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# Evolution of Extended-Spectrum β-Lactam Resistance (SHV-8) in a Strain of *Escherichia coli* during Multiple Episodes of Bacteremia

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**Nine isolates of *Escherichia coli* were recovered from seven blood cultures over a period of 3 months from a 19-month-old female with aplastic anemia. Initial isolates were susceptible to extended-spectrum cephalosporins, including ceftazidime (MIC, ≤0.25 μg/ml), but gradually became resistant to this drug (MICs, ≥128 μg/ml) and other cephalosporins and the monobactam aztreonam. Molecular typing methods, including plasmid profile analysis, pulsed-field gel electrophoresis, and arbitrarily primed PCR, indicated that the nine isolates were derived from a common ancestor. Dot blot hybridization and PCR analysis of total bacterial DNA using $bla_{SHV}$- and $bla_{TEM}$-specific DNA probes and primers identified the presence of a $bla_{TEM}$ β-lactamase gene in all of the isolates and a $bla_{SHV}$ gene in the isolates with elevated ceftazidime MICs. Isoelectric focusing analysis of crude lysates showed that all nine isolates contained an enzyme with a pI of 5.4 corresponding to the TEM-1 β-lactamase, and those isolates containing an SHV-type β-lactamase demonstrated an additional band with a pI of 7.6. The first of the ceftazidime-resistant isolates appeared to hyperproduce the SHV enzyme compared to the other resistant isolates. DNA sequencing revealed a $bla_{SHV-1}$ gene in the first ceftazidime-resistant isolate and a novel $bla_{SHV}$ gene, $bla_{SHV-8}$, with an Asp-to-Asn substitution at amino acid position 179 in the remaining four isolates. Three of the ceftazidime-resistant isolates also showed a change in porin profile. The patient had received multiple courses of antimicrobial agents during her illness, including multiple courses of ceftazidime. This collection of blood isolates from the same patient appears to represent the in vivo evolution of resistance under selective pressure of treatment with various cephalosporins.**

Extended-spectrum β-lactamases (ESBLs) are enzymes capable of hydrolyzing oxyimino cephalosporins, such as cefotaxime (CTX), ceftriaxone, ceftazidime (CAZ), and monobactams (e.g., aztreonam [ATM]), thereby causing resistance to these drugs (9, 18, 42). The enzymes are detected most commonly in *Klebsiella pneumoniae* and *Escherichia coli* but have been noted in other members of the family *Enterobacteriaceae* as well (13, 22, 24, 41, 48). The resistance phenotype mediated by these enzymes can be difficult to detect because MICs of the cephalosporins, although elevated, often remain below the breakpoints used to define resistance by the National Committee for Clinical Laboratory Standards (NCCLS) (19, 32). Since some ESBLs are more active on CTX while others are more active on CAZ (8, 9, 18), the choice of cephalosporins tested can also affect the ability of laboratories to detect resistant strains. β-Lactamase inhibitors, such as clavulanic acid and sulbactam, can be used to distinguish ESBLs from AmpC-type enzymes (9, 21), which can also convey resistance to oxyimino cephalosporins.

The amino acid sequences of ESBLs often differ from those of the parent enzymes, usually TEM-1, TEM-2, and SHV-1, by only 1, 2, or 3 amino acids (5, 17, 36, 49). Extensive use of cephalosporins, particularly in intensive care units in hospitals, may play a role in the development and spread of ESBLs, as suggested by several recent reports (31, 46). ESBLs, however, do not mediate resistance to cephamycins, such as cefoxitin and cefotetan. Rather, elevated MICs of these antimicrobial agents are often the result of changes in an organism's porins, the channels that facilitate entry of β-lactams into the bacterial cell (14, 16, 37). Two porins, designated OmpC and OmpF, have been associated with resistance to cephamycins and other cephalosporins in *E. coli* and other enteric organisms (3, 14, 29, 54).

Here we report the emergence of ESBL activity and concomitant changes in the porins in a strain of *E. coli* isolated multiple times over a 3-month period from the blood of an infant with idiopathic aplastic anemia. The production of large amounts of the SHV-1 β-lactamase due to acquisition of a $bla_{SHV-1}$ gene by the strain was followed by a mutation of that gene that enhanced its CAZ resistance. The infant received several courses of cephalosporin therapy during this period which contributed to the development of resistance in the *E. coli* isolates.

## MATERIALS AND METHODS

**Case history.** The patient was a 19-month-old black female diagnosed with idiopathic aplastic anemia in August 1990, for which she was treated with cyclosporine, antithymocyte globulin, and methylprednisone. Her course was characterized by multiple hospital admissions for fever associated with neutropenia, for which she received numerous courses of broad-spectrum antimicrobial agents. Several febrile episodes were associated with *E. coli* bacteremia; during one episode, *Enterococcus faecium* and *Clostridium clostridiiforme* were also isolated from blood cultures. She also suffered severe gastrointestinal hemorrhage. Although an intestinal ulcer was suspected, this could not be demonstrated. She had persistent neutropenia and thrombocytopenia and died the following January. The history and antimicrobial chemotherapy received are summarized in Fig. 1.

