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Distribution of Extended-Spectrum β-Lactamases in Clinical Isolates of *Enterobacteriaceae* in Vietnam

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Among 730 *Escherichia coli*, 438 *Klebsiella pneumoniae*, and 141 *Proteus mirabilis* isolates obtained between September 2000 and September 2001 in seven hospitals in Ho Chi Minh City, Vietnam, 26.6% were resistant to ceftazidime, 30% were resistant to cefotaxime, 31.5% were resistant to ceftriaxone, 15.9% were resistant to cefoperazone, and 6% were resistant to cefepime. Resistance to imipenem was found in 5.6% of the isolates. In 55 strains producing extended-spectrum β-lactamases (32 *E. coli* isolates, 13 *K. pneumoniae* isolates, and 10 *P. mirabilis* isolates), structural genes for VEB-1 (25.5%), CTX-M (25.5%), SHV (38.1%), and TEM (76.3%) enzymes were detected alone or in combination. Sequencing of the PCR products obtained from the *K. pneumoniae* isolates revealed the presence of $bla_{\text{VEB-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-17}}$, $bla_{\text{SHV-2}}$, and $bla_{\text{TEM-1}}$. Molecular typing of the strains with a similar resistance phenotype to broad-spectrum cephalosporins indicated polyclonal spread. IS*Ecp1* was presumably responsible for dissemination of the $bla_{\text{CTX-M-like}}$ gene.

Resistance to broad-spectrum cephalosporins in members of the family *Enterobacteriaceae* can be secondary to alterations in outer membrane proteins, overproduction of chromosomal or plasmid-mediated cephalosporinases, or production of extended-spectrum β-lactamases (29). Most extended-spectrum β-lactamases in the *Enterobacteriaceae* belong to Ambler class A (1), and among these, the majority are plasmid-encoded TEM and SHV derivatives that remain susceptible to the penicillinase inhibitors (4; G. A. Jacoby and K. Bush [http://www.lahey.org/studies/webt.htm]). However, other families of class A enzymes, such as CTX-M and VEB, are rapidly expanding and may play a significant role in resistance to extended-spectrum cephalosporins in Southeast Asia.

CTX-M β-lactamases are much more active against oxyimino β-lactams, such as cefotaxime and aztreonam, than against ceftazidime (34). To date, the CTX-M family comprises more than 20 members isolated from various enterobacterial species in different geographic areas. CTX-M-17, a recently added member in this group, was detected in a *Klebsiella pneumoniae* clinical isolate from Vietnam (5). It is closely related to $bla_{\text{CTX-M-14}}$ identified in China (accession no. AF252622) and Korea (24). The $bla_{\text{CTX-M-17}}$ gene is flanked downstream by an IS*903*-C copy and upstream by an IS*Ecp1*-like element which provides the promoter and directs the transcription of the gene. The IS*Ecp1*-like copy is also able to mobilize $bla_{\text{CTX-M-17}}$ and has been proposed to be responsible for dissemination of the gene (5).

The VEB-1 β-lactamase was identified recently in an *Escherichia coli* isolated from a Vietnamese patient and is widespread in *Pseudomonas aeruginosa* strains from Thailand (8). Study of its genomic environment indicated that $bla_{\text{VEB-1}}$ was a class 1 integron located in the chromosome (19) or on plasmids (33). The VEB-1 β-lactamase confers a higher level of resistance to ceftazidime than to cefotaxime.

In enterobacteria, extended-spectrum β-lactamases are mainly produced by *E. coli*, *K. pneumoniae*, or *Proteus mirabilis* strains responsible for nosocomial infections (15). These strains are disseminated worldwide (16), but little is known about their prevalence among clinical isolates from Southeast Asia (12). This region faces a serious problem of antibiotic resistance since the drugs are freely available and are used in an indiscriminate fashion.

