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Outbreak of Ceftazidime-Resistant *Klebsiella pneumoniae* in a Pediatric Hospital in Warsaw, Poland: Clonal Spread of the TEM-47 Extended-Spectrum β-Lactamase (ESBL)-Producing Strain and Transfer of a Plasmid Carrying the SHV-5-Like ESBL-Encoding Gene

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In 1996 a large, 300-bed pediatric hospital in Warsaw, Poland, started a program of monitoring infections caused by extended-spectrum β-lactamase (ESBL)-producing microorganisms. Over the first 3-month period eight *Klebsiella pneumoniae* isolates were identified as being resistant to ceftazidime. Six of these were found to produce the TEM-47 ESBL, which we first described in a *K. pneumoniae* strain recovered a year before in a pediatric hospital in Łódź, Poland, which is 140 km from Warsaw. Typing results revealed a very close relatedness among all these isolates, which suggested that the clonal outbreak in Warsaw was caused by a strain possibly imported from Łódź. The remaining two isolates expressed the SHV-5-like ESBL, which resulted from the horizontal transfer of a plasmid carrying the bla_{SHV} gene between nonrelated strains. The data presented here exemplify the complexity of the epidemiological situation concerning ESBL producers typical for large Polish hospitals, in which no ESBL-monitoring programs were in place prior to 1995.

The epidemiological situation concerning extended-spectrum β-lactamase (ESBL)-producing microorganisms is very dynamic and constitutes an increasing problem at health care centers in many countries (9, 22, 39). In a survey conducted in France from 1988 to 1990, the frequency of ESBL in nosocomial *Klebsiella pneumoniae* populations was estimated to be about 13%, but in some hospitals the prevalence of ESBL producers has been found to exceed even 40% of isolates (39). Over the past 15 years ESBLs have substantially diversified, and to date several families of the class A β-lactamases have been distinguished, with the TEM and SHV families predominant in terms of both numbers of isolations and numbers of enzyme variants (10, 11, 27). In several studies the presence of several different ESBLs in a single hospital environment has been documented (8, 13, 14). The first nosocomial outbreaks caused by ESBL-producing strains were recognized in 1985 in France (20, 32, 40), and since that time they have become common worldwide (21, 28, 34, 44). These outbreaks have been convincingly correlated with the extensive use of new β-lactam antibiotics (7, 28, 34).

ESBL genes are usually carried by plasmids, and some of them are located within transposable elements which strongly facilitate their spread between DNA replicons and bacterial strains of even different species (11, 17, 20, 25). Some ESBL outbreaks have been attributed to the dissemination of plasmids among strains of members of the family *Enterobacteriaceae* (20, 21, 35). In other cases, spread of a given ESBL in a single environment has been reported to be due to the appearance of the same gene within unrelated plasmids (7, 33). These plasmids have also frequently been found to carry genes responsible for resistance to other antibiotics, and this has resulted in the growing prevalence of multidrug-resistant organisms (21, 38). ESBL-producing strains can be maintained over prolonged periods of time in hospitals and can cause clonal outbreaks (16, 34, 46). They can be transferred between different wards, as well as between different hospitals or health care institutions and even, with the case of international travel, between different countries (7, 16, 34, 37). Multiple identifications of some ESBL variants in distant geographical regions seem, however, to result more likely from convergent evolutionary events (12, 18, 41).

In Polish hospitals no monitoring of ESBL occurrence was carried out before 1995, despite the increasing use of new broad-spectrum β-lactams. Our preliminary studies of single *K. pneumoniae* and *Escherichia coli* isolates from different hospitals from 1995 allowed us to identify novel, probably endemic variants of TEM ESBLs (14). Here we present the results of the initial phase of ESBL monitoring started in March 1996 in a large pediatric hospital in Warsaw, Poland. Analysis of a small sample of *K. pneumoniae* isolates collected over a short period has revealed the presence of two different ESBLs; one was clonally spread, probably endemic TEM-47 (14), and the other, the SHV-5-type β-lactamase that is found worldwide (27), was transferred with a plasmid. The clonal outbreak concerned three different wards of the hospital and was caused by the TEM-47-producing strain which had probably been imported from another large pediatric hospital, located in Łódź, Poland, a city ca. 140 km from Warsaw.

MATERIALS AND METHODS

Bacterial strains. Eight ceftazidime-resistant *K. pneumoniae* clinical isolates were identified by the microbiological laboratory of the University Children's Hospital in Warsaw from different patients over a 3-month period (March to May 1996). They were isolated from various specimens collected from patients

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TABLE 1. Clinical data, IEF of β-lactamases present in *K. pneumoniae* isolates and *E. coli* transconjugants, ESBLs identified by DNA sequencing, and typing patterns