**Bacterial strains.** Nine isolates of *E. coli* were obtained from seven blood cultures (BACTEC; Becton Dickinson Microbiology Systems, Cockeysville, Md.) taken from the patient over a 3-month period. Dates of isolation are given in Table 1. Organisms were initially identified at Grady Memorial Hospital by the Vitek system (bioMérieux-Vitek, Hazelwood, Mo.) and confirmed at the Centers

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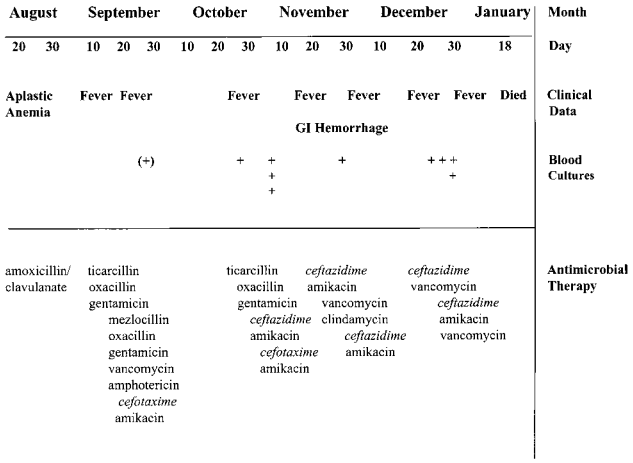


FIG. 1. Time line of patient's hospital course, antimicrobial therapy, and positive blood cultures. Blood cultures positive for *E. coli* are depicted by +. The *E. coli* isolate from the first positive blood culture, designated (+), was not available for testing. GI, gastrointestinal. Extended-spectrum cephalosporins are shown in italics for emphasis.

for Disease Control and Prevention by standard methods (10). Six additional epidemiologically unrelated isolates of *E. coli* from Grady Memorial Hospital were obtained. These were included as controls for strain typing studies.

**MIC testing and confirmation of ESBL activity.** Organisms were tested by broth microdilution with Mueller-Hinton broth (Difco) or agar dilution with Mueller-Hinton agar (Difco) as described in NCCLS standard M7-A3 (32). *E. coli* ATCC 25922 and ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control. MICs were also determined for CTX, CAZ, and ceftriaxone in the presence of 10 μg of clavulanic acid per ml. MICs that decreased by 2 dilutions or more were suggestive of the presence of an ESBL. The isolates were also tested by disk diffusion using a modification of the disk method described by NCCLS standard M2-A5 (33). Zones of inhibition were measured around two sets of disks (Becton Dickinson) containing CTX, CAZ, and ATM; 10 μg of clavulanic acid was added to one set of disks. Zones of inhibition for CTX, CAZ, or ATM that increased by 5 mm or more in the presence of clavulanic acid, compared with the zone sizes obtained in the absence of clavulanic acid for any of the above antimicrobial agents, were assumed to be due to the presence of an ESBL in that isolate (28).

**Isolation and analysis of outer membrane proteins and isoelectric focusing of β-lactamases.** Total membranes were isolated by a modification of the procedure described by Harder et al. (14). Briefly, cells were grown in L broth containing 0.5% NaCl, or nutrient broth, and harvested in mid-log phase. The cells were disrupted by sonication, cell debris was removed by centrifugation at 5,000 × *g* for 10 min, and total cellular membranes were sedimented by centrifugation at 100,000 × *g* for 1 h. Cytoplasmic membranes were solubilized in 2% Sarkosyl, and the insoluble outer membranes were isolated by centrifugation at 100,000 × *g* for 1 h. Outer membrane proteins (10 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through a 9.5 to 12.5% gradient gel.

Isoelectric focusing was performed with polyacrylamide gels as described by Matthew et al. (27). The gel was developed with an overlay of filter paper soaked in 0.05% nitrocefin solution. The β-lactamase activity of selected isolates, expressed as micromolars nitrocefin hydrolyzed per minute per milligram of protein, was determined spectrophotometrically with 100 μM substrate as described by O'Callaghan et al. (35). *E. coli* RT17-121, which contains both the TEM-1 and SHV-1 β-lactamases and is ampicillin resistant but CAZ susceptible, was used as a control strain for these studies.

**Plasmid profile analysis.** Plasmid DNA was isolated as described by Portnoy et al. (45). Plasmids were separated on a 0.7% agarose gel at 200 V for 3 h. Two unrelated *E. coli* strains, F50 and F218, from the culture collection of the Centers for Disease Control and Prevention were used as controls. *E. coli* F218 containing the plasmid pMON38 was provided by R. Levesque (Laval University, Quebec, Quebec, Canada). A supercoiled DNA ladder (BRL Life Technologies) made with 11 plasmids provided molecular size markers.

**Chromosomal DNA analysis by pulsed-field gel electrophoresis (PFGE).** PFGE was performed as described by Maslow et al. (26). Organisms were lysed in situ, the chromosomal DNA was digested with *Xba*I or *Sfi*I, and slices of the plugs were loaded into the wells of a gel. DNA fragments were separated in a 1% agarose gel (Bio-Rad) in 0.5× Tris-borate-EDTA buffer at 14°C by using a contour-clamped homogeneous electric field apparatus (CHEF-DRII; Bio-Rad). The conditions for electrophoresis were 200 V for 24 h with initial and final pulse times of 15 and 60 s, respectively. Eight unrelated *E. coli* strains (including F50 and F218) were used as controls. Fragment patterns were interpreted as described by Tenover et al. (51). Lambda concatemers (Bio-Rad) were used as molecular size standards.

**Arbitrarily primed PCR (AP-PCR).** AP-PCR was carried out as described by Killgore and Kato (20). Total DNA was amplified with the 10-mer primer GTA AGG CCG T. Amplification was conducted for 45 cycles. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. Eight unrelated *E. coli* strains were used as controls. A 1-kb ladder (ϕX174RF DNA/*Hae*III fragments [Bio-Rad]) was used as a molecular size standard.