The aim of this study was (i) to establish the prevalence of resistance to broad-spectrum cephalosporins among *K. pneumoniae*, *P. mirabilis*, and *E. coli* strains recovered during a 1-year period in various hospitals in Ho Chi Minh City, Vietnam, and (ii) to characterize the mechanisms responsible for resistance in representative isolates.

MATERIALS AND METHODS

Clinical isolates. The susceptibilities of 1,309 consecutive isolates, including *E. coli* (730), *K. pneumoniae* (438), and *P. mirabilis* (141), isolated between September 2000 and September 2001 in seven hospitals in Ho Chi Minh City to ceftazidime, cefotaxime, ceftriaxone, cefoperazone, cefepime, and imipenem were determined by E-test (AB BIODISK, Solna, Sweden). The results obtained were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (21). A single isolate per patient was included, and the number of isolates by hospital varied from 57 to 353. Approximately 15 isolates per hospital collected from sporadic cases in intensive care units and medicine, surgery, and pediatric wards, were selected for further analysis; however, a possible link of the sporadic isolates with an outbreak cannot be excluded. The method for susceptibility testing was uniform in all hospitals participating in this study.

One hundred randomly selected isolates resistant to extended-spectrum cephalosporins were studied by the double-disk test (10). The identifications of 55 isolates (*E. coli*, 32 isolates; *K. pneumoniae*, 13 isolates; and *P. mirabilis*, 10 isolates) which displayed synergy between ceftazidime or cefotaxime and clavulanic acid (3, 10) were confirmed with the API 20E test (bioMérieux, Lyon,

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TABLE 1. Sequence of primers for detection of *bla* genes or genotyping of strains

Target	Primer name	Primer sequence (5′-3′)	Position	Reference or accession no.
Detection primers				
bla_{SHV}	OS-5	TTA TCT CCC TGT TAG CCA CC	23–42	Y11069
	OS-6	GAT TTG CTG ATT TCG CTC GG	799–818	
bla_{TEM}	C	TCGGGGAAATGTGCGCG	90–105	32
	D	TGCTTAATCAGTGAGGCACC	1042–1062	
bla_{CTX-M}	MA-1	SCS ATG TGC AGY ACC AGT AA	270–289	X92506
	MA-2	CCG CRA TAT GRT TGG TGG TG	794–813	
$bla_{CTX-M-14,\ 17\ Toho-2}$	M9U	ATG GTG ACA AAG AGA GTG CA	112–131	D89862
	M9L	CCC TTC GGC GAT GAT TCT C	957–975	
bla_{VEB-1}	VEBcas-F	CGA CTT CCA TTT CCC GAT GC	128–151	AF010416
	VEBcas-B	GGA CTC TGC AAC AAA TAC GC	1198–1180	
bla_{OXA-10}	OXA-10scaF	TTA GGC CTC GCC GAA GCG	7331–7348	AF205943
	OXA-10casB	CTTTGTTT TAG CCA CCA ATG ATG	8297–8319	
bla_{PER-1}	PER-A	ATG AAT GTC ATT ATA AAA GC	309–328	22
	PER-B	AAT TTG GGC TTA GGG CAG AA	1233–1214	
bla_{GES-1}	GES-1A	ATG CGC TTC ATT CAC GCA C	1322–1340	28
	GES-1B	CTA TTT GTC CGT GCT CAG G	2095–2077	
bla_{CTX-M} upstream	IS*Ecpl*-L	CCT AGA TTC TAC GTC A	1138–1159	5
	CTX-2S	TTG CTG CAC CGC ACT CGT	3211–3194	
Genotyping primer (rep-PCR)				
	BOX-A1	CTACGGCAAGGCGACGCTGACG		11
	ER1C2	AAGTAAGTGACTGGGGTGAGCG		35

France). Strains were grown in brain-heart infusion broth and agar (Difco) at 37°C.