Isolate Designation	Date of isolation (day.mo.yr)	Ward	Type of specimen	β-Lactamase pI	ESBL	RAPD pattern	RFLP pattern	*Eco*RI plasmid restriction pattern
1042/96	26.03.1996	Surgery	Throat swab	7.6 8.2[a]	SHV-5 type	B	B	B
1289/96	11.04.1996	Surgery	Stool	7.6 8.2	SHV-5 type	C	ND[b]	B
1027/96	23.03.1996	ICU	Blood	6.0 7.6	TEM-47	A	A1	A1
1099/96	09.04.1996	ICU	Tracheostomy tube	6.0 7.6	TEM-47	A	A2	A1
1294/96	13.04.1996	Hematology	Stool	6.0 7.6	TEM-47	A	A1	A1
1298/96	18.04.1996	Cardiology	Urine	6.0 7.6	TEM-47	A	A1	?[c]
1590/96	25.04.1996	ICU	Tracheostomy tube	6.0 7.6	TEM-47	A	A1	A1
1592/96	13.05.1996	ICU	Throat swab	6.0 7.6	TEM-47	A	A3	A2
L-267[d]	18.01.1995[d]	Łódź[d]	Throat swab	6.0 7.6[d]	TEM-47[d]	A[d]	A4	A3

[a] Underlined pI values refer to the pIs for β-lactamases which were also produced by transconjugants.
[b] ND, not determined.
[c] The restriction pattern was not clear; see Fig. 3.
[d] Reference 14.

located in different wards of the hospital. Clinical data concerning the isolates are presented in Table 1. All patients were highly predisposed to infections because all but one were under 1 year of age, were severely debilitated, having undergone various invasive procedures (surgery, insertion of a tube for parenteral nutrition, central line insertion, tracheostomy, and urinary catheterization), and were hospitalized for prolonged periods of time. Six patients were receiving antibiotics prior to *K. pneumoniae* isolation (cefotaxime, ceftazidime, and cefuroxime in two patients each). Species identification was performed by the ID32E ATB test (BioMerieux). All isolates were recognized as ESBL producers by the double-disc test (19). For comparative genotyping analyses, the clinical strain *K. pneumoniae* L-267, which was isolated at the Mother and Child Memorial Hospital in Łódź in January 1995, was used. This strain was shown in a previous study (14) to produce the TEM-47 ESBL. The L-414 (14) and L-934 *K. pneumoniae* strains, isolated in Łódź in January 1995 and in Sosnowiec, Poland, in June 1995, respectively, were used as epidemiologically nonrelated controls in the randomly amplified polymorphic DNA (RAPD) analysis. *E. coli* ATCC 25922 was used as the reference strain for antimicrobial susceptibility testing. The *E. coli* A15 R^- strain, which is resistant to nalidixic acid, was used as a recipient in the mating experiment.

Susceptibility testing. The MICs of various antibiotics were determined by the agar dilution method on Mueller-Hinton agar (Oxoid, Basingstoke, United Kingdom) according to the guidelines of the National Committee for Clinical Laboratory Standards (29). The following antibiotics were used: ampicillin, cefotaxime, and gentamicin (Polfa, Tarchomin, Poland); aztreonam (Bristol-Myers Squibb, New Brunswick, N.J.); ceftazidime (Glaxo Wellcome, Stevenage, United Kingdom); imipenem (Merck, Sharp & Dohme Research, Rahway, N.J.); lithium clavulanate (SmithKline Beecham Pharmaceuticals, Betchworth, United Kingdom); piperacillin (Lederle Piperacillin Inc., Carolina, Puerto Rico); tazobactam (Lederle Laboratories, Pearl River, N.Y.); and tobramycin (Eli Lilly, Indianapolis, Ind.). In all β-lactam–inhibitor combinations the constant concentrations of clavulanate and tazobactam were 2 and 4 μg/ml, respectively.

Transfer of resistance determinants. Equal volumes (1 ml) of cultures of the donor and the recipient strains (10^9 CFU/ml per strain) grown in tryptic soy broth (Oxoid) were mixed and incubated for 18 h at 35°C. Transconjugants were selected on MacConkey agar (Oxoid) supplemented with nalidixic acid (64 μg/ml; Sigma Chemical Company, St. Louis, Mo.), to inhibit the growth of donor strains, and ceftazidime (2 μg/ml), to inhibit the growth of the recipient strain.

IEF of β-lactamases. Crude preparations of β-lactamases (3) from clinical isolates and transconjugants were subjected to isoelectric focusing (IEF) by the procedure described by Matthew et al. (26), with modifications (3), with an LKB Multiphor apparatus (Pharmacia LKB). Following IEF, β-lactamase bands were visualized by staining with nitrocefin (Oxoid). The gels were run over a pH range of 3 to 10.

Assignment of ceftazidimase activity within the lane. After IEF the ceftazidimase activities of separate β-lactamases were detected by the bioassay approach as described previously (3). The ceftazidime concentration used in the bioassay was 2 μg/ml.

Genomic DNA preparation. Cultures of *K. pneumoniae* cells were grown overnight in tryptic soy broth (Oxoid) at 37°C. DNA was extracted from 200 μl of the cultures with the Genomic DNA Prep Plus kit (A & A Biotechnology, Gdańsk, Poland).

