Bacteremia Due to Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae* in a Pediatric Oncology Ward: Clinical Features and Identification of Different Plasmids Carrying both SHV-5 and TEM-1 Genes

L. K. SIU,[1] PO-LIANG LU,[2] PO-REN HSUEH,[2,3] F. M. LIN,[1] SHAN-CHWEN CHANG,[2*]
KWEN-TAY LUH,[2,3] MONTO HO,[1] AND CHIN-YUN LEE[4]

*Division of Clinical Research, National Health Research Institute,[1] and Departments of Internal Medicine,[2]
Laboratory Medicine,[3] and Pediatrics,[4] National Taiwan University Hospital, Taipei, Taiwan*

Thirteen patients who had 16 episodes of bacteremia were observed between 1993 and 1997 in a pediatric oncology ward with a high background isolation rate of cefotaxime- or aztreonam-resistant gram-negative bacteria. Four blood isolates were *Escherichia coli* and 12 were *Klebsiella pneumoniae*, and these isolates harbored extended-spectrum β-lactamases (ESBLs). All episodes of bacteremia were nosocomial, all except one of the episodes occurred in neutropenic patients, and all patients were treated with piperacillin or ceftazidime with amikacin and cefazolin prior to the onset of bacteremia. Nine of 13 patients were receiving extended-spectrum β-lactam treatment when the bacteremias caused by ESBL producers occurred. Molecular studies revealed that four *K. pneumoniae* SHV-2-producing isolates from 1994 were of the same clone. Other ESBL producers, including six that carried both TEM-1 and SHV-5, five that carried SHV-5, and one that carried SHV-2 alone, were unrelated. In conclusion, SHV-5 was present in 11 of the 16 isolates and coexisted with TEM-1 in 6 isolates. Acquisition of resistance genes probably occurred under antibiotic selection pressure. This study highlights the importance of routine checks for and detection of ESBL producers. Effective therapy against ESBL producers should be considered early for children who have malignancies and neutropenia and who are septic, despite treatment with a regimen that includes an extended-spectrum β-lactam, in a clinical setting of an increased incidence of ESBL-producing bacteria.

Neutropenic patients are at high risk for various infectious diseases even if cultures of clinical specimens are not positive. Extended-spectrum β-lactam monotherapy or extended-spectrum β-lactam therapy in combination with an aminoglycoside is generally accepted as the empirical regimen for febrile neutropenia following chemotherapy for a malignancy (6, 7, 17, 27, 40). However, the choice of empirical antimicrobial therapy should be evaluated periodically to prevent treatment failure due to antimicrobial resistance. Combination therapy with an antipseudomonal β-lactam antibiotic such as piperacillin or ceftazidime with an aminoglycoside (amikacin usually) had been used extensively to treat patients with malignancies and infectious diseases in the pediatric oncology ward at the National Taiwan University Hospital (NTUH) in the past 10 years. Such combination therapy was particularly used in febrile neutropenic patients. However, recent reports of treatment failure due to extended-spectrum β-lactamase (ESBL)-producing bacteria were of great concern to clinical practice (3, 21). High incidences of infections caused by ESBL-producing bacteria in intensive care units were reported in different areas (14, 25). Concern about such drug-resistant bacterial infections had extended to nursing homes (43), geriatric (8) and pediatric (10, 12, 13) populations, transplant recipients (12), and oncology patients (16). Those infected were often fragile and unable to tolerate infectious diseases. Failure to identify ESBL producers by routine susceptibility testing may lead to inappro-

priate antimicrobial treatment and may result in increased mortality.

Gram-negative aerobic bacteria accounted for the majority of cases of nosocomial infection at NTUH (5). The frequency of extended-spectrum β-lactam-resistant *Klebsiella pneumoniae* at NTUH increased from 3.4% in 1993 to 10.3% in 1997, as determined by the disk diffusion method (18). An increasing prevalence of extended-spectrum β-lactam-resistant *Escherichia coli*, from 2.5% in 1993 to 6.7% in 1997, was also observed (24). Compared with the average data from a hospital-wide surveillance, the frequencies of extended-spectrum β-lactam-resistant *K. pneumoniae* and *E. coli* isolates in a pediatric ward were found to be three to five times higher (see below). In the current study, a retrospective survey with detailed molecular analysis was conducted to investigate the clinical significance of ESBL-producing *K. pneumoniae* and *E. coli* bacteremia in children with malignancies.

MATERIALS AND METHODS

Patients and bacterial strains. We searched the computerized database at NTUH, an 1,800-bed acute-care medical center, for *E. coli* and *K. pneumoniae* blood isolates resistant or intermediately susceptible to aztreonam or broad-spectrum cephalosporins in a pediatric oncology ward between 1993 and 1997. The pediatric oncology ward has 35 beds, and most patients were admitted for evaluation and management of malignant diseases. The medical records of patients harboring the studied microorganisms were reviewed. Neutropenia was defined as a polymorphonuclear cell count of ≤70°C before retrieval for testing.

Antimicrobial susceptibility testing. Antimicrobial susceptibility was determined by both the agar dilution and disk diffusion tests according to the National Committee for Clinical Laboratory Standards (NCCLS) (30, 31). For susceptibility testing by the agar dilution method, the following antimicrobial agents were obtained as standard reference powders of known potency for laboratory use: amoxicillin, ampicillin, and cephalothin (Sigma Chemical Co., St. Louis, Mo.);

* Corresponding author. Mailing address: Department of Internal Medicine, National Taiwan University Hospital, 7 Chung-Shan South Rd., Taipei, Taiwan. Phone: 886-2-2397-0800, ext. 5045. Fax: 886-2-2397-1412. E-mail: sc4030@ha.mc.ntu.edu.tw.

clavulanic acid (SmithKline Beecham, Brockhans Park, United Kingdom); piperacillin and tazobactam (Lederle Laboratories, Pearl River, N.Y.); cefmetazole (Sankyo Co., Hiratsuka, Japan); imipenem (Merck Sharp & Dohme, West Point, Pa.); cefotaxime (Hoechst Marion Roussel, Frankfurt, Germany); ceftazidime (Glaxo Group Research Limited, Greenford, United Kingdom); cefepime, amikacin, and aztreonam (Bristol-Myers Squibb Laboratories, Princeton, N.J.); meropenem (Sumitomo Pharmaceuticals Co., Osaka, Japan); and ciprofloxacin (Bayer Co., Leverkusen, Germany). All drugs were incorporated into Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) in serial twofold concentrations from 0.03 to 128 μg/ml. Two control strains, *E. coli* ATCC 35218 and ATCC 25922, were included in each test run. Inoculated plates were incubated in ambient air at 35°C for 16 to 18 h. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organism.