Antibiotic susceptibility testing and screening for production of extended-spectrum β-lactamases. The antibiotic susceptibility of the 55 enterobacteria was determined by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France). The MICs of β-lactams were determined, alone or in combination with a fixed concentration of clavulanic acid (2 μg/ml), by agar dilution with an inoculum of 10^4 CFU per spot on Mueller-Hinton medium after 16 h of incubation at 37°C.

DNA manipulations. Total DNA was prepared as described previously (30), and plasmid DNA was purified by using the Wizard Minipreps DNA kit (Promega, Madison, Wis.).

PCR detection of bla_{TEM}, bla_{SHV}, bla_{PER-1}, bla_{VEB-1}, bla_{OXA-10}, bla_{CTX-M}, and bla_{GES-1} was performed with specific oligodeoxynucleotides (Table 1). The combination of primers IS*Ecp1* and CTX-2S, complementary to internal portions of $bla_{CTX-M-17}$, was used to screen for the presence of IS*Ecp1* upstream from $bla_{CTX-M-17}$. PCR was performed in 100-μl reaction mixtures consisting of 1× *Pfu* DNA polymerase buffer, 2 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 50 pmol of each primer, and 25 ng of DNA in a GeneAmp PCR system 2400 (Perkin-Elmer Cetus, Norwalk, Conn.). The PCR mixture was submitted to a denaturation step (2 min at 94°C), which was followed by 30 cycles of amplification (45 s of denaturation at 94°C, 1 min of annealing at 52°C, 1 min of elongation at 72°C) and 10 min at 72°C for the last step. The PCR products were analyzed by electrophoresis in a 1.2% agarose gel.

The PCR-*Nhe*I method was used to discriminate between $bla_{SHV-BLSE}$ and $bla_{SHV-nonBLSE}$ genes (23).

The amplification products were purified with the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France) and sequenced directly on both strands using a CEQ 2000 DNA analysis system automatic sequencer (Beckman Instruments, Inc., Palo Alto, Calif.).

Colony hybridization. The search for bla_{VEB-1} by colony hybridization was carried out as follows. Bacteria spotted with a multiple inoculator on sterile nitrocellulose filters were lysed after 3 h of incubation on Mueller-Hinton agar, and hybridization was performed in 50% formamide at 42°C as described previously (30). The amplification product internal to bla_{VEB-1} used to generate the probe was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Radiochemical Center, Amersham, England) using a nick translation kit (Amersham).

Computer analysis of sequence data. Nucleotide and amino acid sequences were analyzed with the Genetics Computer Group (Madison, Wis.) sequence analysis software package (version 7). The GenBank and SwissProt databases were screened for sequence similarity.

Strain typing. Total DNA was amplified by repetitive extragenic palindromic PCR (rep-PCR) with primers ERIC2 or BOX-A1 (Table 1) as described previously (11). PCR products were electrophoresed in 1.2% agarose, stained with ethidium bromide, and visualized using a UV transilluminator and a digital image capture system (Gel Doc; Bio-Rad, Hercules, Calif.)

RESULTS AND DISCUSSION

Prevalence of resistance to broad-spectrum cephalosporins in *Enterobacteriaceae*. During a 1-year period, from September 2000 to September 2001, the susceptibilities to broad-spectrum cephalosporins of a total of 1,309 clinical isolates of *K. pneumoniae*, *P. mirabilis*, and *E. coli* were tested in seven hospitals in Ho Chi Minh City (Table 2). Strains resistant or intermediate to ceftazidime were more predominant in *E. coli* (32%) and *P. mirabilis* (30%) than in *K. pneumoniae* (17%). These figures are similar to those recently reported from Thailand, where 35% of enterobacteria were resistant to ceftazidime (6), but much higher than those in European countries (9, 25). Resistance to cefotaxime and cefpirome ranged from 25 to 35% and was equally distributed in all three groups. Imipenem and cefepime were the most active, but resistance was detected in