Plasmid DNA preparation. Plasmid DNA was purified from wild-type or transconjugant cells by the alkaline method (6) with the QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany), according to the manufacturer's procedure, as described previously (4).

PCR amplification of bla_{SHV} and bla_{TEM} genes. Primers SHV-A (5′-ACTGA ATGAGGCGCTTCC-3′) and SHV-B (5′-ATCCCGCAGATAAATCACC-3′) were used for partial amplification of bla_{SHV} genes (297 bp). Primers TEM-A and TEM-B (24) were used for amplifications of the entire bla_{TEM} genes. Plasmid DNA preparations from transconjugants were used as templates for bla_{SHV} amplifications, and total DNA preparations from clinical isolates were used for bla_{TEM} amplifications. The reactions were run as described previously (14). The resulting PCR products were run in 1% agarose gels (SeaKem; FMC Bioproducts, Rockland, Maine) and were purified for sequencing reactions by using a QIAquick PCR Purification Kit (QIAGEN).

DNA sequencing. Specific PCR products were subjected to direct sequencing reactions by the dideoxy chain termination method of Sanger et al. (36) with an automatic sequencer (373A; Applied Biosystems, Weiterstadt, Germany). Primers TEM-A, TEM-B, TEM-C, TEM-D, TEM-E, and TEM-F (24) were used for sequencing of the amplified bla_{TEM} genes. SHV-A and SHV-B primers (see above) were used for sequencing of the amplified parts of the bla_{SHV} genes. (Full sequences of the bla_{SHV} genes were not obtained due to the lack of DNA sequencing facilities in the Sera & Vaccines Research Laboratory in Warsaw, in which this work was continued; the first phase of the project, including studies of β-lactamases and sequencing, was performed in the Max von Pettenkofer-Institut in Munich, Germany).

RAPD typing. RAPD analysis was performed with the RAPD-7 (45) primer under the conditions described previously (14).

Genomic DNA RFLP typing. For the restriction fragment length polymorphism (RFLP) analysis, total DNA preparations embedded in 1% agarose plugs (InCert Agarose; FMC Bioproducts) were digested with the *Xba*I restriction enzyme (MBI Fermentas, Vilnius, Lithuania) and were separated in a 1% agarose gel (pulsed field certified; Bio-Rad, Hercules, Calif.) with a CHEF DRII pulsed-field gel electrophoresis system (Bio-Rad). The procedure was performed as described by Struelens et al. (42).

Plasmid fingerprinting. For the fingerprinting analysis, about 5 μg of plasmid DNA was digested with 10 U of the *Eco*RI or *Pst*I restriction enzyme (MBI Fermentas) for 2 h at 37°C. The resulting DNA fragments were electrophoresed in 1% agarose gels (Sigma Chemical Company).

RESULTS

Susceptibility testing of clinical isolates. The MICs of the different antibiotics obtained for the clinical isolates are presented in Table 2, together with the MICs for strain L-267 and its transconjugant R^+[*K. pneumoniae* L-267] (14). The MICs of aminoglycosides for the L-267 and R^+[*K. pneumoniae* L-267] strains were not presented before. All the isolates were uniformly resistant to ampicillin (MICs, > 512 μg/ml), piperacillin (MICs, ≥512 μg/ml), ceftazidime (MICs, 32 to 256 μg/ml), and aztreonam (MICs, 64 to 512 μg/ml). They were intermediate or fully resistant to cefotaxime (MICs, 16 to 64 μg/ml). All were susceptible to imipenem (MICs, 0.125 to 0.25 μg/ml). β-Lactam inhibitors reduced the MICs of piperacillin, ceftazidime, cefotaxime, and aztreonam; all isolates were proven to be susceptible to the combination of piperacillin and tazobactam (MICs, 2 to 8 μg/ml). The patterns of resistance to aminoglycosides discriminated two sets of isolates; all but two (isolates 1042/96 and 1289/96) were resistant to gentamicin

TABLE 2. Antimicrobial susceptibilities of clinical *K. pneumoniae* isolates and their *E. coli* transconjugants