Screening tests for ESBL-producing strains. The double-disk synergy test and the Etest for ESBLs were used as screening tests to detect ESBL-producing strains. In the double-disk synergy test, the following antimicrobial disks were placed on Mueller-Hinton agar (BBL Microbiology Systems) adjacent to an amoxicillin-clavulanic acid disk (20 μg of amoxicillin plus 10 μg of clavulanate): cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), and cefepime (30 μg). All disks were purchased from Becton Dickinson Microbiology System (Sparks, Md.). The procedures and interpretation of the double-disk synergy test were as described previously (19).

The Etest ESBL screen (PDM Epsilometer; AB Biodisk, Solna, Sweden), based on the recognition of a reduction in the ceftazidime MIC in the presence of clavulanic acid, was performed according to the manufacturer's instructions.

Genomic fingerprinting by PFGE. Total DNA was prepared and pulsed-field gel electrophoresis (PFGE) was performed as described previously (1, 41). The restriction enzyme *Xba*I (New England Biolabs, Beverly, Mass.) was used at the manufacturer's suggested temperature. Restriction fragments were separated by PFGE in a 1% agarose gel (Bio-Rad, Hercules, Calif.) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA [pH 8.0]) with the Bio-Rad CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.). The initial pulse time of 1 s was increased linearly to 35 s in 20 h at 200 V at 4°C. The gels were then stained with ethidium bromide and photographed under UV light.

The band patterns were visually compared and were classified as indistinguishable (clonal), closely related (clonal variants, three or fewer band differences), possibly related (four to six band differences), and unrelated according to previously described criteria (42).

Plasmid isolation and resistance transfer. Plasmid profile analysis was performed by the alkaline extraction method (20). Resistance transfer was carried out by conjugation. A rifampin-resistant strain of *E. coli* (strain JP-995) was used as the recipient. Recipients and donors were separately inoculated into brain heart infusion broth (Oxoid, Basingstoke, England) and were incubated at 37°C for 4 h. They were then mixed at a volume ratio of 1:1 for overnight incubation at 37°C. A 0.01-ml volume of the overnight broth mixture was then spread onto a MacConkey agar plate containing rifampin (100 μg/ml) and either ceftazidime (1 μg/ml) or aztreonam (2 μg/ml).

Isoelectric focusing. The isoelectric focusing procedure was generally performed as described previously (28). Bacteria were harvested from a 20-h brain heart infusion broth culture by centrifugation, and the pellet was resuspended in 1 ml of phosphate buffer (0.05 M; pH 7). The enzymes were released by two cycles of freezing (−70°C) and thawing (room temperature) and by sonication for 5 min in a sonicator in ice-cold water. Isoelectric focusing was performed in an ampholine gel (pH 3.0 to 10.0; Pharmacia, Uppsala, Sweden). Preparations from standard strains known to harbor TEM-1, SHV-1, and SHV-5 were used as standards. After isoelectric focusing, β-lactamases were detected by spreading nitrocefin (50 μg/ml) on the gel surface.

Plasmid profile by restriction enzyme digestion. Plasmid DNA from the transconjugant was prepared as described previously (1). Restriction enzyme analysis of the plasmids from the transconjugants was performed according to the manufacturer's instructions. The restriction enzymes *Pvu*II and *Pst*I (Gibco BRL, Grand Island, N.Y.) were used. *Hin*dIII- and *Eco*RI-digested λ phage DNA was used as a molecular weight marker.

PCR amplification for *bla*TEM and *bla*SHV and direct DNA sequencing. The oligonucleotide primers (Gibco BRL) used for the PCR assay were as follows: 5′-ATAAAATTCTTGAAGACGAAA (primer A), 5′-GACAGTTACCAATG CTTAATCA (primer B), 5′-GGGTAATTCTTATTTGTCGC (primer C), and 5′-TTAGCGTTGCCAGTGCTC (primer D). Primers A and B were known to be specific for *bla*TEM (26). Primers C and D were known to be specific for *bla*SHV (37). Reactions were performed in a DNA thermal cycler (Bio-Rad) in 50-μl mixtures containing 2.5 U of *Taq* polymerase (Promega, Madison, Wis.) and 1× buffer consisting of 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01 μg of gelatin, each deoxynucleoside triphosphate at a concentration of 200 μM, and each oligonucleotide primer at a concentration of 2 μM. Thirty-five cycles were performed for each reaction, with the following temperature profile for each cycle: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min.

For direct DNA sequencing, PCR products were purified with MicroSpin S-300 HR PCR purification columns (Pharmacia). Sequencing reactions were performed with corresponding primers specific for the *bla*TEM and *bla*SHV genes (26, 37) by the method of Sanger et al. (38). An automated sequencer (377; ABI Prism, Perkin-Elmer, Norwalk, Conn.) was used.

RESULTS

Bacterial strains and clinical features. Forty-nine (56.3%) of 87 isolates of *K. pneumoniae* and 21 (18.6%) of 113 *E. coli* isolates obtained from all unselected clinical specimens from the pediatric oncology ward submitted to the clinical microbiology laboratory between 1993 and 1997 were resistant or intermediately susceptible to aztreonam or broad-spectrum cephalosporins by the disk diffusion method. Of the blood isolates, 46.2% (12 of 26) of the *K. pneumoniae* isolates and 23.1% (6 of 26) of the *E. coli* isolates were not susceptible to the extended-spectrum β-lactams. Of the 18 resistant blood isolates recovered from storage, one *E. coli* isolate was missing and another *E. coli* isolate was excluded because the MICs of aztreonam and the broad-spectrum cephalosporins for the strain were in the susceptible range (MICs, ≤2 μg/ml) when the MICs were determined by the agar dilution method. Twelve clinical isolates of *K. pneumoniae* (isolates kp1 to kp12) and four isolates of *E. coli* (isolates ec13 to ec16) were included in this study. Two isolates of *E. coli* (isolates ec13 and ec14) were obtained from the same patient on different occasions (2 days apart). Similarly, two pairs of *K. pneumoniae* isolates (isolates kp1 and kp2 and isolates kp10 and kp11) were from two individual patients and were recovered 2 and 4 days apart, respectively. A total of three episodes of *E. coli* bloodstream infection and 10 episodes of *K. pneumoniae* bloodstream infection in the pediatric ward from 1993 to 1997 were reviewed. Clinical data on 13 patients are summarized in Table 1. All patients had malignancies and received chemotherapy before their bacteremia occurred. Only one (patient 6) was not neutropenic when the multiple-drug-resistant strains were isolated. All these infection episodes were nosocomial. The infection focus could not be determined in most cases except for two episodes of catheter-related infection and two episodes of urinary tract infection (patients 1, 3, 4, and 12).