TABLE 2. Prevalence of resistance to cephalosporins and imipenem among enterobacteria

Strain (no. of isolates)	% (no.) of isolates resistant to[a]:					
	CAZ	CTX	CRO	CFP	FEP	IMP
E. coli (730)	32 (233)	30 (219)	30 (219)	15 (109)	3 (22)	3 (22)
P. mirabilis (141)	30 (42)	25 (35)	28 (40)	11 (16)	9 (13)	4 (7)
K. pneumoniae (438)	17 (74)	32 (140)	35 (153)	19 (83)	10 (44)	10 (44)
Total (1,309)	26 (349)	30 (394)	31.5 (412)	15.9 (208)	6.0 (79)	5.6 (73)

[a] Abbreviations: CAZ, ceftazidime; CFP, cefoperazone, CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime, IMP, imipenem.

the three species, in particular in *P. mirabilis*, with resistance to cefepime and to imipenem of 9 and 4%, respectively.

β-Lactam susceptibilities of strains producing extended-spectrum β-lactamases. Fifty-five randomly selected isolates resistant to cephalosporins, including 32 *E. coli*, 13 *K. pneumoniae*, and 10 *P. mirabilis* isolates, were studied further. Synergy between a disk impregnated with ceftazidime or cefotaxime and a disk containing clavulanate was observed for all strains, suggesting the production of an extended-spectrum β-lactamase by every isolate (10). The MICs of β-lactams for the strains of *K. pneumoniae* are listed in Table 3. All isolates were resistant to amoxicillin, cephalothin, and cefuroxime but displayed various degrees of resistance to ceftazidime and cefotaxime. Resistance (MIC ≥ 16 μg/ml) to ceftazidime was observed in 5 out of 13 strains (38.4%), and resistance to cefotaxime was observed in 8 of 13 strains (61.5%). Production of an extended-spectrum β-lactamase was confirmed in all strains based on an 8- to 16-fold reduction in the MIC of the cephalosporins when combined with clavulanic acid (2 μg/ml).

K. pneumoniae is intrinsically resistant to amino-, carboxy-, and acylureido-penicillins due to the chromosomal $bla_{\text{LEN-1-like}}$ gene, whereas despite low expression of chromosomal *ampC*, *E. coli* remains susceptible. By contrast, *P. mirabilis* is naturally susceptible to these antibiotics. Taking into account the natural characteristics of these species, the resistance genotypes of the 55 strains were analyzed.

Characterization of genes for extended-spectrum β-lactamases and of their environment. PCR experiments with primers specific for bla_{TEM}, bla_{SHV}, $bla_{\text{VEB-1}}$, $bla_{\text{OXA-10}}$, $bla_{\text{CTX-M}}$, $bla_{\text{GES-1}}$, and $bla_{\text{PER-1}}$ genes were performed on total DNA as a template (Table 4). Five out of the seven genes were found alone or in various combinations. $bla_{\text{TEM-like}}$ and $bla_{\text{SHV-like}}$ genes were found in 42 of 55 and in 21 of 55 of the strains, respectively. $bla_{\text{VEB-1-like}}$ and $bla_{\text{CTX-M-like}}$ genes were detected in 14 out of the 55 isolates.

One *K. pneumoniae* and two *E. coli* isolates were resistant to broad-spectrum cephalosporins but did not give any rise to PCR product, suggesting the presence of new β-lactamases in these isolates, and are being studied further.

Sequence determination of all the PCR products obtained from the *K. pneumoniae* isolates confirmed the identity of the genes. The MICs of β-lactams and the enzyme contents of the strains are summarized in Table 3.