Antibiotics[a]	MIC (μg/ml) for the following isolates:												
	1027/96	1099/96	1294/96	1298/96	1590/96	1592/96	1042/96	1289/96	R+[b] [1042/96]	R+ [1289/96]	L-267[c]	R+ [L-267][c]	A15 R^-
Ampicillin	>512	>512	>512	>512	>512	>512	>512	>512	512	512	>512	>512	2
Pip	>512	>512	>512	512	>512	>512	>512	>512	64	32	>512	256	0.5
Pip + Taz	2	2	2	2	2	4	8	4	0.5	0.5	8	0.5	0.5
Ctx	64	32	32	16	32	64	64	32	4	2	32	8	≤0.03
Ctx + Clav	0.06	0.06	0.125	0.06	0.06	0.125	≤0.03	0.06	≤0.03	≤0.03	0.25	≤0.03	≤0.03
Ctx + Taz	0.06	0.06	0.125	0.06	0.06	0.125	0.125	0.125	≤0.03	≤0.03	0.25	≤0.03	≤0.03
Caz	128	128	64	32	64	128	256	128	32	16	128	32	0.06
Caz + Clav	0.5	0.5	0.5	0.25	0.5	1	0.5	0.5	0.125	0.06	0.5	0.125	0.06
Caz + Taz	0.5	0.5	0.25	0.25	0.25	0.5	1	1	0.25	0.125	0.5	0.125	0.06
Atm	256	256	128	64	128	256	512	256	64	32	128	32	0.06
Atm + Clav	0.25	0.125	0.125	0.06	0.06	0.125	0.06	0.06	0.06	≤0.03	0.125	0.06	≤0.03
Atm + Taz	0.25	0.125	0.125	0.06	0.06	0.125	0.5	0.25	0.125	0.06	0.25	0.06	≤0.03
Imipenem	0.125	0.125	0.25	0.125	0.125	0.25	0.125	0.125	0.125	0.25	0.125	0.25	0.25
Gentamicin	64	64	64	64	64	128	0.25	0.125	0.125	0.125	64	16	0.125
Tobramycin	8	8	8	8	8	16	0.5	0.5	0.125	0.125	16	2	0.25

[a] Pip, piperacillin; Taz, tazobactam; Ctx, Cefotaxime; Clav, clavulanate; Caz, ceftazidime.
[b] R+, transconjugant strain.
[c] Reference 14 (excluding the MICs of aminoglycosides).

(MICs, 64 to 128 μg/ml) and fully or intermediately resistant to tobramycin (MICs, 8 to 16 μg/ml). The group of isolates resistant to the aminoglycosides tested was characterized by similar resistance patterns except for the pattern for isolate 1298/96, for which the MICs of β-lactam antibiotics were slightly lower. Antimicrobial MICs for these isolates were also found to be similar to those characterizing strain L-267 from Łódź (14).

Resistance transfer and susceptibility testing of transconjugants. The ceftazidime resistance transfer experiment was carried out with all the clinical isolates. Transconjugants selected on the medium supplemented with ceftazidime and nalidixic acid were obtained for only two of them; isolates 1042/96 and 1289/96. The efficiencies of transfer were 2.3×10^{-3} and 5.1×10^{-4} recombinants per donor cell, respectively. Results of the subsequent susceptibility testing of the R^+[*K. pneumoniae* 1042/96] and R^+[*K. pneumoniae* 1289/96] strains are listed in Table 2. The MICs obtained for the transconjugant strains were substantially lower but reflected very well the data obtained for the respective donor strains. As in the case for the clinical isolates, the MICs of ceftazidime (16 to 32 μg/ml) and aztreonam (32 to 64 μg/ml) were higher than those of cefotaxime (2 to 4 μg/ml).

IEF of β-lactamases and detection of ceftazidimase activity. The pI values of β-lactamases identified by analytical IEF in extracts of all clinical isolates and transconjugants are presented in Table 1. Isolates 1042/96 and 1289/96 produced two β-lactamases with pIs of 7.6 and 8.2. The transconjugant R^+[*K. pneumoniae* 1042/96] and R^+[*K. pneumoniae* 1289/96] strains were found to express an enzyme with a pI of 8.2, which was subsequently demonstrated to possess the ceftazidimase activity in the bioassay experiment (data not shown). This experiment was performed only with extracts of transconjugant strains. The remaining isolates, isolates 1027/96, 1099/96, 1294/96, 1298/96, 1590/96, and 1592/96, were found to produce another set of β-lactamases consisting of two enzymes with pI values of 6.0 and 7.6.

Identification of ESBL-encoding genes by specific PCR and DNA sequencing. Plasmid DNA preparations from the R^+[*K. pneumoniae* 1042/96] and R^+[*K. pneumoniae* 1289/96] transconjugant strains were used in specific PCRs with SHV-A and SHV-B primers (see Materials and Methods). PCR products of the expected size of about 0.3 kb representing the part of bla_{SHV} genes in which some of the mutations characterizing the SHV family of β-lactamases occur (5) were obtained in both cases (data not shown). For technical reasons (see Materials and Methods), only sequences of about 230 bp encompassing the regions between codons Thr180 and Arg259 (numbering according to Ambler et al. [1]) were determined and contained only a doublet of mutations responsible for the Gly238Ser and Glu240Lys amino acid substitutions when compared with the sequence of the p453 SHV-1 β-lactamase (2), which is characteristic for the corresponding parts of the bla_{SHV-5} (5), bla_{SHV-7} (8), or bla_{SHV-12} (31) gene.

