. coli* 1 and *K. pneumoniae* 18 on the other hand. It is likely that, in some cases, plasmid transfer may explain interspecies transfer of bla_{VEB-1}. Additionally, *E. cloacae* 11 and 12 were clonally related and harbored identically sized plasmid conferring identical non-β-lactam antibiotic resistance phenotypes in their transformants (Table 4).

Identification of bla_{VEB-1} and integron structure determination. From each of the 18 bla_{VEB-1}-positive strains, cassette-located external primers for the bla_{VEB-1} gene were used to PCR amplify and sequence the entire bla_{VEB-1} genes. An identical bla_{VEB-1} gene was identified in all cases, showing the widespread distribution of this ESBL gene and underlining the genetic stability of this coding sequence and its cassette (data not shown). Since the bla_{VEB-1} gene cassette had been reported as integron located, PCR amplifications were performed to identify its precise location. Using 5′-CS, 3′-CS, and bla_{VEB-1} primers, PCR fragments of various sizes were ob-

TABLE 3. MICs of β-lactams for bla_{VEB-1}-positive clinical isolates, their transformants in *E. coli* DH10B, and the *E. coli* DH10B reference strain

Isolate	MIC (μg/ml)[a]									
	AMX	PIP	TZB	CEF	FOX	CAZ	CAZ-CLA	CTX	CTX-CLA	AZT
E. coli DH10B	2	2	1	4	8	0.12	0.12	0.06	0.06	0.12
E. coli 1	>512	256	4	128	8	128	0.25	8	0.12	128
Transformant	>512	128	8	64	4	128	0.25	4	0.06	64
E. coli 2	>512	64	4	256	32	128	0.25	16	0.25	256
Transformant	512	32	4	64	8	256	0.25	2	0.06	64
E. coli 3	>512	128	4	256	8	128	0.25	8	0.06	128
Transformant	512	16	4	32	8	128	0.25	2	0.06	64
E. coli 4	>512	64	2	64	4	128	0.12	2	0.06	64
Transformant	512	16	4	64	8	256	0.25	4	0.06	64
E. coli 5	>512	64	4	64	4	128	0.12	2	0.06	64
Transformant	512	16	4	32	4	128	0.25	2	0.06	32
E. coli 6	>512	64	2	128	4	64	0.25	8	0.06	64
Transformant	512	16	2	32	4	128	0.25	2	0.06	64
E. coli 7	>512	>512	2	128	4	128	0.12	8	0.06	64
Transformant	512	16	4	32	4	128	0.12	4	0.12	64
E. coli 8	>512	256	4	64	4	128	0.5	4	0.06	32
Transformant	256	16	2	32	4	128	0.25	2	0.06	64
E. coli 9	>512	32	2	32	2	64	0.06	4	0.06	32
Transformant	512	16	2	32	4	128	0.25	4	0.12	64
E. coli 10	>512	>512	32	128	4	128	0.25	4	0.06	64
Transformant	256	16	2	32	4	128	0.25	4	0.06	32
E. cloacae 11	>512	64	32	>512	512	256	32	64	32	64
Transformant	256	16	4	128	4	64	0.25	1	0.06	16
E. cloacae 12	>512	64	64	>512	>512	128	32	64	64	64
Transformant	256	8	2	128	4	64	0.25	1	0.06	16
E. cloacae 13	>512	>512	2	>512	64	64	0.5	16	0.5	256
Transformant	>512	128	2	256	4	64	0.25	1	0.06	128
E. cloacae 14	>512	>512	256	>512	256	512	128	256	256	128
Transformant	>512	256	2	512	8	>512	0.5	64	0.06	>512
E. sakazakii 15	>512	512	8	>512	64	512	8	16	16	128
Transformant	>512	512	4	64	16	256	0.25	4	0.06	64
K. pneumoniae 16	>512	64	4	256	8	256	0.12	8	0.06	128
Transformant	512	16	4	32	8	18	0.25	4	0.06	64
K. pneumoniae 17	>512	128	8	128	8	512	0.5	8	0.12	256
Transformant	>512	32	2	64	8	128	0.5	4	0.06	64
K. pneumoniae 18	>512	64	4	256	4	256	0.12	16	0.06	128
Transformant	>512	64	2	128	8	128	0.25	4	0.06	64