***bla*$_{\text{VEB-1}}$.** The recently identified $bla_{\text{VEB-1}}$ gene (27), which mediates resistance to ceftazidime and aztreonam, was found in the three species studied, in particular in 6 out of 10 *P. mirabilis* isolates (20). The sequence of two PCR products obtained from *K. pneumoniae* was identical to that published for $bla_{\text{VEB-1}}$ (27), confirming the structural conservation of this gene observed in Thailand (6, 8). The $bla_{\text{OXA-10}}$ gene has been found associated with $bla_{\text{VEB-1}}$ in the same integron (19), and the *K. pneumoniae* isolate containing $bla_{\text{VEB-1}}$ also harbored $bla_{\text{OXA-10}}$ or a variant thereof.

***bla*$_{\text{CTX-M-like}}$.** In contrast to $bla_{\text{VEB-1}}$, $bla_{\text{CTX-M-like}}$ was detected predominantly in *K. pneumoniae*, in 8 out of 13 isolates (61.5%). Sequencing of the eight amplification products revealed the presence of $bla_{\text{CTX-M-17}}$ in two isolates and the presence of $bla_{\text{CTX-M-14}}$ in the remaining strains. The genes differ by two mutations, leading to the single Glu289→Lys substitution. $bla_{\text{CTX-M-like}}$ was found in only 6 of 32 (18.7%) *E. coli* isolates and not in *P. mirabilis*. It has been shown that IS*Ecp1* can provide the promoter and direct the transcription of the $bla_{\text{CTX-M-17}}$ gene in *K. pneumoniae* BM4493 (5). Sequence analysis of the region upstream from the $bla_{\text{CTX-M-like}}$ genes of *K. pneumoniae* indicated the presence of IS*Ecp1* in six out of eight strains.

***bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$.** bla_{TEM} genes were found in all *P. mirabilis* isolates, in 27 of 32 (84.3%) *E. coli* isolates, and in 5 of 13

TABLE 3. *bla* genotypes and β-lactam resistance phenotypes of *K. pneumoniae* isolates

K. pneumoniae isolate	Gene content				MIC (μg/ml)[a]											
	bla_{TEM}	bla_{SHV}	bla_{VEB}	bla_{CTX}	PIP	CF	CXM	FOX	CTX	CAZ	ATM	CAZ + CA	FOX + CA	CF + CA	PIP + CA	CTX + CA	AMX
BM4498	−	−	−	+	128	>256	>256	8	64	4	16	0.25	2	16	4	<0.125	>256
BM4499	−	+	−	−	128	256	16	8	8	8	4	0.5	8	4	8	<0.125	256
BM4500	−	+	−	+	128	>256	>256	4	32	2	4	0.25	8	16	4	<0.125	>256
BM4501	+	+	−	+	64	>256	>256	4	16	1	8	<0.125	4	16	1	<0.125	>256
BM4502	+	−	+	+	64	256	128	2	8	128	32	0.25	2	4	2	<0.125	>256
BM4503	−	+	−	−	128	>256	32	4	8	16	8	1	4	4	>4	<0.125	>256
BM4504	−	+	−	+	128	>256	>256	4	32	2	8	0.5	4	8	4	<0.125	>256
BM4505	−	−	+	−	32	64	64	4	8	256	256	0.5	4	4	4	<0.25	>256
BM4506	+	−	−	+	128	>256	>256	16	64	16	32	0.25	32	32	1	<0.125	>256
BM4507	+	−	−	+	256	>256	>256	8	32	4	8	0.5	4	32	4	<0.125	>256
BM4508	−	−	−	−	128	>256	32	4	16	16	8	1	4	4	4	<0.125	>256
BM4509	+	+	−	−	64	256	8	4	4	4	1	<0.125	4	4	0.25	<0.125	>256
BM4510	−	+	−	+	256	>256	>256	4	64	8	8	0.25	2	>16	4	0.25	>256

[a] Abbreviations: AMX, amoxicillin; ATM, aztreonam; CA, clavulanic acid at a fixed concentration of 2 μg/ml; CAZ, ceftazidime; CF, cephalothin; CTX, cefotaxime; CXM, cefuroxime; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; PIP, piperacillin.