Total genomic DNA preparations of isolates 1027/96, 1099/96, 1294/96, 1298/96, 1590/96, and 1592/96 (all of which produced the β-lactamase with a pI of 6.0) were used in PCRs with primers TEM-A and TEM-B (24) specific for the bla_{TEM} family genes. PCR products of the expected size of about 1.1 kb (the entire coding sequence of a bla_{TEM} gene together with the promoter region) were detected in all but one case. In the reaction with DNA extracted from isolate 1298/96, a product that was ca. 1 kb longer (total length, ca. 2.1 kb) was amplified (data not shown). All these products were subsequently sequenced and found to represent amplified genes coding for the TEM-47 enzyme (Table 1) identified in the previous study (14) in *K. pneumoniae* L-267 from the pediatric hospital in Łódź. The bla_{TEM-47} gene is characterized by three amino acid residue substitutions, G914→A (Gly238Ser), G917→A (Glu240Lys), and C990→T (Thr265Met), and four silent mutations, C226→T, C436→T, T682→C, and G925→A, when compared to the sequence of the bla_{TEM-1a} gene (43). (Numbering of the nucleotide positions is according to Sutcliffe 43].)

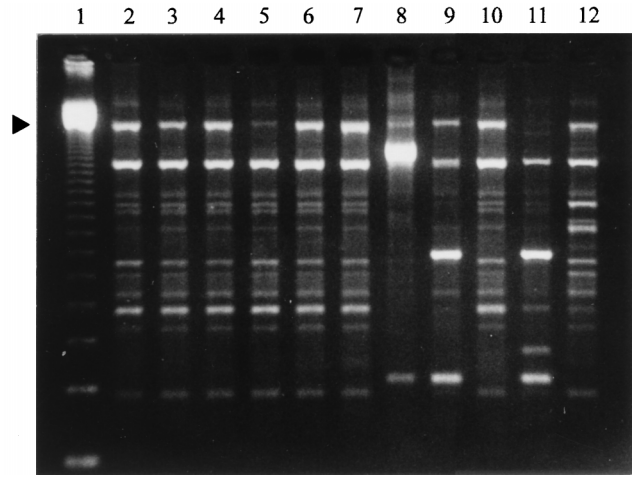

FIG. 1. RAPD analysis of *K. pneumoniae* isolates. Amplification patterns obtained with the RAPD-7 primer were very similar within the group of TEM-47-producing isolates, including the L-267 strain from Łódź. RAPD patterns of SHV-5-type ESBL-producing strains were different from those of TEM-47 producers and from each other. The arrowhead indicates the RAPD band which, in the case of isolate 1298/96 (lane 5), was repeatedly amplified at a lower efficiency compared to the efficiencies of amplification for the other TEM-47 producers. Lanes: 1, 123-bp DNA ladder (Gibco BRL); 2, *K. pneumoniae* 1027/96; 3, *K. pneumoniae* 1099/96; 4, *K. pneumoniae* 1294/96; 5, *K. pneumoniae* 1298/96; 6, *K. pneumoniae* 1590/96; 7, *K. pneumoniae* 1592/96; 8, *K. pneumoniae* 1042/96; 9, *K. pneumoniae* 1289/96; 10, *K. pneumoniae* L-267 (14); 11, *K. pneumoniae* L-414 (nonrelated control) (14); 12, *K. pneumoniae* L-934 (nonrelated control).

Typing by RAPD analysis. Figure 1 and Table 1 present the results of the RAPD analysis done with the RAPD-7 primer (45) and DNA preparations from the clinical isolates, the original TEM-47-producing L-267 *K. pneumoniae* strain from Łódź (Fig. 1, lane 10) (14), and two nonrelated control strains (Fig. 1, lanes 11 and 12). SHV-5-like β-lactamase-producing isolates 1042/96 (Fig. 1, lane 8) and 1289/96 (Fig. 1, lane 9) were reproducibly characterized by unique RAPD patterns (patterns B and C, respectively). All six TEM-47-expressing isolates, isolates 1027/96, 1099/96, 1294/96, 1298/96, 1590/96, and 1592/96 (Fig. 1, lanes 2 to 7, respectively), were found to produce the same RAPD pattern (pattern A), which was also identical to the one obtained for strain L-267. The only difference observed was the repeatedly lower efficiency of amplification of one of the DNA bands of higher molecular mass in the RAPD pattern of isolate 1298/96 (Fig. 1, lane 5). The nonrelated control strains revealed RAPD patterns different from those observed for the analyzed isolates; however, some degree of similarity between the patterns of isolate 1289/96 (Fig. 1, lane 9) and the L-414 control strain (Fig. 1, lane 11) was observed.

Typing by genomic DNA RFLP analysis. Figure 2 and Table 1 present the results of the macrorestriction genomic DNA analysis carried out with TEM-47-producing isolates 1027/96, 1099/96, 1294/96, 1298/96, 1590/96, and 1592/96; the original TEM-47 producer L-267, and SHV-5-like ESBL-expressing isolate 1042/96, used here as the nonrelated control (selected according to RAPD analysis results). (As seen in Fig. 2, the DNA preparation from isolate 1590/96 underwent a partial degradation during the experimental procedure.) The *Xba*I restriction patterns of DNAs from isolates 1027/96 (Fig. 2, lane 2), 1294/96 (Fig. 2, lane 4), 1298/96 (Fig. 2, lane 5), and 1590/96 (Fig. 2, lane 6) were revealed to be identical (pattern A1). Isolate 1099/96 (Fig. 2, lane 3) produced a pattern which differed from the predominant one by four DNA bands (pattern A2), and isolate 1592/96 (Fig. 2, lane 7) produced a pattern which differed by four bands (pattern A3), while strain L-267 (lane 8) produced a pattern which differed by two bands (pattern A4). Isolate 1042/96 (Fig. 2, lane 9) gave a pattern which was completely different from those produced by TEM-47-expressing isolates (pattern B).