**DNA probes and hybridization procedures.** The $bla_{TEM}$ DNA probe was a 298-bp *Hin*cII/*Pst*I restriction fragment of plasmid pBR322 that is specific for the $bla_{TEM}$ gene (52). The probe was labeled by random priming with digoxigenin with the Genius system (Boehringer Mannheim). The $bla_{SHV}$ DNA probe was a 275-bp digoxigenin-labeled PCR product generated with amplification primers 4 and 13 (Table 2). For dot blot hybridization, bacterial cells grown overnight in brain heart infusion broth (Difco) were lysed by the method of Birnboim and Doly (7) and 10 μl of cell lysate was deposited onto nylon filters (Zeta-probe; Bio-Rad). Hybridization was carried out as described by Gootz et al. (12). Colorimetric detection of digoxigenin-labeled probes was performed with the Genius kit (Boehringer Mannheim). For Southern blot hybridization, plasmid DNA was transferred from the agarose gel onto a nylon membrane and hybrid-

TABLE 1. MICs (in micrograms per milliliter) of selected antimicrobial agents against *E. coli* blood isolates from a single patient[a]

| Strain | Date[c] | MIC (μg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ATM | CAZ | CTX | CXM | CTN | FOX | TET | GENT | SUL |
| 2-80 | 10/28 | 0.12 | 0.25 | 0.06 | 4 | <0.25 | 4 | 2 | 2 | 16 |
| 7-29[b] | 11/6 | 0.12 | 0.25 | 0.06 | 8 | <0.25 | 4 | 2 | 4 | 16 |
| 7-30[b] | 11/6 | 0.12 | 0.25 | 0.06 | 8 | <0.25 | 8 | 32 | >16 | >512 |
| 2-78 | 11/6 | 0.12 | 0.25 | 0.06 | 4 | <0.25 | 4 | 4 | 4 | 16 |
| 2-77 | 11/29 | 4 | 32 | 0.5 | 32 | 4 | 16 | 4 | 2 | 32 |
| 2-75 | 12/23 | 16 | >128 | 16 | >32 | >32 | >32 | 4 | 2 | 64 |
| 2-76 | 12/26 | 32 | >128 | 16 | >32 | >32 | 16 | 1 | 2 | 64 |
| 7-35[b] | 12/27 | 16 | >128 | 16 | >32 | 16 | 16 | 2 | 4 | 32 |
| 7-36[b] | 12/27 | 2 | 64 | 2 | 16 | 8 | 8 | 2 | 4 | 16 |

[a] CXM, cefuroxime; CTN, cefotetan; FOX, cefoxitin; TET, tetracycline; GENT, gentamicin; SUL, sulfonamides.
[b] Both strains isolated from the same blood culture vial.
[c] Month/day.

TABLE 2. Nucleotide sequences of the oligonucleotides used for PCR amplification and DNA sequencing

| Primer | Nucleotide sequence 5′ to 3′ | Position |
|---|---|---|
| $bla_{TEM}$ | | |
| Amplification primer 1 | ATG AGT ATT CAA CAT TTC CG | 208[a] |
| Amplification primer 2 | CTG ACA GTT ACC AAT GCT TA | 1075 |
| $bla_{SHV}$ | | |
| Amplification primer 3 | GGG TTA TTC TTA TTT GTC GC | 58[b] |
| Amplification primer 4 | GGT TAT GCG TTA TAT TCG CC | 121 |
| Amplification primer 5 | TTA GCG TTG CCA GTG CTC | 988 |
| Sequencing primers | | |
| Leading strand 6 | GAA CAG CTG GAG CGA AAG AT | 377 |
| Leading strand 7 | CAG ATC GGC GAC AAC GTC AC | 572 |
| Leading strand 8 | CTG CAG TGG ATG GTG GAC GA | 737 |
| Leading strand 9 | CCT GCT TGG CCC GAA TAA CA | 856 |
| Lagging strand 10 | GGG CCA AGC AGG GCG ACA AT | 867 |
| Lagging strand 11 | TCG TCC ACC ATC CAC TGC AG | 756 |
| Lagging strand 12 | GTG ACG TTG TCG CCG ATC TG | 591 |
| Lagging strand 13 | ATC TTT CGC TCC AGC TGT TC | 396 |
| Lagging strand 14 | TAA TTT GCT CAA GCG GCT GC | 212 |

[a] Position number corresponds to the first 5′ base of the oligonucleotide according to the Sutcliffe numbering system (50).
[b] Position number corresponds to the first 5′ base of the oligonucleotide according to the coding sequence of SHV-1 (30).

TABLE 3. Effect of clavulanic acid on zone diameters and MICs of selected antibiotics

| No. | Date (mo/day) | Zone size increase/MIC decrease[a] | | |
|---|---|---|---|---|
| | | ATM + CA | CAZ + CA | Ceftriaxone + CA |
| 2-80 | 10/28 | 2 mm/1 dilution | 3 mm/0 dilutions | 2 mm/1 dilution |
| 7-30[b] | 11/6 | 2/0 | 2/0 | 3/1 |
| 7-29[b] | 11/6 | 2/0 | 4/0 | 1/1 |
| 2-78 | 11/6 | 1/0 | 3/0 | 1/1 |
| 2-77 | 11/29 | **3/4**[c] | **13/2** | **1/3** |
| 2-75 | 12/23 | **7/4** | **22/3** | **8/5** |
| 2-76 | 12/26 | **10/5** | **22/4** | **12/5** |
| 7-35[b] | 12/27 | **5/5** | **21/6** | **6/5** |
| 7-36[b] | 12/27 | **3/4** | **21/6** | **5/5** |

[a] Numbers represent the increased size of the zone diameters (in millimeters) around disks containing β-lactam plus clavulanic acid (CA) compared with the zone diameters around disks that have not been supplemented with clavulanic acid. A zone diameter change of ≥5 mm is a presumptive indication of an ESBL; decrease in MICs is shown in numbers of doubling dilutions.

[b] Isolates 7-30 and 7-29 and 7-35 and 7-36 were recovered from the same blood culture vial.

[c] Boldface indicates a positive result.

ized with the probe as previously described (47). The DNA was cross-linked to the filters by UV irradiation (GS Gene Linker UV chamber; Bio-Rad).