Patients had previously received piperacillin and/or ceftazidime with amikacin and/or cefazolin. Nine of the 10 patients were receiving an extended-spectrum β-lactam when they developed bacteremia caused by an ESBL-producing member of the family *Enterobacteriaceae*. Thereafter, nine patients received imipenem-containing regimens. Of these nine patients, four patients recovered, three died of causes not attributable to infection with an ESBL producer, and two died of mixed bacteremia or *K. pneumoniae* infection within 1 day of blood culturing. The other 4 patients (of a total of 13 patients) were treated with an extended-spectrum β-lactam-containing regimen, and only one of these patients died as a result of the infection with an ESBL-producing *K. pneumoniae* strain. One patient survived, one died of fungemia, and one died of *Stenotrophomonas maltophilia* infection.

Three patients (patients 1, 3, and 4) who did not die as a result of ESBL-producing *K. pneumoniae* bacteremia and who received extended-spectrum β-lactams had identified sources of infection, i.e., urinary tract infection or catheter-related infection. Their infected catheters were removed, and their antimicrobial regimens contained effective antibiotics. For two patients (patients 1 and 4) with catheter-related ESBL-producing *K. pneumoniae* infection, cefmetazole (MIC = 1 μg/ml for kp1) or amikacin (MIC = 0.5 μg/ml for kp5) was used. For patient 3, who had a urinary tract infection and bacteremia due to ESBL-producing *K. pneumoniae*, the isolate was susceptible to amikacin (MIC = 16 μg/ml), and he was successfully treated with amikacin with cefazolin and ceftazidime.

Three patients (patients 1, 9, and 11) had bacteremias 2 to 4 days after a prior bacteremic episode. When the second episode of bacteremia occurred, one patient (patient 1) was not

TABLE 1. Clinical features and review of antimicrobial agent use by patients with respect to detection of ESBL-producing bacteremia[a]

Patient	Isolate	Age (yr)	Sex	Infection focus	Malignancy	Antibiotic used when bacteremia occurred	Antibiotic used after detection (timing after bacteremia)[b]	Outcome
P1	kp1	14	F	Catheter-related infection	Ewing's sarcoma	Cefazolin, amikacin, piperacillin, amphotericin B	No change	Died of *Candida tropicalis* fungemia on day 34 after *K. pneumoniae* kp2 bacteremia
	kp2 (isolated 2 days after kp1 was isolated)						Cefmetazole, aztreonam, amphotericin B (within 1 day)	
P2	kp3	2	M	FUO	AML	Piperacillin, cefazolin, amikacin	Imipenem, gentamicin, amphotericin B (within 1 day)	Recovered
P3	kp4	1	F	Urinary tract infection	Neuroblastoma	Nil	Cefazolin, amikacin, ceftazidime (2 days)	Recovered
P4	kp5	9	M	Catheter-related infection	ALL	Cefazolin, amikacin, piperacillin, ceftazidime	Vancomycin, amikacin, ceftazidime, amphotericin B (within 1 day)	Died of *S. maltophilia* infection on day 36 after *K. pneumoniae* kp5 bacteremia
P5	kp6	6	M	FUO	AML	Cefazolin, amikacin, ceftazidime, amphotericin B, vancomycin	Imipenem, cefmetazole, amphotericin B (1 day)	Died of *S. maltophilia* infection on day 74 after *K. pneumoniae* kp6 bacteremia
P6	kp7	13	M	FUO	Ewing's sarcoma	Amikin, piperacillin, amphotericin B, cefazolin	Imipenem, gentamicin (4 days)	Died of seizure and apnea on day 34 after *K. pneumoniae* kp7 bacteremia
P7	kp8	16	M	FUO	ALL	Cefazolin, ceftazidime, amikacin	Vancomycin, imipenem, ciprofloxacin (within 1 day)	Died on day 2 after *K. pneumoniae* bacteremia
P8	kp9	13	M	FUO	ALL	Cefazolin, netilmicin, piperacillin, amphotericin B	Imipenem, cefmetazole, amphotericin B (2 days)	Recovered
P9	kp10	2	F	FUO	AML	Cefazolin, ceftazidime, amikacin	Imipenem (3 days)	Died on day 2 after *K. pneumoniae* kp11 and *Citrobacter freundii* bacteremia (*K. pneumoniae* and *C. freundii* were isolated on 2 separate days)
	kp11 (isolated 4 days after kp10 was isolated)					Imipenem	Imipenem, amphotericin B (within 1 day)	

Patient	Isolate	Age	Sex	Diagnosis	Underlying disease	Antibiotics before	Antibiotics after[b]	Outcome
P10	kp12	4	M	FUO	AML	Cefazolin, amikacin, ceftazidime, amphotericin B	No change	Died on day of *K. pneumoniae* bacteremia
P11	ec13	5	F	FUO	ALL	Ceftazidime	Imipenem (1 day)	Recovered
	ec14 (isolated 2 days after kp13 was isolated)					Imipenem	Imipenem, gentamicin (1 day)	
P12	ec15	3	M	Urinary tract infection	ALL	Nil	Imipenem, amikacin (2 days)	Recovered
P13	ec16	2	M	FUO	AML	Nil	Cefazolin, piperacillin, and tobramycin (within 1 day) and then imipenem and vancomycin (3 days)	Died of sepsis caused by unidentified pathogen on day 116 after *E. coli* ec16 bacteremia

[a] Abbreviations: F, female; M, male; FUO, fever of unknown origin; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia.

[b] The antibiotics used after the detection of bacteremia, including the antibiotics used empirically and antibiotics used after the culture report became available. The timing of the change in antibiotic when antibiotics were adjusted on the day of bacteremia is presented as "within 1 day."

receiving therapy effective against an ESBL producer and two patients (patients 9 and 11) had just begun (within 1 day) to receive imipenem.