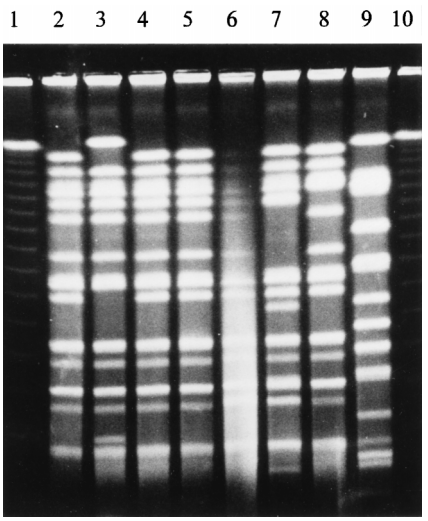

FIG. 2. Genomic DNA RFLP analysis. RFLP patterns of the group of TEM-47-producing *K. pneumoniae* isolates including the L-267 strain from Łódź were found to be very similar to each other. Lanes: 1, λ DNA ladder (Bio Rad); 2, *K. pneumoniae* 1027/96; 3, *K. pneumoniae* 1099/96; 4, *K. pneumoniae* 1294/96; 5, *K. pneumoniae* 1298/96; 6, *K. pneumoniae* 1590/96 (partially degraded); 7, *K. pneumoniae* 1592/96; 8, *K. pneumoniae* L-267; 9, *K. pneumoniae* 1042/96 (nonrelated control); 10, λ DNA ladder (Bio-Rad).

Plasmid fingerprinting analysis. Figure 3 and Table 1

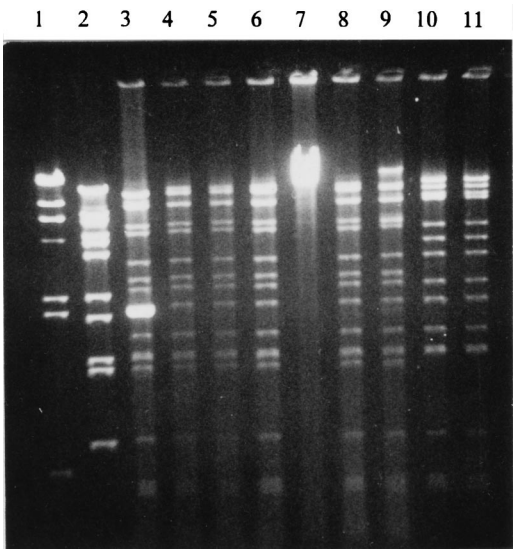

FIG. 3. Plasmid fingerprinting analysis. Large plasmids purified from TEM-47-producing isolates were found to have very similar *Eco*RI restriction patterns. Plasmids purified from transconjugants of SHV-5-type ESBL-producing isolates had identical *Eco*RI patterns. Lanes: 1, λ DNA/*Hin*dIII ladder (Kucharczyk, Warsaw, Poland); 2, λ DNA/*Bst*EII Ladder (Kucharczyk, Warsaw, Poland); 3, *K. pneumoniae* L-267; 4, *K. pneumoniae* 1027/96; 5, *K. pneumoniae* 1099/96; 6, *K. pneumoniae* 1294/96; 7, *K. pneumoniae* 1298/96; 8, *K. pneumoniae* 1590/96; 9, *K. pneumoniae* 1592/96; 10, R^+[*K. pneumoniae* 1042/96]; 11, R^+[*K. pneumoniae* 1289/96].