Five control strains were used for hybridization studies: *E. coli* F50 (negative for both $bla_{SHV}$ and $bla_{TEM}$); *E. coli* (pBR322), the $bla_{TEM}$ control; and three *E. coli* isolates containing the $bla_{SHV-1}$, $bla_{SHV-3}$, and $bla_{SHV-5}$ genes.

**Amplification of DNA.** Both $bla_{SHV}$ and $bla_{TEM}$ gene products were each amplified in 100-μl reaction mixtures containing 1 μl of crude cellular lysate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1 μM oligonucleotide primers, 200 μm deoxynucleoside triphosphates, and 2.5 U of native *Taq* DNA polymerase (Boehringer Mannheim). For the digoxigenin-labeled $bla_{SHV}$ probe, an alternate deoxynucleoside triphosphate mix containing substituted nucleosides was used. PCR assays for $bla_{TEM}$ (using primers 1 and 2) and $bla_{SHV}$ (using primers 4 and 5) were performed in a Perkin-Elmer model 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) with the following cycling parameters: denaturation at 96°C for 5 min; 35 cycles of 96°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final extension period of 72°C for 10 min. Synthesis of the digoxigenin-labeled $bla_{SHV}$ probe (using primers 4 and 13) and a second $bla_{SHV}$ amplification reaction (using primers 3 and 5) for DNA sequencing used similar cycling parameters except that the annealing temperatures were 60 and 56°C, respectively.

**Direct sequencing of PCR products.** PCR products were purified with QIAquick PCR purification columns (Qiagen, Inc., Chatsworth, Calif.). Cycle sequencing of these products was performed on a GeneAmp PCR System 9600 (Perkin-Elmer) utilizing the Applied Biosystems Prism Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Perkin-Elmer/Applied Biosystems Division, Foster City, Calif.) according to directions provided by the manufacturer. Primers used for cycle sequencing of $bla_{SHV}$ are shown in Table 2; those for $bla_{TEM}$ were described previously (23). Excess dye-labeled terminators were removed from extension products by using spin columns (CentriSep; Princeton Separations, Adelphia, N.J.). The extension products were then evaporated to dryness (SpeedVac; Savant Instruments, Holbrook, N.Y.) and stored at −20°C. Each sample was resuspended in 6 μl of loading buffer (5:1 deionized formamide–50 mM EDTA [pH 8] plus dextran blue [30 mg/ml]), heated for 2 min at 95°C, and applied to an automated Model 373A Sequencer (Perkin-Elmer/Applied Biosystems, Inc.).

## RESULTS

**Antimicrobial susceptibility patterns of *E. coli* blood isolates for eight antimicrobial agents.** The MICs for the nine *E. coli* isolates are shown in Table 1. An increase in the MICs of CAZ, CTX, cefuroxime, cefotetan, cefoxitin, and ATM was first noted with isolate 2-77 recovered on 29 November. The five isolates showed three phenotypic patterns; isolates 2-75, 2-76, and 7-35 had a similar pattern. The last isolate, 7-36, demonstrated a slightly different antibiogram from 7-35, which was a coisolate from the same blood culture vial. The MICs of the extended-spectrum cephalosporins and ATM were reduced by 2 to 6 dilutions when incubated in the presence of 10 μg of clavulanic acid per ml (Table 3). Resistance to gentamicin, tetracycline, and sulfonamides was demonstrated only for isolate 7-30, which was recovered from the same blood culture vial as isolate 7-29.

An effect of clavulanic acid on the size of the zones of inhibition around ATM, CAZ, and CTX disks in a disk diffusion assay was also noted (Table 3). Increased zone diameters in the presence of clavulanic acid were observed first with CAZ for isolate 2-77 and then for all subsequent isolates. This, along with the concomitant decrease in the cephalosporin MICs with clavulanic acid noted above, was suggestive of ESBL activity for these five isolates. Although no augmentation of zone sizes or changes in MICs were noted with cefoxitin, clavulanate did apparently decrease the MICs of cefotetan by as much as 3 dilutions, an effect which was confirmed on subsequent testing. This effect is unusual for ESBLs.

**PCR analysis, DNA hybridization studies, and plasmid fingerprinting.** PCR analysis revealed that all nine isolates contained a $bla_{TEM}$ β-lactamase gene, but only isolates with elevated CAZ MICs were positive for $bla_{SHV}$ (Fig. 2). Eight of the nine isolates had the same plasmid pattern, which showed three high-molecular-weight plasmids, two of which have similar molecular sizes of ~90 kb and appeared as a single band and the third of which was approximately 120 kb (Table 4). In isolate 7-30, the largest of the three plasmids was increased slightly in size. Unlike the other eight isolates, isolate 7-30 was resistant to gentamicin, tetracycline, and sulfonamides. While