Plasmid profile and transfer of resistance determinants. Comparison of plasmids isolated from clinical strains revealed that each contained one or two plasmids with molecular sizes of >90 kb. The gene encoding the ESBL could be transferred with either ceftazidime or aztreonam selection in most donors except for four *K. pneumoniae* isolates (isolates kp1 to kp4). Only one plasmid was transferred for each strain, and the resistance gene in each transconjugant was found to be located in a plasmid of >90 kb (data not shown).

PFGE analysis of donors and plasmid profiles of transconjugants. Two *E. coli* isolates (isolates ec13 and ec14) from the same patient had exactly the same total DNA profile by PFGE after *Xba*I digestion (Fig. 1A, lanes 13 and 14). Among the three patients with *E. coli* bacteremia, three different DNA profiles were identified by PFGE (Fig. 1A, lanes 15, 13 and 14, and 16). Regarding the *K. pneumoniae* isolates, four isolates (isolates kp1 to kp4) had indistinguishable PFGE patterns. The other eight *K. pneumoniae* isolates (Fig. 1B, lanes 5 to 12, respectively), including isolates kp10 and kp11 from the same patient, had unrelated PFGE patterns (Fig. 1B). Restriction enzyme digestion of plasmids showed that all four *E. coli* transconjugants (Fig. 2A) and eight *K. pneumoniae* transconjugants (isolates kp5 to kp12) (Fig. 2B) had different *Pst*I or *Puv*II digestion profiles, indicating that their plasmids were all different.

Antimicrobial susceptibility of clinical isolates and transconjugants. Results of the in vitro antimicrobial susceptibility tests are shown in Table 2. All 4 *E. coli* isolates and all 12 *K. pneumoniae* isolates were resistant to ampicillin and cefazolin and susceptible to cefepime, cefmetazole, imipenem, meropenem, and ciprofloxacin. The proportions of isolates susceptible to amoxicillin-clavulanic acid and piperacillin-tazobactam were 66.6 and 93.8%, respectively. The MICs of extended-spectrum β-lactams were not above the resistance breakpoint according to NCCLS criteria (31) for all clinical isolates. A total of 20, 26.6, 46.6, and 100% of the clinical isolates were susceptible to aztreonam, ceftazidime, cefotaxime, and cefepime, respectively. However, when clavulanic acid at a fixed concentration of 4 μg/ml was combined with ceftazidime, cefotaxime, aztreonam, or cefepime individually for susceptibility testing, the MICs decreased by more than 16 times for all isolates and all isolates became susceptible. The MICs of various antimicrobial agents for the 12 transconjugants were similar to those for the clinical isolates from which they were derived.

All 16 isolates from 13 patients yielded positive results by the double-disk synergy test and the Etest ESBL screening test.

Isoelectric focusing. Isoelectric focusing of sonic extracts of the strains followed by nitrocefin screening revealed two major bands with pIs of 5.4 and 8.2 for both the clinical isolates and the transconjugants of two *K. pneumoniae* isolates (isolates kp9 and kp12) and all four *E. coli* isolates. Five isolates (isolates kp1 to kp4 and kp10) had only one band with a pI of 7.6 and five isolates (isolates kp5 to kp8 and kp11) had a band with a pI of 8.2 (Table 3).

PCR amplification and sequencing of PCR products for *bla*$_{TEM}$ and *bla*$_{SHV}$. All four *E. coli* transconjugants were positive for both *bla*$_{TEM}$ and *bla*$_{SHV}$ by PCR amplification. The entire *bla*$_{TEM}$ sequence including the promoter region from the four *E. coli* isolates was found to be identical to the *bla*$_{TEM-1}$-encoding Tn*2* sequence (11). Comparison of the sequences at the *bla*$_{SHV}$ region of the four *E. coli* strains to the published SHV-1 gene sequence (29) revealed three nucleotide substitutions that caused two amino acid changes (position 238, Gly [GGC]→Ser [AGC]; position 240, Glu [GAG]→Lys [AAG]),
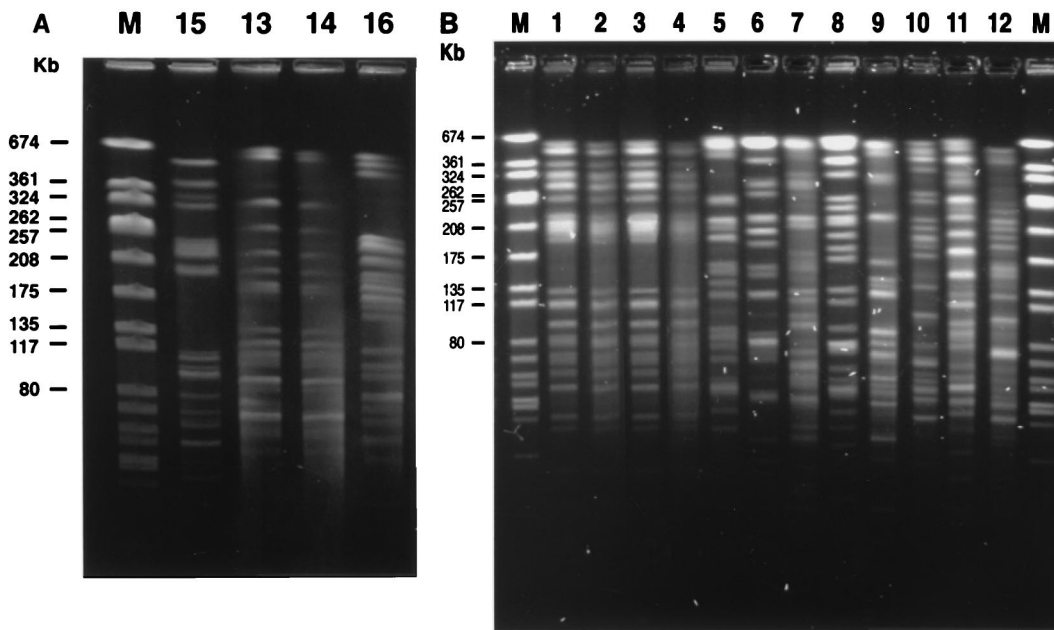
FIG. 1. Profiles produced by PFGE of macrorestriction fragments of ESBL-producing *E. coli* (A) and *K. pneumoniae* (B) isolates after digestion with *Xba*I. Lanes M, *Sma*I-digested *Staphylococcus aureus* NCTC 8325 DNA ladder as a molecular size marker. See Table 1 for the origins of the isolates. Lanes 1 to 12, isolates from patients 1 to 10, respectively; lane 15, isolate from patient 12; lanes 13 and 14, isolates from patient 11; lane 16, isolate from patient 13.