[a] Abbreviations: AMX, amoxicillin; PIP, piperacillin; TZB, piperacillin and tazobactam at a fixed concentration of 4 μg/ml; CEF, cephalothin; FOX, cefoxitin; CAZ, ceftazidime; CLA, clavulanic acid at a fixed concentration of 2 μg/ml; CTX, cefotaxime; AZT, aztreonam.

tained (Table 4), indicating that bla_{VEB-1} was part of class 1 integrons. Taking into account the size of the amplified fragments, it could be deduced that the surrounding sequences upstream of bla_{VEB-1} varied from 0.1 to 4.2 kb. Since the MICs of β-lactams were similar for each of the transformants (except *E. cloacae* 14 [Table 4]), the expression of VEB-1 was not related to the position of the bla_{VEB-1} cassette relative to that of the 5′-CS where the integron promoter sequences are located.

Using primers located in the 3′-CS and in the bla_{VEB-1} gene, amplimers were obtained ranging from 0.8 to 8.2 kb. Thus, the sizes of the bla_{VEB-1}-containing integrons were on the average around 10 kb, a size that fits with that previously described for other bla_{VEB-1}-containing integrons (23, 29, 38). Interestingly, in some cases (transformants *E. coli* 1 and 3 and *K. pneumoniae* 18), two PCR fragments were obtained using primers for bla_{VEB-1} and 3′-CS. This result may indicate the presence of two different bla_{VEB-1}-containing integrons within the same plasmid or spontaneous deletion of the gene cassette within integrons.

Since bla_{OXA-10} is associated with bla_{VEB-1} in *P. aeruginosa* JES isolates (23), PCR experiments using bla_{OXA-10} primers were performed. In all cases but three (*E. cloacae* 11, 12, and 14 and their transformants), a bla_{OXA-10}-like gene was identified (Table 4). A correlation was established between expression of a β-lactamase with a pI value of 6.3 and bla_{OXA-10}-like positive PCR results (Table 3). These bla_{OXA-10}-like genes likely coded for the narrow-spectrum clavulanic acid-resistant β-lactamase OXA-10 because (i) the MICs of ceftazidime,

TABLE 4. Genotypic characterization of enterobacterial isolates and their transformants, β-lactamase isoelectric points and plasmid DNA sizes of transformants, and phenotypic characterization of non-β-lactam antibiotic resistance markers