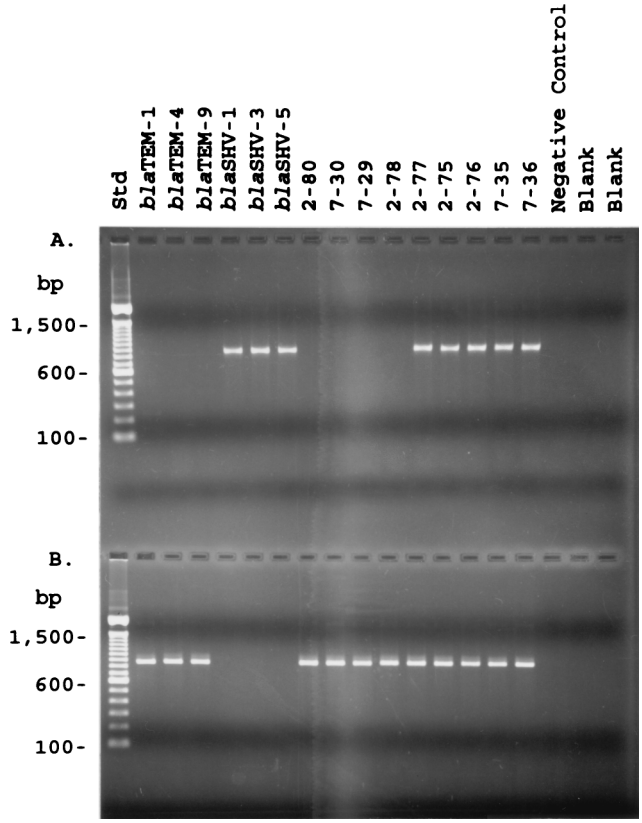


FIG. 2. Agarose gel showing products of PCR amplification from the nine *E. coli* isolates with $bla_{SHV}$ (A)- and $bla_{TEM}$ (B)-specific primers. The $bla_{SHV}$ positive controls were *E. coli* isolates containing $bla_{SHV-1}$, $bla_{SHV-3}$, and $bla_{SHV-5}$. The positive controls for $bla_{TEM}$ were *E. coli* isolates containing $bla_{TEM-1}$, $bla_{TEM-4}$, and $bla_{TEM-9}$. Negative controls were reagent blanks with no DNA and *E. coli* F50, which is ampicillin susceptible. The molecular size standard is a 100-bp DNA ladder.

TABLE 4. Molecular characteristics of *E. coli* blood isolates

| No. | Date (mo/day) | $bla_{TEM}$ PCR[a] | $bla_{SHV}$ PCR | Plasmid profile[b] | PFGE type[c] | IEP result[d] | Porin result (no. of bands) | *bla* gene content |
|---|---|---|---|---|---|---|---|---|
| 2-80 | 10/28 | + | − | 120, 95, 90 | A | 5.4 | 3 | $bla_{TEM-1}$ |
| 7-29 | 11/6 | + | − | 120, 95, 90 | A | 5.4 | 3 | $bla_{TEM-1}$ |
| 7-30 | 11/6 | + | − | 135, 95, 90 | A1 | 5.4 | 3 | $bla_{TEM-1}$ |
| 2-78 | 11/6 | + | − | 120, 95, 90 | A | 5.4 | 3 | $bla_{TEM-1}$ |
| 2-77 | 11/29 | + | + | 120, 95, 90 | A2 | 5.4, 7.6 | 2 | $bla_{TEM-1}$, $bla_{SHV-1}$ |
| 2-75 | 12/23 | + | + | 120, 95, 90 | A3 | 5.4, 7.6 | 2 | $bla_{TEM-1}$, $bla_{SHV-8}$ |
| 2-76 | 12/26 | + | + | 120, 95, 90 | A4 | 5.4, 7.6 | 2 | $bla_{TEM-1}$, $bla_{SHV-8}$ |
| 7-35 | 12/27 | + | + | 120, 95, 90 | A5 | 5.4, 7.6 | 3 | $bla_{TEM-1}$, $bla_{SHV-8}$ |
| 7-36 | 12/27 | + | + | 120, 95, 90 | A6 | 5.4, 7.6 | 3 | $bla_{TEM-1}$, $bla_{SHV-8}$ |

[a] +, presence of $bla_{TEM}$ gene by PCR; −, absence of $bla_{TEM}$ gene by PCR.
[b] Plasmid sizes given in kilobases.
[c] All isolates showed subtypes of pattern A.
[d] IEP, isoelectric focusing; pIs of bands indicated by nitrocefin staining of gels.

gentamicin and sulfonamide resistance could be transferred to a recipient *E. coli* strain via conjugation, resistance to ampicillin, CAZ, and other cephalosporins could not be transferred even during extended mating times. Plasmid DNA was transferred to nylon filters and hybridized with a $bla_{TEM}$-specific probe which confirmed the presence of a $bla_{TEM}$ gene on one of the 90-kb plasmids in each of the nine isolates (data not shown). However, a $bla_{SHV}$-specific DNA probe failed to hybridize to plasmid DNA but did hybridize to the chromosomal band on the plasmid fingerprinting gels from the latter five isolates (data not shown). Hybridization studies to identify the gentamicin, sulfonamide, and tetracycline resistance genes were not undertaken.

**Chromosomal DNA analysis by PFGE.** The PFGE restriction profiles of the DNA from the nine isolates after cleavage with *Xba*I are shown in Fig. 3 (lanes 3 to 11). The patterns for the nine *E. coli* isolates, each consisting of 16 to 18 bands ranging from 48.5 to ~600 kb, have many bands in common in the size range from 50 to 388 kb. However, differences of one to four bands can be seen among the isolates, especially in the large-molecular-size bands. The overall patterns appear similar, particularly compared with the two epidemiologically unrelated *E. coli* strains included as controls (lanes 1 and 2) and additional unrelated isolates from the same hospital (data not shown), which had very different patterns. Isolate 2-77 (lane 7) shows an apparent increase in the size of a single band compared to the first four isolates and is similar to the pattern of isolate 2-76 (lane 9). Isolates 7-35 and 7-36 (lanes 10 and 11), obtained from the same blood culture vial, appear to have patterns similar to isolate 2-75 (lane 8). The PFGE profiles of the DNA from the nine isolates after cleavage with *Sfi*I showed less diversity, with only one to three band differences (data not shown).

