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

Van Cao,1,2 Thierry Lambert,1,3,* Duong Quynh Nhu,2 Huynh Kim Loan,2 Nguyen Kim Hoang,2 Guillaume Arlet,4 and Patrice Courvalin1

Unité des Agents Antibactériens, Institut Pasteur, 75724 Paris Cedex 15,1 Centre d’Etude Pharmaceutiques, Châtenay-Malabry,3 and Service de Bactériologie, Hôpital Tenon, U.F.R Saint-Antoine 75970 Paris Cedex 20,4 France, and Institut Pasteur d’Ho Chi Minh Ville, Ho Chi Minh City, Vietnam2

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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 cepoferezine, 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 blaVEB-1, blaCTX-M-14, blaCTX-M-17, blaSHV-2, and blaTEM-1. Molecular typing of the strains with a similar resistance phenotype to broad-spectrum cephalosporins indicated polyclonal spread. ISEcp1 was presumably responsible for dissemination of the β-lactamase genes.

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 penicillinas 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 blaCTX-M-14, identified in China (accession no. AF252622) and Korea (24). The blaCTX-M-17 gene is flanked downstream by an IS903-C copy and upstream by an ISEcp1-like element which provides the promoter and directs the transcription of the gene. The ISEcp1-like copy is also able to mobilize blaCTX-M-14 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 are widespread in Pseudomonas aeruginosa strains from Thailand (6).

Study of its genomic environment indicated that blaVEB-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, cecepin, 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,
TABLE 1. Sequence of primers for detection of bla genes or genotyping of strains

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
<th>Position</th>
<th>Reference or accession no.</th>
</tr>
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<tr>
<td>Detection primers</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;</td>
<td>OS-5</td>
<td>TTA TCT CCC TGT TAG CCA CC</td>
<td>23–42</td>
<td>Y11069</td>
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<tr>
<td></td>
<td>OS-6</td>
<td>GAT TTG CTG ATT TCG CTC GG</td>
<td>799–818</td>
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</tr>
<tr>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;</td>
<td>C</td>
<td>TCGGGGAAAAATGGCAGGCC</td>
<td>90–105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>TGCTTTATCGTGAGGCACC</td>
<td>1042–1062</td>
<td></td>
</tr>
<tr>
<td>bla&lt;sub&gt;CTX-M&lt;/sub&gt;</td>
<td>MA-1</td>
<td>SCS ATG TGC AGY ACC AGT AA</td>
<td>270–289</td>
<td>X92506</td>
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<tr>
<td></td>
<td>MA-2</td>
<td>CCG CTA TAT GRT TGG TGG TG</td>
<td>794–813</td>
<td></td>
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<td>bla&lt;sub&gt;CTX-M&lt;/sub&gt;-&lt;sub&gt;M1&lt;/sub&gt;</td>
<td>M9U</td>
<td>ATG GTG ACA AAG AGA GTT GA</td>
<td>112–131</td>
<td>D89862</td>
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<tr>
<td></td>
<td>M9L</td>
<td>CCC TTC GGC GAT GAT TCT C</td>
<td>957–975</td>
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<tr>
<td>bla&lt;sub&gt;VEB&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
<td>VEBcas-F</td>
<td>CGA CTT CCA TTT CCC GAT GC</td>
<td>128–151</td>
<td>AF010416</td>
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<tr>
<td></td>
<td>VEBcas-B</td>
<td>GGA CTC TGC AAG AAA TAC GC</td>
<td>1198–1180</td>
<td></td>
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<tr>
<td>bla&lt;sub&gt;OXA&lt;/sub&gt;-&lt;sub&gt;10&lt;/sub&gt;</td>
<td>OXA-10casF</td>
<td>TTT GGC TCG GCC GAA GGG</td>
<td>7331–7348</td>
<td>AF205943</td>
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<td>OXA-10casB</td>
<td>CTTTGTTT TAC GCA CCA ATG ATG</td>
<td>8297–8319</td>
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<tr>
<td>bla&lt;sub&gt;PER&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
<td>PER-A</td>
<td>ATG AAT GTC ATT AAT AAA GC</td>
<td>309–328</td>
<td>22</td>
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<tr>
<td></td>
<td>PER-B</td>
<td>ATG AAT GTC ATT AAT AAA GC</td>
<td>1233–1214</td>
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<td>bla&lt;sub&gt;GES&lt;/sub&gt;-&lt;sub&gt;1&lt;/sub&gt;</td>
<td>GES-1A</td>
<td>ATG CGC TTT ATT CAC GCA C</td>
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<td></td>
<td>GES-1B</td>
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<td>2095–2077</td>
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<tr>
<td>bla&lt;sub&gt;CTX-M&lt;/sub&gt;-&lt;sub&gt;L&lt;/sub&gt;</td>
<td>IS&lt;sup&gt;cl&lt;/sup&gt;A-&lt;sub&gt;L&lt;/sub&gt;</td>
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<td></td>
<td>CTX-2S</td>
<td>TTG CTT CAC GCG ACT CGT</td>
<td>3211–3194</td>
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<td>Genotyping primer (rep-PCR)</td>
<td>BOX-A1</td>
<td>CTACCGCAAGGCGACGCTAGCG</td>
<td>11</td>
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<tr>
<td></td>
<td>ERIC2</td>
<td>AAGTTAAGTGCCTGGGGTGAGCG</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

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-lactamasess. 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<sup>6</sup> CFU per spot on Mueller-Hinton medium after 18 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 Miniprep DNA kit (Promega, Madison, Wis.).