Isolate	PCR[a]				PCR (kb)[a]				Isoelectric point(s)	Plasmid size (kb)	Associated non-β-lactam antibiotic resistance markers[b]
	bla$_{TEM}$	*bla*$_{SHV}$	*bla*$_{OXA-10}$	*arr*-2	5′ CS-*bla*$_{VEB-1}$	*bla*$_{VEB-1}$-3′ CS	*bla*$_{VEB-1}$-*arr-2*	*bla*$_{VEB-1}$-*bla*$_{OXA-10}$			
E. coli 1	+	−	+	+							Sp Ka Tb Ss Sx Cl Na Pe Ra
Transformant	+	−	+	+	0.1	6.3; 0.8	0.7	3	7.4; 6.3; 5.4	210	Sp Ka Ss Cl Ra
E. coli 2	+	−	+	+							Sp Ka Tb Ss Sx Cl Te Na Pe Ra
Transformant	−	−	+	+	2.3	6.5	0.7	4.5	7.4; 6.3	150	Sp Ka Tb Ss Cl Ra
E. coli 3	−	+	+	+							Sp Ka Tb Ss Sx Cl Te Na Pe Ra
Transformant	−	−	+	+	2.3	8.2; 2.3	1.8	5	7.4; 6.3	150	Sp Ka Tb Ss Cl Ra
E. coli 4	+	−	+	+							Sp Ka Tb Ss Sx Cl Na Pe Ra
Transformant	−	−	+	+	4.2	6.5	0.7	3	7.4; 6.3	150	Sp Ka Tb Ss Cl Ra
E. coli 5	+	−	+	+							Sp Ka Tb Ss Sx Cl Te Na Pe Ra
Transformant	−	−	+	+	2.3	6.5	0.7	3	7.4; 6.3	210	Sp Ka Tb Ss Sx Cl Te Ra
E. coli 6	+	−	+	+							Sp Ka Tb Ss Sx Cl Ra
Transformant	−	−	+	+	2.3	6.5	0.7	3	7.4; 6.3	150	Sp Ka Tb Ss Cl Ra
E. coli 7	+	−	+	+							Sp Ka Tb Ss SX Cl Te Ra
Transformant	−	−	+	+	2.3	6.5	0.7	3	7.4; 6.3	150	Sp Ka Tb Ss Cl Ra
E. coli 8	+	−	+	+							Sp Ka Tb Ss Sx Cl Te Na Pe Ra
Transformant	−	−	+	+	2.3	8.6	−	5	7.4; 6.3	150	Sp Ka Tb Ss Te
E. coli 9	−	−	+	+							Sp Ka Tb Ss Cl Te Ra
Transformant	−	−	+	+	2.3	6.5	0.7	3	7.4; 6.3	150	Sp Ka Tb Ss Cl Ra
E. coli 10	+	−	+	+							Sp Ka Tb Ss Sx Cl Te Ra
Transformant	−	−	+	+	2.3	6.5	0.7	3	7.4; 6.3	210	Sp Cl Ra
E. cloacae 11	−	−	−	+							Ka Tb Ra
Transformant	−	−	−	+	1.3	3.9	−	−	7.4	150	Ka
E. cloacae 12	−	−	−	+							Ka Tb Ra
Transformant	−	−	−	+	1.3	3.9	−	−	7.4	150	Ka
E. cloacae 13	+	+	+	+							Sp Ka Tb Ss Sx Cl Te Ra
Transformant	+	−	−	+	0.1	0.8	−	3	7.4; 6.3; 5.4	100	Ka Tb Ss Sx Ra
E. cloacae 14	+	−	−	+							Sp Ka Tb Ss Sx Cl Ra
Transformant	−	−	−	+	0.1	0.8	−	−	7.4; 6.5	24	Ss
E. sakazakii 15	+	−	+	+							Sp Ka Tb Ss Sx Cl Te Na Pe Ra
Transformant	+	−	+	+	1.7	4.8	0.7	3	7.4; 6.3; 5.4	150	Ka Tb Te Ra
K. pneumoniae 16	−	+	+	+							Sp Ka Tb Ss Sx Ra
Transformant	−	−	+	+	2.3	6.5	0.7	3	7.4; 6.3	150	Sp Ka Tb Ss Ra
K. pneumoniae 17	−	+	+	+							Sp Ka Tb Ss Sx Cl Te Na Pe Ra
Transformant	−	−	+	+	1.4	4.8	0.7	3	7.4; 6.3	210	Sp Ka Tb Ss Sx Cl Te Ra
K. pneumoniae 18	+	+	+	+							Sp Ka Tb Ss Sx Cl Te Ra
Transformant	+	−	+	+	0.1	6.3; 0.8	0.7	3	7.4; 6.3; 5.4	210	Sp Ka Ss Cl Ra

[a] Minus (−) indicates that no PCR product was obtained.

[b] Antibiotic resistance markers: Sp, spectinomycin; Ka, kanamycin; Tb, tobramycin; Ss, sulfonamides; Sx, trimethroprim-sulfamethoxazole; Cl, chloramphenicol; Te, tetracycline; Na, nalidixic acid; Pe, pefloxacin; Ra, rifampin.