with the remaining one being silent (position 268, Thr [ACG]→Thr [ACC]). The amino acid sequences at the bla_{SHV} region of all four *E. coli* isolates were found to be identical to the published SHV-5 sequence (2). Two isolates of *K. pneumoniae* (isolates kp9 and kp12) also harbored both TEM-1 and SHV-5 genes, as did the four *E. coli* isolates. The other 10 *K. pneumoniae* isolates had only one SHV-type gene (Table 3). Five isolates had the SHV-5 gene. Five isolates had an SHV-2

gene with the characteristic of one nucleotide substitution that caused one amino acid change (position 238, Gly [GGC]→Ser [AGC]). Four SHV-2-producing *K. pneumoniae* isolates (isolates kp1 to kp4) did not have the silent mutation (position 268, Thr [ACG]→Thr [ACC]), whereas another SHV-2 producer (isolate kp10) and all the SHV-5-containing isolates had the same silent mutation that the *E. coli* isolates had.
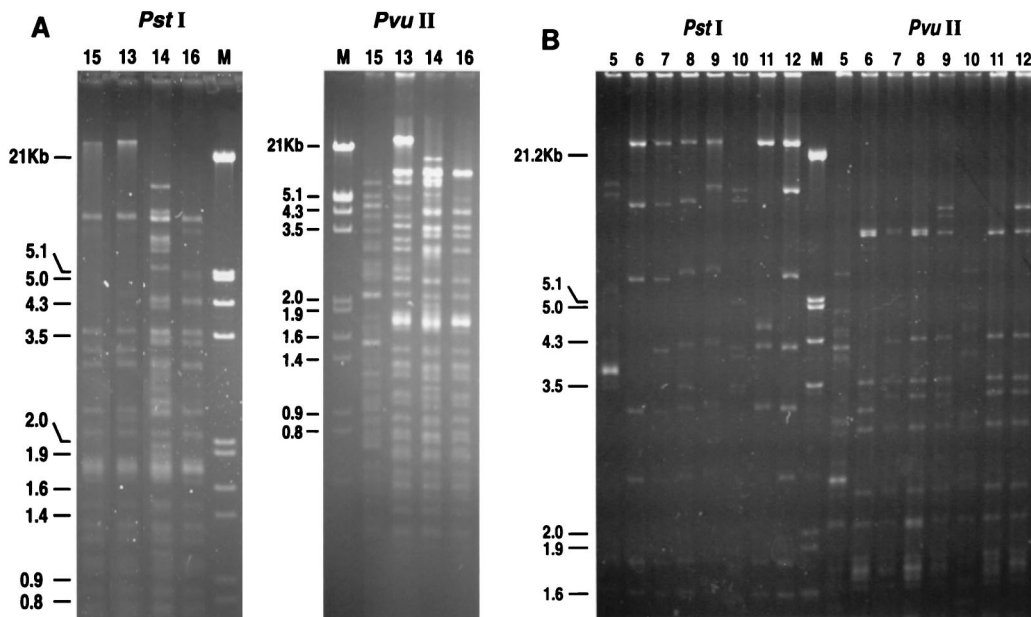

FIG. 2. *Pst*I and *Pvu*II digestion profiles of plasmids from transconjugants of *E. coli* (A) and *K. pneumoniae* (B). Lanes M, *Eco*RI-*Hin*dIII-digested λ phage DNA ladder; lanes 5 to 12 and 13 to 16, transconjugants of clinical isolates kp5 to 12 and ec13 to 16, respectively. See Table 1 for the origins of the clinical isolates.

TABLE 2. In vitro susceptibilities of the 4 *E. coli* isolates, 12
K. pneumoniae isolates, and their transconjugants determined
by the agar dilution method

Antibiotic[a]	MIC (μg/ml)[b]			% Susceptible strains
	Range	50%	90%	
ATM	2–64	16	64	25
CAZ	4–≥256	128	≥256	25
CAZ+CLA	0.06–4	0.5	4	
CTX	2–32	16	32	43.8
CTX+CLA	≤0.03–2	0.5	2	
FEP	0.12–2	1	2	100
AMP	16–≥256	≥256	≥256	0
AMX+CLA	2–32	8	32	62.5
CFZ	16–≥256	64	128	0
CMZ	0.5–16	2	16	100
AMK	0.5–16	4	16	100
IPM	0.06–0.5	0.12	0.25	100
CIP	≤0.03–1	≤0.03	0.5	100
PIP+TZB	1–128	2	16	93.8

[a] ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; AMP, ampicillin; AMX, amoxicillin; CFZ, cefazolin; CMZ, cefmetazole; AMK, amikacin; IPM, imipenem; CIP, ciprofloxacin; PIP, piperacillin; CLA, clavulanic acid (which was used at a fixed concentration of 4 μg/ml when tested with ceftazidime, cefotaxime, aztreonam, and cefepime and at a ratio of 2:1 when it was tested with amoxicillin); TZB, tazobactam (fixed concentration of 4 μg/ml).
[b] 50% and 90%, MICs at which 50 and 90%, respectively, are inhibited.

DISCUSSION

In the hospital studied, routine susceptibility testing was performed by the Kirby-Bauer disk diffusion test, and cefotaxime was the antibiotic representative of broad-spectrum cephalosporins, as recommended by NCCLS (30), unless a special request was made to test for susceptibility to another antimicrobial agent or the isolate was resistant to cefotaxime. The double-disk synergy test was not routinely used in our clinical microbiology laboratory. Since some ESBL producers may not be detectable by the routine use of only one extended-spectrum β-lactam, the prevalence of ESBL-producing members of the family *Enterobacteriaceae* might have been even higher than what was reported.

In this study, clonal spread was detected by PFGE in four isolates of *K. pneumoniae* from 3 patients (patients 1 to 3) that produced SHV-2 in 1994. The four isolates also shared the characteristic of not transferring their resistance by conjugation and lacked a silent mutation (position 268, Thr [ACG]→Thr [ACC]). The other *K. pneumoniae* isolates from different patients proved to be unrelated by PFGE. The differences in their plasmid digestion profiles also ruled out the possibility of plasmid dissemination in the ward studied.