present the results obtained by *Eco*RI digestion of plasmid DNA purified from isolates 1027/96, 1099/96, 1294/96, 1298/96, 1590/96, and 1592/96; isolate L-267; and transconjugant strains R^+[*K. pneumoniae* 1042/96] and R^+[*K. pneumoniae* 1289/96]. Single, large-molecular-mass (ca. 80 kb) plasmid molecules purified from the SHV-5-like-producing R^+[*K. pneumoniae* 1042/96] and R^+[*K. pneumoniae* 1289/96] transconjugants (Fig. 3, lanes 10 and 11, respectively) were found to have identical *Eco*RI restriction patterns (pattern B). All the TEM-47-expressing clinical isolates contained one large plasmid (ca. 60 to 80 kb), but isolate 1298/96 (Fig. 3, lane 7) carried at least two plasmids (ca. 15 to 20 kb) and strain L-267 (Fig. 3, lane 3) carried one additional smaller plasmid of about 2 kb (plasmid profiles are not shown). In the case of isolate 1298/96, smaller plasmids (with only single *Eco*RI sites, if any at all) copurified repeatedly with the large plasmid with such a high efficiency that it was impossible to reveal its restriction pattern in a relatively simple way (digestions with other enzymes, hybridization with nonradioactive probes). Large plasmids purified from isolates 1027/96, 1099/96, and 1294/96 (Fig. 3, lanes 4 to 6, respectively) and isolate 1590/96 (Fig. 3, lane 8) (ca. 65 kb) demonstrated identical *Eco*RI restriction maps (pattern A1). Very similar *Eco*RI digestion patterns (with differences only in the sizes of the longest fragments) were also shown by plasmids of isolate 1592/96 (ca. 70 kb) (Fig. 3, lane 9; pattern A2) and strain L-267 (ca. 60 kb) (pattern A3). These data were confirmed by digestion with *Pst*I, an enzyme which recognizes many more restriction sites in the analyzed DNA (data not shown). The *Eco*RI digestion patterns of plasmids purified from SHV-5-like and TEM-47-producing isolates (patterns B and A1-3, respectively) demonstrated some degree of similarity.

DISCUSSION

From the beginning of March 1996 the Infection Control Committee of the University Children's Hospital in Warsaw started monitoring infections caused by ESBL-producing strains of members of the family *Enterobacteriaceae*. Over the first period of 3 months, eight ceftazidime-resistant *K. pneumoniae* isolates were collected from different patients hospitalized in four different wards including the intensive care unit (ICU), surgery, cardiology, and hematology, which indicated that the problem was of hospital-wide importance (Table 1). All the patients were at increased risk of infection due to young age, prolonged period of hospitalization, the use of various invasive diagnostic-therapeutic procedures, and prior antibiotic therapy. Such factors have been reported to contribute to infection with ESBL-producing organisms (27). Susceptibility testing of ceftazidime-resistant *K. pneumoniae* isolates (Table 2) revealed patterns typical for class A ESBL producers (11, 23). Higher MICs of ceftazidime and aztreonam compared to those of cefotaxime suggested the ceftazidimase activities of the ESBLs in the isolates. For six of the isolates (isolates 1027/96, 1099/96, 1294/96, 1298/96, 1590/96, and 1592/96) the MICs of all β-lactams tested were similar (with lower MICs observed for isolate 1298/96) and the isolates were uniformly resistant to gentamicin and tobramycin. The remaining two isolates (isolates 1042/96 and 1289/96) were found to be susceptible to the aminoglycosides tested, and in general the MICs of β-lactams were higher for these isolates. Only these two isolates produced transconjugants in the ceftazidime resistance transfer experiment which proved that the ESBL-encoding genes of these isolates were carried by conjugative plasmids.

The analysis of the β-lactamase contents of the isolates (Table 1), performed by analytical IEF, confirmed initial suggestions from the susceptibility testing and mating results, which indicated the existence of at least two different clusters of isolates. All the isolates produced a β-lactamase with a pI of 7.6 which was likely to be a chromosomally encoded *K. pneumoniae* enzyme. Additionally, isolates 1042/96 and 1289/96 expressed an enzyme that had a pI value of 8.2 and that comigrated in IEF gels with the SHV-5 ESBL standard. This β-lactamase was also produced by their transconjugants and was found to possess ceftazidimase activity by the bioassay approach. Specific PCR followed by sequencing of PCR products revealed two identical partial sequences of the bla_{SHV} gene(s) with two mutations determining amino acid substitutions in respect to the corresponding region (Thr180 to Arg259) of the p453 SHV-1 β-lactamase (2). The combination of Gly238Ser and Glu240Lys mutations alone within the analyzed region plus the pI value of 8.2 characterizes SHV-5 (5) and SHV-12 (31). Therefore, it is considered that isolates 1042/96 and 1289/96 produced one of these or another closely related enzyme.

All the other six isolates were found to express an additional β-lactamase with a pI value of 6.0 (Table 1). Sequencing of the PCR products obtained with bla_{TEM} gene-specific primers revealed six identical sequences of regions coding for the TEM-47 β-lactamase which was described and which was shown to possess the ESBL activity in a previous study (14). In the case of isolate 1298/96, the PCR product resulting from the amplification with the TEM-A and TEM-B primers (24) was found to be much longer than the PCR products from the other isolates (data not shown). This suggests an insertion of a DNA fragment of about 1 kb between the TEM-A priming site and the promoter sequences of the bla_{TEM-47} gene in this particular isolate; this insertion has not influenced in a significant way the expression of the gene. The TEM-47 ESBL was previously postulated to belong, together with TEM-48 and TEM-49, to a group of very closely related β-lactamases and to have evolved from the TEM-48 ESBL by a single cross-over event (14).