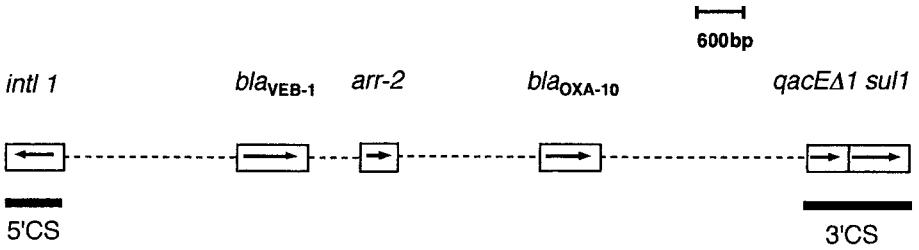

FIG. 1. Schematic representation of the most common class 1 integron that carries the bla_{VEB-1} gene cassette as found in transformants of *E. coli* 5, 6, 7, 9, and 10 and *K. pneumoniae* 16. The coding regions are represented as boxes, with arrows indicating their transcription direction. Dashed lines represent undetermined nucleotide sequences but are proportional to the gene distance.

cefotaxime, and aztreonam for the transformants were decreased by the addition of clavulanic acid (almost at the level for wild-type *E. coli* DH10B) and (ii) the pI value of 6.3 corresponded to OXA-10. In the case of transformant *E. cloacae* 14, the β-lactamase with a pI value of 6.5 did not correspond to any of the class A ESBL genes tested nor to *oxa-1*, *oxa-2*, and *pse-1* derivatives (data not shown).

PCR detection of the *arr-2* gene that conferred resistance to rifampin was found for all clinical isolates and transformants, thus indicating a plasmid location for this gene. Expression of rifampin resistance was found for all transformants except *E. coli* 8 and *E. cloacae* 11, 12 and 14. These transformants and transformant *E. cloacae* 13 gave a weakened PCR signal compared to the other transformants (data not shown).

By performing PCR experiments with primers for bla_{VEB-1}, bla_{OXA-10}, and *arr-2* genes and comparing the results with those obtained with primers for bla_{VEB-1}, 5′-CS, and 3′-CS, the structures of integrons were deduced. The most common structure of the integrons was the downstream location of the *arr-2* gene from bla_{VEB-1} followed by a bla_{OXA-10}-like gene (transformants *E. coli* 1, 2, 3, 4, 5, 6, 7, 9, and 10, *E. cloacae* 15, and *K. pneumoniae* 16, 17, and 18) (Table 4 and Fig. 1). In one case (transformant *E. cloacae* 13), a bla_{OXA-10}-like gene was located outside the 3′-CS sequence (Table 4). For transformants *E. coli* 8 and *E. cloacae* 11, 12, and 13, which gave a weak PCR signal for the *arr-2* gene, no bla_{VEB-1}–*arr-2* fragment was amplified by PCR. In these cases, an *arr2*-like gene may be involved in conferring either no or a low level of resistance to rifampin (Table 4). Although the *sul1* gene is associated with class 1 integrons, expression of resistance to sulfonamides was not found in several transformants (*E. coli* 10, *E. cloacae* 11 and 12, and *E. sakazakii* 15 [Table 4]). In these cases, the *sul1* gene is either lacking or not expressed, as reported previously (4, 5, 32).

Different groups of *Enterobacteriaceae* with integrons of identical structure were found: (i) transformants *E. coli* 1 and *K. pneumoniae* 18 and (ii) *E. coli* 5, 6, 7, 9, and 10 and *K. pneumoniae* 16 (Fig. 1). The presence of a similar-sized plasmid conferring identical non-β-lactam antibiotic-resistance markers in the transformants and similar integrons was found for *E. coli* 1 and *K. pneumoniae* 18 on the one hand and for *E. coli* 6, 7, and 9 on the other hand (Table 4).

Thus, the spread of the bla_{VEB-1} gene among these nosocomial isolates may be explained rarely by the spread of clonally related strains and often by similar plasmids and integrons.

DISCUSSION

Taking into account the total number of enterobacterial isolates in the studied period, the prevalence of ESBL-producing organisms was 4.5% for *E. coli*, 9.6% for *K. pneumoniae*, and 26% for *Enterobacter* sp. These values ranged within those reported for isolates of North American and European hospitals (12, 18, 20, 36, 46). In a multicenter study performed in Thailand, the prevalence of ceftazidime-resistant *E. coli* strains ranged from 0 to 62.5% (37). In this same study, the prevalence of ceftazidime-resistant *K. pneumoniae* was 45% on average, and that is higher than the value reported here. In Korea, the prevalence of ESBL-producing *E. coli* was 4.8% with mostly TEM- and SHV-type ESBLs and was 22% in *K. pneumoniae* isolates (14). In Taiwan, the prevalence of ESBL-producing *K. pneumoniae* was quite high (30%), mostly involving TEM-type ESBLs (17, 26, 45). On the contrary, in Japan ESBL-producing organisms are rarely encountered (<0.001%) and the ESBLs are mostly of the TOHO-2 type and rarely of the TEM and SHV type (44). In China, ESBLs have been reported but their prevalence is unknown (34).