Two plasmid-mediated β-lactamases, TEM-1 and SHV-5, were simultaneously found in two *K. pneumoniae* isolates (isolates kp9 and kp12) and in all four *E. coli* isolates. These resistance determinants were conjugatively transferable, and a plasmid of >90 kb was identified in all six transconjugants. However, the plasmid digestion profiles of these isolates revealed six different patterns, even for the two isolates from the same patient (isolates ec13 and ec14). The circulation of one resistant plasmid harboring two β-lactamase genes was thus excluded.

It is estimated that worldwide about 50% of clinical *E. coli* isolates produce a TEM-1 β-lactamase (23, 36), and TEM-1 accounts for about 80% of all plasmid-encoded β-lactamases in clinical members of the family *Enterobacteriaceae* (9). Although the prevalence of TEM-1 in Taiwan was not studied, considering the worldwide distribution of TEM-1 and the finding that, in 1998, 82% of the *E. coli* isolates in our hospital were ampicillin resistant, as determined by the routine disk diffusion method, the existence of TEM-1 in our *E. coli* isolates seems reasonable. The gene for SHV-5 has been reported to be the most common ESBL gene in *K. pneumoniae* isolates in another Taiwan hospital (22) and was the predominant ESBL gene in our *K. pneumoniae* isolates as well. That the spread of ESBL is mainly by patient-to-patient transfer rather than by direct selection of point mutation derivatives has been postulated on the basis of a clinical intervention study (34). The coexistence of TEM-1 and SHV-5 instead of the occurrence of TEM-1 mutant ESBLs in our *E. coli* isolates suggests a greater ease of acquisition of another ESBL gene compared with the development of a TEM-1 ESBL gene with a mutation.

There were three pairs of sequential isolates from three patients (Table 3). Two *E. coli* isolates from patient 11 recovered 2 days apart were of the same clone but had different plasmids. Although the existence of completely different plasmids in the two isolates was not impossible, this finding suggests the possible existence of a unit smaller than a plasmid, such as integrons or transposons (15), or the existence of an unstable plasmid that may change easily. The two *K. pneumoniae* isolates from patient 1 (isolates kp1 and kp2) and another two strains (isolates kp3 and kp4) had the same macrorestriction pattern by PFGE, suggesting a clonal origin. As for isolates kp10 and kp11, which were isolated from patient 9 4 days apart, the totally different macrorestriction patterns, ESBL genes, and plasmid digestion patterns suggest that these two strains were acquired independently of each other. There were thus three different bacteriologic patterns in patients with bacteremia caused by consecutive ESBL producers.

In the current study, most of the ESBL producers had initially been reported to be intermediately susceptible or resistant to cefotaxime. With even further determination of MICs by the agar dilution method, some isolates were susceptible to cefotaxime, ceftazidime, or aztreonam. According to the new recommendations by NCCLS in 1999 (32) for susceptibility testing of *E. coli* and *K. pneumoniae*, strains that are inhibited to a lesser degree than the normal susceptible population, even

TABLE 3. Molecular characterization of ESBL-producing *E. coli*
and *K. pneumoniae* isolates

Patient	Isolate	SHV	TEM	Macrorestriction genotype[a]	Plasmid digestion profile[b]	β-Lactamase pI[c]
P1	kp1	SHV-2		B	ND	7.6
P1	kp2	SHV-2		B	ND	7.6
P2	kp3	SHV-2		B	ND	7.6
P3	kp4	SHV-2		B	ND	7.6
P4	kp5	SHV-5		E	e	8.2
P5	kp6	SHV-5		F	f	8.2
P6	kp7	SHV-5		G	g	8.2
P7	kp8	SHV-5		H	h	8.2
P8	kp9	SHV-5	TEM-1	I	i	5.4, 8.2
P9	kp10	SHV-2		J	j	7.6
P9	kp11	SHV-5		K	k	8.2
P10	kp12	SHV-5	TEM-1	L	l	5.4, 8.2
P11	ec13	SHV-5	TEM-1	A	a	5.4, 8.2
P11	ec14	SHV-5	TEM-1	A	c	5.4, 8.2
P12	ec15	SHV-5	TEM-1	C	b	5.4, 8.2
P13	ec16	SHV-5	TEM-1	D	d	5.4, 8.2

[a] Macrorestriction genotypes were determined by PFGE after digestion with *Xba*I.
[b] Plasmid digestion profile for tranconjugants digested with *Pvu*II and *Pst*I. ND, not determined.
[c] β-Lactamase pIs were determined by isoelectric focusing.

4026 SIU ET AL.

though the MIC is lower than the standard breakpoint for resistance, should be screened for a potential ESBL. Resistance should be reported if the strain shows a positive inhibition test result (32). Since the SHV-5 β-lactamase is generally classified as a ceftazidimase and hydrolyzes cefotaxime less efficiently (4), if organisms harboring this enzyme are not tested according to the guidelines stated above but are tested only by a cefotaxime disk test, an inability to detect resistance and treatment failure might ensue (21).

Schiappa et al. (39) found that patients with bacteremia caused by ESBL-producing *E. coli* strains were more likely to survive if they received appropriate treatment within 3 days of the onset of the infection. A pediatric patient with leukemia who developed bacteremia caused by a cefotaxime-susceptible but ceftazidime-resistant *E. coli* strain died after receiving cefotaxime for less than 1 day (39). In addition, another study suggested that mortality was significantly lower when a carbapenem regimen instead of a noncarbapenem regimen was used in the first 5 days of bacteremia (35). Delays in the use of antibiotics effective against ESBL producers may result in delayed clearance of bacteremia, as demonstrated in the three patients (patients 1, 9, and 11) with two episodes of bacteremia. For patient 9 the outcome of the second bacteremia episode was fatal because of mixed ESBL-producing *K. pneumoniae* and *Citrobacter freundii* infections. Thus, timely identification of an ESBL producer is important, and policies for laboratory performance and antimicrobial therapy may need to be reevaluated to take this into account, especially when the prevalence of an ESBL producer is increasing in a specific setting.