The β-lactamase data along with the typing and plasmid fingerprinting results demonstrate a complex view of the epidemiological situation of ESBL-producing *K. pneumoniae* in the hospital. At least two different ESBLs were present in the population of strains at the time of the study; this multiplicity of ESBLs is seen more commonly in hospital environments today (8, 13, 14). The two isolates expressing SHV-5 or a related enzyme (isolates 1042/96 and 1289/96) were found to be different by RAPD analysis (Fig. 1; Table 1) but were found to carry the same conjugative plasmid. Thus, the plasmid carrying the bla_{SHV} gene appears to have spread among strains. Both isolates were collected from patients hospitalized in the surgical ward, which may provide a potential source of spread of SHV-5-like ESBL-expressing strains of members of the family *Enterobacteriaceae* in the hospital.

The other aspect of the epidemiological situation concerns the six isolates producing TEM-47, all of which were demonstrated to be very closely related by methods which have been reported to be reliable in epidemiological studies of *K. pneumoniae* (15, 16, 34). All of the isolates showed identical RAPD patterns (Fig. 1; Table 1) and isolates 1027/96, 1294/96, and 1590/96 were also indistinguishable by genomic RFLP analysis (Fig. 2; Table 1) and plasmid fingerprinting (Fig. 3, Table 1). Two of these were isolated from ICU patients and one was isolated from a patient hospitalized on the hematology ward (Table 1). Isolate 1298/96 from the cardiology ward produced the same RFLP pattern but had a different plasmid DNA profile and the DNA insertion adjacent to the bla_{TEM-47} gene.

Isolates 1099/96 and 1592/96 collected from ICU patients were characterized by distinct although highly similar RFLP patterns; one of them (isolate 1099/96) had identical plasmids and the other one (1592/96) had a slightly different, larger plasmid compared to the plasmids from isolates 1027/96, 1294/96, and 1590/96. All these results suggest the clonal spread of a single TEM-47-producing *K. pneumoniae* strain. Such an ESBL outbreak, in which clonally related isolates are characterized by different plasmid profiles, has already been described (34). It is impossible to determine when the original strain could have appeared in the hospital because the monitoring of ESBLs commenced only with the collection of these isolates. Various genetic changes found within the isolates (chromosomal rearrangements, acquisition or loss of plasmids, plasmid DNA recombination events) may suggest that spread had already started a relatively long time before March 1996 and that the TEM-47-producing *K. pneumoniae* strain had been maintained in the environment, causing infections in predisposed patients. The ICU may be postulated to be the source of spread of this strain within the hospital.

Typing results have also revealed a very close relatedness of the original TEM-47-producing strain, *K. pneumoniae* L-267, to the population of isolates in this study. This strain was isolated a year before (January 1995) in the pediatric hospital in Łódź and was characterized by a β-lactamase content (14) and RAPD pattern (Fig. 1) that were the same as those of the Warsaw isolates and RFLP patterns (Fig. 2) and large plasmid restriction patterns (Fig. 3) that were very similar to those of the Warsaw isolates. In opposition to the Warsaw isolates, this strain also carried a small plasmid of approximately 2 kb (Fig. 3) and produced recombinants in the conjugation experiment (14). The R^+[*K. pneumoniae* L-267] transconjugant was revealed to carry only the large plasmid (14), and the MICs of both aminoglycosides and β-lactams for this isolate were elevated (Table 2). It is likely that the large plasmids purified from the Warsaw isolates have origins similar to that of the plasmid which was found in the strain from Łódź but may have lost conjugative functions due to some DNA rearrangements (reflected by differences in the fingerprinting pattern). Because the exchange of patients between the University Children's Hospital in Warsaw and the Mother and Child Memorial Hospital in Łódź is a common practice, it may be proposed that the TEM-47-producing *K. pneumoniae* strain bearing the large conjugative plasmid with ESBL and aminoglycoside resistance genes has been transferred from one hospital to the other. From this study, the more likely scenario would be the transfer from Łódź to Warsaw, the loss of conjugative functions by the plasmid, and the consequent clonal spread of the strain in the Warsaw hospital.

The results of the analysis reported here reflect very well the epidemiological situation of infections caused by ESBL-producing strains in a large, specialized hospital in Poland. With a small sample of isolates recovered over a short period of time and belonging to only one bacterial species, it was possible to demonstrate several previously described epidemiological phenomena. Similar data describing both the clonal spread of the ESBL-producing strain and the horizontal spread of the ESBL gene-carrying plasmid at the same time in one center have been reported but have usually concerned a single ESBL variant (30, 46). Factors which in our case may have facilitated the development of such a situation include the size of the hospital (nine wards, 300 beds, 10,000 patients admitted per year), transfer of patients between wards and other hospitals, and the long-term use of expanded-spectrum cephalosporins which has not been accompanied by proper monitoring and control of infections caused by ESBL-producing organisms. In 1995 about 1.5 kg of cefuroxime, 1.4 kg of ceftriaxone, 0.5 kg of cefotaxime, and 0.5 kg of ceftdazidime were used for therapy of infections and prophylaxis. In fact, the situation in this particular hospital is much more complex if other bacterial species are to be considered and is certainly representative of other, comparable hospitals in Poland (14; unpublished data).

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