PCR detection of bla<sub>TEM</sub>, bla<sub>SHV</sub>, bla<sub>PER</sub>-<sub>1</sub>, bla<sub>VEB</sub>-<sub>1</sub>, bla<sub>OXA</sub>-<sub>10</sub>, and bla<sub>GES</sub>-<sub>1</sub> was performed with special oligodeoxynucleotides (Table 1). The combination of primers IS<sup>cl</sup><sup>cl</sup>-<sup>cl</sup> and CTX-2S, complementary to internal portions of bla<sub>CTX-M</sub>-<cl>, was used to screen for the presence of IS<sup>cl</sup><sup>cl</sup>-<sup>cl</sup> upstream from bla<sub>CTX-M</sub>-<cl>. PCR was performed in 100-μl reaction mixtures consisting of 1X Pfu DNA polymerase buffer, 2 U of Pfu DNA polymerase (Stratagene, La Jolla, Calif.), 1.5 mM MgCl<sub>2</sub>, 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-Norel method was used to discriminate between bla<sub>SHV-M</sub>-<sup>cl</sup> and bla<sub>AVI</sub>-<sub>SHV-M</sub>-<sup>cl</sup> genes (23).

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

Strain hybridization. The search for bla<sub>VEB</sub>-<sub>1</sub> 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<sub>VEB</sub>-<sub>1</sub> used to generate the probe was labeled with [α-<sup>32</sup>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 ERIC<sub>2</sub> or BOX<sub>-</sub>A<sub>1</sub> (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
the three species, in particular in *P. mirabilis*, with resistance to cefepime and of imipenem of 9 and 4%, respectively.

**β-Lactam susceptibilities of strains producing extended-spectrum β-lactamas.** 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 ceftaxime 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 compared with clavulanic acid (2 µg/ml).

*K. pneumoniae* is intrinsically resistant to amino-, carboxy-, and acylureido-penicillins due to the chromosomal *bla* gene 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 compared with clavulanic acid (2 µg/ml).

**Characterization of genes for extended-spectrum β-lactamas and of their environment.** PCR experiments with primers specific for *bla*,-*blaps*, *blavb*, *blaxa*, *blact*, *blag*, and *blav/f* 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* and *blas* genes were found in 42 of 55 and in 21 of 55 of the strains, respectively. *blavb* and *blav/f* 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-lactamas 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*/-*vi*. The recently identified *bla*/ 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*/ (27), confirming the structural conservation of this gene observed in Thailand (6, 8). The *bla* gene has been found associated with *bla*/ in the same integron (19), and the *K. pneumoniae* isolate containing *bla*/ also harbored *bla* or a variant thereof.

**bla*/ In contrast to *bla*/* and *bla*/ 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*/ in two isolates and the presence of *bla*/ in the remaining strains. The genes differ by two mutations, leading to the single Glu289 substitution, *bla*/ was found in only 6 of 32 (18.7%) *E. coli* isolates and not in *P. mirabilis*. It has been shown that ISEcp1 can provide the promoter and direct the transcription of the *bla*/ gene in *K. pneumoniae* (5). Sequence analysis of the region upstream of the *bla*/ genes of *K. pneumoniae* indicated the presence of ISEcp1 in six out of eight strains.

**bla** and **bla** genes were found in all *P. mirabilis* isolates, in 27 of 32 (84.3%) *E. coli* isolates, and in 5 of 13

<table>
<thead>
<tr>
<th>Strain</th>
<th>% (no.) of isolates resistant to β-lactams</th>
</tr>
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<tbody>
<tr>
<td><em>E. coli</em> (213)</td>
<td>32 (233) 30 (219) 30 (219) 15 (109) 3 (22) 3 (22)</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>30 (42) 25 (35) 28 (40) 11 (16) 9 (13) 4 (7)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>17 (34) 32 (140) 35 (153) 19 (83) 10 (44) 10 (44)</td>
</tr>
</tbody>
</table>

**Abbreviations:** CAZ, ceftazidime; CF, cefotaxime; CRO, ceftriaxone; CTX, cefotaxime; FEP, cepafempe; IMP, imipenem; PIP, piperacillin.
(38.4%) K. pneumoniae isolates. Sequencing showed the presence of bla<sub>TEM-1</sub> in all K. pneumoniae isolates. bla<sub>SHV-4</sub> 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<sub>SHV-2</sub> with mutation Gly238—Ser relative to bla<sub>SHV-1</sub> (7, 13, 17). The incidence of bla<sub>SHV-2</sub> 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<sub>GES-1</sub> and bla<sub>PBR-1</sub> 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 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 cefazidime, 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 cefazidime 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<sub>VEB-1</sub> (8, 27) and the presence of IS<i>Ecpl</i> and IS903 flanking bla<sub>CTX-M-14/17</sub> (5) are consistent with this notion.

This study revealed a high prevalence of resistance to broad-spectrum cephalosporins among <i>Enterobacteriaceae</i> 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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