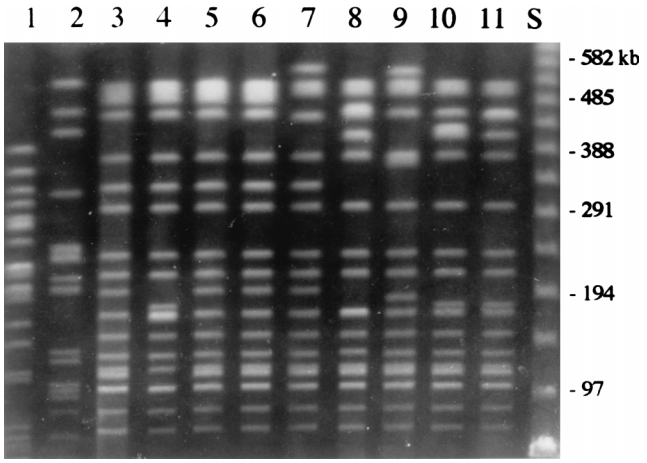


FIG. 3. Agarose gel demonstrating the patterns generated by PFGE of *Xba*I-digested chromosomal DNA obtained from the nine *E. coli* blood isolates. Lanes 1 and 2, unrelated *E. coli* isolates; lane 3, isolate 2-80; lane 4, 7-30; lane 5, 7-29; lane 6, 2-78; lane 7, 2-77; lane 8, 2-75; lane 9, 2-76; lane 10, 7-35; lane 11, 7-36. S, molecular size standards.

**AP-PCR typing.** The AP-PCR profiles for the nine *E. coli* isolates had five identical major bands and several additional minor bands that are similar in size but different in their intensity of staining (data not shown). The overall profile of the blood isolates is different from the profiles of the unrelated *E. coli* strains.

**Isoelectric focusing and nitrocefin hydrolysis studies.** Isoelectric focusing of crude cell lysates demonstrated that the CAZ-susceptible isolates contained only a β-lactamase with a

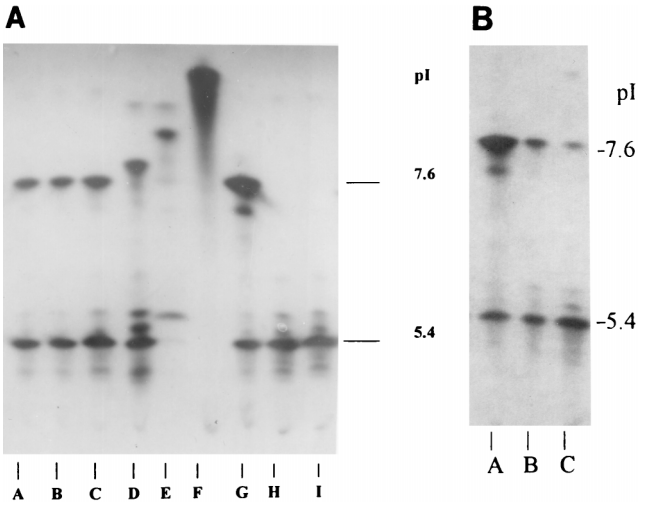


FIG. 4. (A) Isoelectric focusing patterns of β-lactamases produced by *E. coli* clinical isolates and controls. Lane A, isolate 2-75 (CAZ resistant); lane B, 2-76 (CAZ resistant); lane C, 7-35 (CAZ resistant); lane D, *E. coli* containing the $bla_{TEM-1}$ gene (pI, 5.4) and $bla_{SHV-4}$ (pI, 7.75); lane E, *E. coli* containing $bla_{SHV-5}$ (pI, 8.2); lane F, *E. coli* 87120702 containing AmpC (pI, 8.5); lane G, isolate 2-77 (CAZ resistant); lane H, 2-78 (CAZ susceptible); lane I, 2-80 (CAZ susceptible). (B) Isoelectric focusing patterns of β-lactamases produced by *E. coli* isolate 2-77 (CAZ resistant) (lane A), 7-35 (CAZ resistant) (lane B), and *E. coli* RT17-121 producing TEM-1 and SHV-1 (CAZ susceptible) (lane C). Each lane was standardized to contain equivalent units of β-lactamase activity.

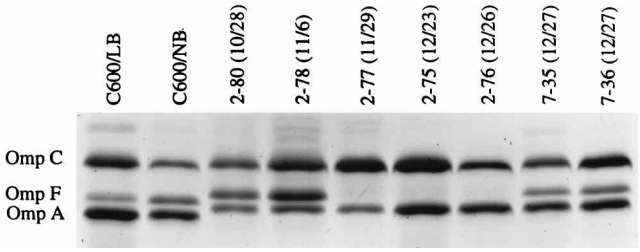


FIG. 5. Porin profiles of *E. coli* clinical isolates and controls. Lane 1, *E. coli* C600 grown in L broth (LB); lane 2, *E. coli* C600 grown in nutrient broth (NB); lane 3, isolate 2-80 (CAZ susceptible); lane 4, 2-78 (CAZ susceptible); lane 5, 2-77 (CAZ resistant); lane 6, 2-75 (CAZ resistant); lane 7, 2-76 (CAZ resistant); lane 8, 7-35 (CAZ resistant); lane 9, 7-36 (CAZ resistant).

pI of 5.4 (Fig. 4A, lanes H and I) while the isolates with elevated CAZ MICs demonstrated two β-lactamases with pIs of approximately 5.4 (TEM-1) and 7.6 (SHV-like) (Fig. 4A, lanes A, B, C, and G). The bands with pIs of 7.6 from isolates 2-75, 2-76, 2-77, and 7-35 have the same migration profile as those of SHV-1 when run in tandem on other gels (data not shown). Isolate 2-77 (lane G), the first resistant isolate, appears to produce more of the SHV β-lactamase (pI, 7.6) than of TEM-1. In Fig. 4B, the isoelectric patterns of 2-77 (lane A) and 7-35 (lane B) are compared to the ampicillin-resistant, CAZ-susceptible *E. coli* strain RT17-121, which also produces both TEM-1 and SHV-1 (lane C). All three lanes contained protein concentrations that were standardized to equivalent units of activity. This clearly shows that 2-77 produces greater amounts of the SHV-1 enzyme. The β-lactamase activity of the three isolates was measured by determining their rates of nitrocefin hydrolysis. The rates of hydrolysis were 7.82 ± 0.22 μM/min/mg of protein for strain 2-77, 2.85 ± 0.1 μM/min/mg of protein for 7-35, and 3.94 ± 0.1 μM/min/mg of protein for RT17-121. This is consistent with the hypothesis that increased production of the SHV-1 enzyme in 2-77 contributed to its CAZ resistance.