Two of nine patients receiving imipenem-containing regimens died within 1 day of septicemia, and three of four patients receiving noncarbapenem therapy did not die as a result of bacteremia caused by an ESBL producer. No increased mortality among patients treated with antibiotics to which ESBL producers are susceptible was found. However, we cannot provide a definite suggestion about the appropriate treatment regimen for bacteremia caused by ESBL producers from the results of this study due to the small number of cases and the lack of a case-control design. Among the four patients receiving antibiotics to which ESBL producers are susceptible, two had catheter-related infections and one had a urinary tract infection. For the patients with catheter-related bacteremia caused by ESBL producers, immediate removal of the infected catheter and the use of an active antimicrobial agent other than imipenem may still produce a good outcome. Naumovski et al. (33) reported that seven children with nonbacteremic urinary tract infections were successfully treated with ceftazidime therapy, and they proposed that a high level of ceftazidime in urine was the reason for successful therapy. Ceftazidime and amikacin therapy for bacteremic urinary tract infection was shown to be effective for one patient in the study.

Prior administration of any antibiotic and prior ceftazidime or aztreonam administration were reported to be risk factors for the acquisition of ESBL-producing organisms (39). Every patient included in current study had received the standardized regimen containing an antipseudomonal β-lactam, amikacin, and cefazolin 3 weeks prior to bacteremia. However, the retrospective nature of this study could not prove definitely that prior antimicrobial administration was the risk factor. Nevertheless, the finding that 9 of 13 patients developed bacteremia caused by an ESBL-producing strain while under extended-spectrum β-lactam therapy argues for the occurrence of selection pressure.

In conclusion, we report on the clonal spread of ESBL-producing *K. pneumoniae* and the identification of *E. coli* and

K. pneumoniae isolates from blood with transferable resistance plasmids carrying both extended-spectrum (SHV-5) and restricted-spectrum (TEM-1) β-lactamase genes in a pediatric oncology ward. SHV-5 was the predominant ESBL detected in this study. The possibility that the ESBL gene may disseminate to other members of the family *Enterobacteriaceae* necessitates close monitoring of the ESBL producer to prevent such an occurrence. In a ward with high percentages of ESBL-producing *E. coli* and *K. pneumoniae* isolates, if symptomatic improvement is not seen during empirical treatment with a combination of an antipseudomonal β-lactam and an aminoglycoside, a change in the antibiotic treatment regimen to include agents that are active against ESBL producers, such as imipenem, should be promptly considered. The new recommendation by NCCLS to screen for ESBLs in *E. coli* and *K. pneumoniae* isolates should also be strictly enforced to avoid a false designation of susceptibility to broad-spectrum cephalosporins and a resulting clinical failure.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Morre, J. G. Seidman, and J. A. Smith.** 1995. Miniprep of bacterial genomic DNA. *In* Current protocols in molecular biology, unit 2.4.2. Massachusetts General Hospital and Harvard Medical School, Boston, Mass.
2. **Billot-Klein, D., L. Gutmann, and E. Collatz.** 1990. Nucleotide sequence of the SHV-5 β-lactamase gene of a *Klebsiella pneumoniae* plasmid. Antimicrob. Agents Chemother. **34:**2439–2441.
3. **Brun-Buisson, C., P. Legrand, A. Philippon, F. Montravers, M. Ansquer, and J. Duval.** 1987. Transferable enzymatic resistance to third-generation cephalosporins during nosocomial outbreak of multiresistant Klebsiella pneumoniae. Lancet **ii:**302–306.
4. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
5. **Chen, M. L., Y. C. Chen, H. J. Pan, S. C. Chang, L. S. Yang, S. W. Ho, K. T. Luh, W. C. Hsieh, and C. Y. Chuang.** 1995. Secular trends in the etiology of nosocomial infection at a teaching hospital in Taiwan, 1981–1994. Chin. J. Microbiol. Immunol. **28:**203–217.
6. **Chong, C. Y., A. M. Tan, and J. Lou.** 1998. Infections in acute lymphoblastic leukaemia. Ann. Acad. Med. Singapore. **27:**491–495.
7. **Cordonnier, C., R. Herbrecht, J. L. Pico, M. Gardembas, A. Delmer, M. Delain, P. Moreau, S. Ladeb, V. Nalet, C. Rollin, and J. J. Gres.** 1997. Cefepine/amikacin versus ceftazidime/amikacin as empirical therapy for febrile episodes in neutropenic patients: a comparative study. Clin. Infect. Dis. **24:**41–51.
8. **Cukier, L., P. Lutzler, A. Bizien, and J. L. Avril.** 1999. Investigation of an epidemic of an extended spectrum beta-lactamase producing *Escherichia coli* in a geriatrics department. Pathol. Biol. **47:**440–444.
9. **Du Bois, S. K., M. S. Marriott, and S. G. B. Amyes.** 1995. TEM- and SHV-derived extended-spectrum β-lactamases: relationship between selection, structure and function. J. Antimicrob. Chemother. **35:**7–22.
10. **Gniadkowski, M., A. Palucha, P. Grzesiowski, and W. Hryniewicz.** 1998. Outbreak of ceftazidime-resistant *Klebsiella pneumoniae* in a pediatric hospital in Warsaw, Poland: clonal spread of the TEM-47 extended-spectrum beta-lactamase (ESBL)-producing strain and transfer of a plasmid carrying the SHV-5-like ESBL-encoding gene. Antimicrob. Agents Chemother. **42:**3079–3085.
11. **Goussard, S., and P. Courvalin.** 1991. Sequence of the genes blaT-1B and blaT-2. Gene **15:**71–73.
12. **Green, M., and K. Barbadora.** 1998. Recovery of ceftazidime-resistant *Klebsiella pneumoniae* from pediatric liver and intestinal transplant recipients. Pediatr. Transplant. **2:**224–230.
13. **Grogan, J., H. Murphy, and K. Butler.** 1998. Extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in a Dublin paediatric hospital. Br. J. Biomed. Sci. **55:**111–117.
14. **Gunseren, F., L. Mamikoglu, S. Ozturk, M. Yucesoy, K. Biberoglu, N. Yulug, M. Doganay, B. Sumerkan, S. Kocagoz, S. Unal, S. Cetin, S. Calangu, I. Koksal, H. Leblebicioglu, and M. Gunaydin.** 1999. A surveillance study of antimicrobial resistance of gram-negative bacteria isolated from intensive care units in eight hospitals in Turkey. J. Antimicrob. Chemother. **43:**373–378.
15. **Heritage, J., P. M. Hawkey, N. Todd, and I. J. Lewis.** 1992. Transposition of the gene encoding a TEM-12 extended-spectrum beta-lactamase. Antimicrob. Agents Chemother. **36:**1981–1986.
16. **Hibbert-Rogers, L. C., J. Heritage, D. M. Gascoyne-Binzi, P. M. Hawkey, N. Todd, I. J. Lewis, and C. Bailey.** 1995. Molecular epidemiology of ceftazidime resistant Enterobacteriaceae from patients on a paediatric oncology ward. J. Antimicrob. Chemother. **36:**65–82.