In Bangkok, as reported previously in other countries (39), *Enterobacter* sp. but not *K. pneumoniae* may represent the main reservoir of ESBL-producing enteric isolates. This may result from either an outbreak or a situation in which such organisms are endemic. The bla_{VEB-1} gene seemed to be highly prevalent in these ceftazidime-resistant Thai isolates since it accounted for 60% of the ESBL-possessing isolates. However, frequent identifications of bla_{TEM} and bla_{SHV} derivatives may correspond also to additional ESBL genes in the same clinical isolates. The epidemiological analysis at the strain and plasmid levels indicated that bla_{VEB-1} had spread among various enterobacterial species. Its dissemination was not due to a single strain or a single plasmid type. Most of the bla_{VEB-1}-positive isolates harbored a self-conjugative plasmid of large size, as found for *E. coli* MG-1 from Vietnam (30).

The present work established the dissemination of an Ambler class A ESBL gene via various structures of class 1 integrons. A dissemination of an unusual Ambler class A ESBL gene has been reported for bla_{PER-1} in Turkey (40, 41). However, in this case the plasmid location but not the integron location of bla_{PER-1} is known. In the case of the Ambler class B IMP-1 β-lactamase, its integron-located gene has been reported extensively in Japan (10). However, as opposed to bla_{IMP-1} (10), the bla_{VEB-1} location on a class 1 integron is always followed by its expression.

Structure analysis of the bla_{VEB-1}-containing integrons showed their variability compared to the three other known bla_{VEB-1}-containing integrons, which are from *E. coli* MG-1 (from Vietnam) and *P. aeruginosa* JES-1 and Thl-1 (from Thailand) (23, 30, 38). Various structures of class 1 integrons have been detected in *Vibrio cholerae* isolates from Thailand carrying a carbenicillinase gene not related to bla_{VEB-1} (6). Spread of another class A β-lactamase (bla_{PSE-1}) has also been reported to be related to integron location in *Salmonella enterica* serotype Typhimurium DT104 (28).

In the present study, most of the integrons containing the bla_{VEB-1} gene cassette possessed *arr-2* and bla_{OXA-10} gene cassettes. This result raises the question of how these genes have evolved and if they have been transferred among soil organisms such as mycobacteria, *Enterobacteriaceae*, and *Pseudomonas* species. Indeed, *arr-2* shares a structural relationship with *arr-1* from *Mycobacterium smegmatis* (38) and bla_{OXA-10}, and their extended-spectrum derivative genes are mostly found in *P. aeruginosa* isolates (22). Once located on integrons, these resistance genes may have been transferred in block through a transposition-related mechanism. It would be interesting to test if bla_{VEB-1}-carrying integrons are located on transposons such as Tn*21* derivatives, as described for some class 1 integrons (4).

Our results also raise the question of whether antibiotic selective pressure in hospitals in Thailand may have led to dissemination of this integron-located gene. β-Lactam as well as aminoglycoside, sulfonamide, and disinfectant resistance genes are associated with class 1 integrons that may enhance the in vivo dissemination of these integrons. Restricted clinical use of broad-spectrum antibiotics and rigorous hygiene measures are the most important means to prevent the spread of this ESBL gene as much as possible. Since bla_{VEB-1} has been detected also in two *P. aeruginosa* isolates from Thailand, a further study would evaluate its spread among other gram-negative rods.

The important prevalence of bla_{VEB-1} among enterobacterial isolates may lead to heavy use of carbapenems in this Bangkok hospital, which in turn may favor the selection of, at least, carbapenem-resistant *P. aeruginosa* isolates.

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