**Porin analysis.** Outer membrane proteins were prepared from all nine *E. coli* isolates and compared to the proteins isolated from *E. coli* C600 (Fig. 5). The porin profiles of two of the four CAZ-susceptible isolates (2-80 and 2-78) show three bands resembling the profile of the *E. coli* C600 control. The other two CAZ-susceptible isolates had identical porin profiles (data not shown). The porin profiles of the first three CAZ-resistant isolates, 2-77, 2-75, and 2-76, however, show loss of one of the major porins. Interestingly, the porins of the latter two CAZ-resistant isolates, 7-35 and 7-36, recovered from the same blood culture vial, appear similar to the CAZ-susceptible isolates, although the respective quantities of the bands corresponding to OmpA and OmpF may have changed.

**DNA sequence analysis.** DNA sequence analysis of the PCR products generated by amplification of the open reading frames of the $bla_{TEM}$ and $bla_{SHV}$ determinants with specific primers demonstrated that all nine isolates contained a $bla_{TEM-1}$ determinant (50). In addition, the five CAZ-resistant isolates each contained a $bla_{SHV}$-type gene. Isolate 2-77, the first of the CAZ-resistant isolates (MIC, 32 μg/ml), contained a $bla_{SHV-1}$ gene. The latter four isolates contained a novel gene, designated $bla_{SHV-8}$ (Fig. 6), which has a novel Asp-to-Asn mutation at position 179 (by the numbering scheme of Ambler et al. [1]), the site that anchors the conserved Ω loop opposite Arg-164 in class A β-lactamases (21, 40, 53). This is a result of a single base mutation from G to A at position 1453

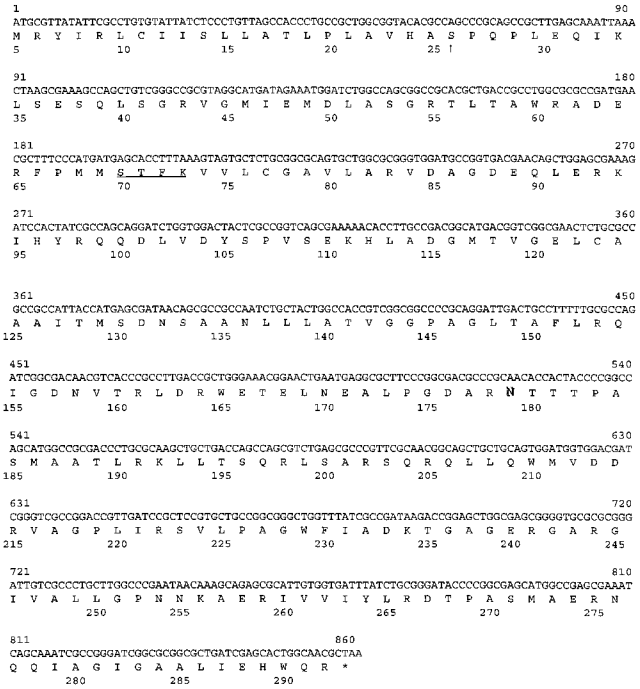


FIG. 6. Nucleotide sequence and predicted amino acid sequence of the $bla_{SHV-8}$ β-lactamase gene. Amino acid numbering is according to the consensus numbering of Ambler et al. (1). Amino acids 238 and 240 are adjacent because of a deletion observed within the class A consensus sequence. ↑, start of the mature protein, as determined previously (5, 38). The underlined portion of the amino acid sequence indicates the active site Ser-X-X-Lys motif. Amino acid 179 (shadowed and slightly enlarged) represents the only significant change from the amino acid sequence of SHV-1 (4, 5).

(designated 523 in Fig. 6) of the $bla_{SHV-1}$ gene (43). A comparison of key amino acid sites is shown in Table 5.

## DISCUSSION

This study demonstrates the evolution of extended-spectrum β-lactam resistance in a strain of *E. coli* recovered from multiple blood cultures obtained from the same patient over a 3-month period. The first four isolates were susceptible to oxyimino cephalosporins, ATM, and cephamycins whereas the last five isolates demonstrated resistance to these classes of drugs. The child had received several courses of CAZ and CTX

TABLE 5. Comparative amino acid sequences of SHV-type β-lactamases at selected positions

| β-Lactamase | pI of β-lactamase | Amino acid at position[a]: | | | | | | Reference(s) |
|---|---|---|---|---|---|---|---|---|
| | | 8 | 43 | 179 | 205 | 238 | 240 | |
| SHV-1 | 7.6 | Ile | Arg | Asp | Arg | Gly | Glu | 4 |
| SHV-2 | 7.6 | Ile | Arg | Asp | Arg | **Ser** | Glu | 5, 11, 17, 43, 44 |
| SHV-3 | 7.0 | Ile | Arg | Asp | **Leu** | **Ser** | Glu | 34 |
| SHV-4 | 7.8 | Ile | Arg | Asp | **Leu** | **Ser** | **Lys** | 38 |
| SHV-5 | 8.2 | Ile | Arg | Asp | Arg | **Ser** | **Lys** | 6 |
| SHV-7[b] | 7.6 | **Phe** | **Ser** | Asp | Arg | **Ser** | **Lys** | 8 |
| SHV-8 | 7.6 | Ile | Arg | **Asn** | Arg | Gly | Glu | This study |

[a] Location of selected amino acids according to the consensus numbering of Ambler et al. (1). Amino acids in boldface type represent changes from SHV-1 at these positions (4).