17. **Hughes, W. T., D. Armstrong, G. P. Bodey, A. E. Brown, J. E. Edwards, R. Feld, P. Pizzo, K. V. Rolston, J. L. Shenep, and L. S. Young.** 1997. 1997 guidelines for the use of antimicrobial agents in neutropenic patients with unexplained fever. Clin. Infect. Dis. **25:**551–573.

18. **Jan, I. S., P. R. Hsueh, T. J. Teng, S. W. Ho, and K. T. Luh.** 1998. Antimicrobial susceptibility testing for *Klebsiella pneumoniae* isolates resistant to extended spectrum beta-lactam antibiotics. J. Formos. Med. Assoc. **97:**661–666.

19. **Jarlier, V., M. H. Nicolas, G. Fournier, and A. Philippon.** 1998. Extended broad-spectrum β-lactamases conferring resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. **10:**867–878.

20. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.

21. **Karas, J. A., D. G. Pillay, D. Muckart, and A. W. Sturm.** 1996. Treatment failure due to extended spectrum beta-lactamase. J. Antimicrob. Chemother. **37:**203–204.

22. **Liu, P. Y., J. C. Tung, S. C. Ke, and S. L. Chen.** 1998. Molecular epidemiology of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates in a district hospital in Taiwan. J. Clin. Microbiol. **36:**2759–2762.

23. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.

24. **Lu, P. L., P. R. Hsueh, C. C. Hung, S. C. Chang, and K. T. Luh.** 1998. Clinical feature of extended spectrum beta-lactam antibiotics resistant *Escherichia coli* bacteremia, p. 47. *In* Abstracts of the 1998 Annual Meeting of the Infectious Disease Society of Republic of China.

25. **Lucet, J. C., S. Chevret, D. Decre, D. Vanjak, A. Macrez, J. P. Bedos, M. Wolff, and B. Regnier.** 1996. Outbreak of multiply resistant Enterobacteriaceae in an intensive care unit: epidemiology and risk factors for acquisition. Clin. Infect. Dis. **22:**430–436.

26. **Mabilat, C., and S. Goussard.** 1993. PCR detection and identification of genes for extended-spectrum β-lactamases, p. 553–563. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.

27. **Maschmeyer, G., W. Hiddemann, H. Link, O. A. Cornely, D. Buchheidt, B. Glass, and D. Adam.** 1997. Management of infections during intensive treatment of hematologic malignancies. Ann. Hematol. **75:**9–16.

28. **Matthew, M., and A. M. Harris.** 1976. Identification of beta-lactamases by analytical isoelectric focusing: correlation with bacterial taxonomy. J. Gen. Microbiol. **94:**55–67.

29. **Mercier, J., and R. C. Levesque.** 1990. Cloning of SHV-2, OHIO-1, and OXA-6 beta-lactamases and cloning and sequencing of SHV-1 beta-lactamase. Antimicrob. Agents Chemother. **34:**1577–1583.

30. **National Committee for Clinical Laboratory Standards.** 1997. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.

31. **National Committee for Clinical Laboratory Standards.** 1997. Performance standards for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.

32. **National Committee for Clinical Laboratory Standards.** 1999. Performance standards for antimicrobial susceptibility testing: ninth information supplement, (M100-S9) vol. 19, no. 36. M2-A6 and M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.

33. **Naumovski, L., J. P. Quinn, D. Miyashiro, M. Patel, K. Bush, S. B. Singer, D. Graves, T. Palzkill, and A. M. Arvin.** 1992. Outbreak of ceftazidime resistance caused by extended-spectrum beta-lactamase in isolates from cancer patients. Antimicrob. Agents Chemother. **36:**1991–1996.

34. **Nordmann, P.** 1998. Trends in beta-lactam resistance among Enterobacteriaceae. Clin. Infect. Dis. **27**(Suppl. 1)**:**S100–S106.

35. **Paterson, D. L., W. Ko, A. V. Gottberg, S. Mohapatra, J. M. Casellas, L. Mulazimoglu, H. Goossens, F. Trenholme, K. Klugman, L. B. Rice, R. A. Bonomo, and V. L. Yu.** 1998. In vitro susceptibility and clinical outcome of bacteremia due to extended spectrum beta-lactamase (ESBL) producing *K. pneumoniae*, abstr. 188. *In* Abstracts of the 36th Annual Meeting of the Infectious Diseases Society of America.

36. **Philippon, A., G. Arlet, and P. H. Lagrange.** 1994. Origin and impact of plasmid-mediated extended-spectrum beta-lactamases. Eur. J. Clin. Microbiol. Infect. Dis. **13:**S17–S29.

37. **Rasheed, J. K., C. Jay, B. Metchock, F. Berkowitz, L. Weigel, J. Crellin, C. Steward, B. Hill, A. A. Medeiros, and F. C. Tenover.** 1997. Evolution of extended-spectrum beta-lactam resistance (SHV-8) in a strain of *Escherichia coli* during multiple episodes of bacteremia. Antimicrob. Agents Chemother. **41:**647–653.

38. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

39. **Schiappa, D. A., M. K. Hayden, M. G. Matushek, F. N. Hashemi, J. Sullivan, K. Y. Smith, D. Miyashiro, J. P. Quinn, R. A. Weinstein, and G. M. Trenholme.** 1996. Ceftazidime-resistant *Klebsiella pneumoniae* and *Escherichia coli* bloodstream infection: a case-control and molecular epidemiologic investigation. J. Infect. Dis. **174:**529–536.

40. **Schimpff, S. C.** 1986. Empiric antibiotic therapy for granulocytopenic cancer patients. Am. J. Med. **80:**13–20.

41. **Schoonmaker, D., T. Heimberger, and G. Birkhead.** 1992. Comparison of ribotyping and restriction enzyme analysis using pulsed-field gel electrophoresis for distinguishing *Legionella pneumophila* isolates obtained during a nosocomial outbreak. J. Clin. Microbiol. **30:**1491–1498.

42. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.

43. **Wiener, J., J. P. Quinn, P. A. Bradford, R. V. Goering, C. Nathan, K. Bush, and R. A. Weinstein.** 1999. Multiple antibiotic-resistant *Klebsiella* and *Escherichia coli* in nursing homes. JAMA **281:**517–523.