TABLE 4. *bla* gene content of enterobacteria as detected by PCR

Strain (no. of isolates)	No. (%) of strains containing:			
	$bla_{\text{CTX-M-like}}$	$bla_{\text{VEB-1-like}}$	$bla_{\text{TEM-like}}$	$bla_{\text{SHV-like}}$
E. coli (32)	6	6	27	14
P. mirabilis (10)	0	6	10	0
K. pneumoniae (13)	8	2	5	7
Total (55)	14 (25.5)	14 (25.5)	42 (76.3)	21 (38.1)

(38.4%) *K. pneumoniae* isolates. Sequencing showed the presence of $bla_{\text{TEM-1}}$ in all *K. pneumoniae* isolates. $bla_{\text{SHV-like}}$ genes were also found at high frequencies: in 7 of 13 (54%) *K. pneumoniae* isolates and in 14 of 32 (44%) *E. coli* isolates but not in *P. mirabilis*. DNA sequencing indicated the presence of $bla_{\text{SHV-2}}$ with mutation Gly238→Ser relative to $bla_{\text{SHV-1}}$ (7, 13, 17). The incidence of $bla_{\text{SHV-2}}$ producers appears to be higher in European countries than in the United States, and they are very common in African countries (2, 26).

The $bla_{\text{GES-1}}$ and $bla_{\text{PER-1}}$ genes were not detected.

Molecular characterization of *K. pneumoniae*. The relationship between the 13 *K. pneumoniae* isolates was studied by rep-PCR using independently BOX-A1 and ERIC2 (enterobacterial repetitive intergenic consensus) primers. Amplification with ERIC2 primer provided poorly reproducible results, and only BOX-A1 gave discriminant DNA profiles of the strains (data not shown). Among the isolates resistant to ceftazidime or to cefotaxime, the various profiles obtained indicated polyclonal dissemination of resistance to broad-spectrum cephalosporins

The prevalence of resistance to antibiotics varies greatly from one geographic area to another as well as between hospitals within a community, mainly because of the differences in antimicrobial usage and infection control practices (18). In Taiwan, the prevalence of *K. pneumoniae* producing extended-spectrum β-lactamase is quite high (30%), involving mostly TEM-type and SHV-12 enzymes (14, 36). By contrast, in Japan, organisms producing such β-lactamases are rarely encountered, and the enzymes are mostly Toho-2 (37). In China, extended-spectrum β-lactamases have been reported, but their prevalence is unknown (31). The distribution of TEM-1, VEB-1, and SHV-like (SHV-2a, SHV-5, and SHV-12) enzymes in Thailand has been reported very recently (6).

Two highly prevalent resistance phenotypes, to cefotaxime or to ceftazidime, associated with the respective production of CTX-M-14/17 and VEB-1, were detected in *K. pneumoniae* (Table 3). These isolates also produced SHV-2 and TEM-1 penicillinases. The association of enzymes, up to four β-lactamases in a single strain, including the combination of VEB-1 and CTX-M-14 in one *K. pneumoniae* isolate, resulted in high-level resistance to both ceftazidime and cefotaxime and also to aztreonam. Enzymes VEB-1 and CTX-M-14/CTX-M-17 are newly detected extended-spectrum β-lactamases, and their origins remain unknown. The observation that strains harboring identical genes are not related clonally suggests dissemination of resistance determinants by mobile elements. The integron environment of $bla_{\text{VEB-1}}$ (8, 27) and the presence of IS*Ecp1* and IS*903* flanking $bla_{\text{CTX-M-14/17}}$ (5) are consistent with this notion.

This study revealed a high prevalence of resistance to broad-spectrum cephalosporins among *Enterobacteriaceae* in Vietnam. It also indicated the particular widespread presence of VEB-1 and CTX-M-like extended-spectrum β-lactamases associated with TEM-1 and SHV-2 penicillinases in this country.

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