[b] SHV-6 has been described biochemically, but sequencing data are not yet available (2).

therapy that provided the selective pressure for the emergence of resistance.

The results of plasmid fingerprinting, PFGE, and AP-PCR typing studies and the similarity of the porin profiles suggest that these isolates are clonal. The variability of the *Xba*I PFGE patterns was surprising since we had anticipated that the patterns would be identical based on the plasmid fingerprinting and AP-PCR data. Even among the first four CAZ-susceptible isolates, minor variations in banding patterns were observed. The variability of the banding patterns probably reflects the changes in the genomic sequences resulting from acquisition of the $bla_{SHV}$ gene and the changes in the porin genes of the latter five isolates. The *Sfi*I restriction patterns did show less variability, however, reinforcing our hypothesis that these organisms were isolates of the same strain. Such variability in PFGE patterns among closely related isolates has been reported previously by Gouby et al. for *K. pneumoniae* (13) and for *E. coli* by Maslow et al. (25). The selective pressure provided by the prolonged intensive antimicrobial chemotherapy may also have contributed to these variations by causing changes not reflected in the phenotypes assayed.

The increase in CAZ MICs and the MICs of the other cephalosporins in the five resistant isolates was due to the acquisition of a $bla_{SHV}$ gene, which was suggested by the modulation of both MICs and disk diffusion zone sizes by clavulanic acid, and in three isolates by concomitant porin changes. Decreases in MICs but not augmentation of zone sizes was also observed for the cephamycin cefotetan, which is unusual for known ESBLs (9, 18, 36). The mechanism of this is unknown.

While a $bla_{TEM-1}$ gene was detected in all nine isolates, a $bla_{SHV-1}$ gene was detected in the first CAZ-resistant isolate (2-77), and the novel $bla_{SHV-8}$ gene was identified in the latter four resistant isolates. The $bla_{SHV-8}$ gene contains the GGC codon, which results in a Gly at position 238, which is typical of the $bla_{SHV-1}$ and different from the other $bla_{SHV}$ genes, which have a Ser at that position (Table 5), and a single nucleotide change of G to A at position 1453 (1, 43). The latter base change predicts an amino acid change of Asp to Asn at position 179. Our predicted amino acid sequence matches the SHV-1 sequence of Barthélémy et al. (5) with the exception of the reversal of Ala and Thr at positions 140 and 141 (according to the numbering system of Ambler et al. [1]) previously noted by Podbielski and Melzer (43) and the change at position 179. A change of Asp to Asn at position 179, when introduced by Vakulenko et al. into the TEM-1 β-lactamase produced by pUC19, resulted in an increase in CAZ resistance and decrease in the ampicillin MIC for the host organism (53). Knox has suggested that the decrease in the ampicillin MIC is due to the decrease in the catalytic efficiency of the enzyme (21), which is likely due to alteration of the salt bridge formed by amino acids 164 and 179 which links the two ends of the conserved Ω loop of the β-lactamase. This change results in increased movement of the destabilized loop, which expands the binding site allowing hydrolysis of cephalosporins, such as CAZ, resulting in their inactivation (21, 40). A similar change has been detected in the staphylococcal β-lactamase PC1 (15) but not, to date, in clinical isolates of staphylococci or in enzymes from gram-negative organisms. While many mutants of $bla_{TEM-1}$ have substitutions at residue 164 that destabilize the Ω loop, this is the first example of an Ω-loop-loosening mutation in an SHV-type enzyme. As Herzberg et al. (15) note, the fact that amino acid 179 is buried within the fold of the enzyme may explain why the isoelectric points for both SHV-1 and SHV-8 are the same.

The increase in the MICs of the cephamycins was intriguing and suggested a concurrent change in the organism's porins. Porin changes in an ESBL-producing strain of *K. pneumoniae* that manifested cefoxitin resistance have been reported previously (37). Porin changes also are associated with increases in CAZ resistance (3). Thus, in isolate 2-77, which contained a $bla_{TEM-1}$ and a $bla_{SHV-1}$ gene, the low-level CAZ resistance is likely attributable to the porin changes and to the hyperproduction of the SHV-1. Such hyperproduction, as suggested by the nitrocefin hydrolysis studies, is consistent with the report of Petit et al. (39), who noted that CAZ resistance in particular could be caused by large quantities of SHV-1. Changes in porin profiles were also noted for isolates 2-75 and 2-76, which may have contributed to the high-level CAZ resistance in addition to the change at Asp-179, which is known to significantly enhance CAZ resistance (40, 53). Interestingly, the porins appeared unchanged in the last two isolates (7-35 and 7-36), the latter of which also had a lower CAZ MIC. However, it is unclear whether the structure or function of the porins may have been modified, which may have affected the MICs of CAZ. This will require further study.

While it was possible to transfer gentamicin, sulfonamide, and tetracycline resistance genes in isolate 7-30 to a recipient strain of *E. coli* via conjugation, we were unable to transfer CAZ resistance with any of the latter five isolates via conjugal matings in broth or on nylon filters. Hybridization studies using $bla_{SHV}$-specific probes suggested that the genes were introduced into the chromosome of the parent organism and not onto plasmid DNA. The chromosomal location of the $bla_{SHV}$ genes is also supported by the conjugation experiments, although this negative data is recognizably less compelling. Nonetheless, the acquisition of new resistance genes and the simultaneous change in the organisms' porins show the tremendous ability of organisms to adapt under the selective pressure of antimicrobial therapy.

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