ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2001, p. 88–95 Vol. 45, No. 1
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.1.88–95.2001

Complexity and Diversity of *Klebsiella pneumoniae* Strains with Extended-Spectrum β-Lactamases Isolated in 1994 and 1996 at a Teaching Hospital in Durban, South Africa

SABIHA Y. ESSACK,[1] LUCINDA M. C. HALL,[2] DEVADAS G. PILLAY,[3] MARGARET LYNN MCFADYEN,[1] AND DAVID M. LIVERMORE[4]*

Department of Pharmacy and Pharmacology, University of Durban-Westville, Durban, 4000,[1] and Department of Medical Microbiology, University of Natal, Congella, 4013,[3] South Africa, and Department of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, London E1 2AD,[2] and Antibiotic Resistance Monitoring and Reference Laboratory, Central Public Health Laboratory, London NW9 5HT,[4] United Kingdom

Received 10 January 2000/Returned for modification 25 April 2000/Accepted 10 October 2000

β-Lactamase production was investigated in cultures of 25 *Klebsiella pneumoniae* isolates isolated at a hospital in Durban, South Africa, in 1994 and 1996. Twenty of these isolates gave ceftazidime MIC/ceftazidime plus clavulanate MIC ratios of ≥8, implying production of extended-spectrum β-lactamases (ESBLs), and DNA sequencing identified an ESBL gene (bla_{TEM-53}) in a further two isolates. Pulsed-field gel electrophoresis (PFGE) defined 4 distinct strains among the 12 isolates collected in 1994 and 9 distinct strains among the 13 isolates collected in 1996. In three cases, multiple isolates from single patients varied in their PFGE profiles and antibiograms, implying mixed colonization or infection. Isoelectric focusing and DNA hybridization found both TEM and SHV enzymes and their genes in all 25 isolates. Many isolates had multiple identical or different β-lactamase gene variants, with at least 84 bla_{SHV} and bla_{TEM} gene copies among the 25 organisms. Sequencing identified the genes for the SHV-1, -2, and -5 enzymes and for four new SHV types (SHV-19, -20, -21, and -22). These new SHV variants had novel mutations remote from sites known to affect catalytic activity. Sequencing also found the genes for TEM-1, TEM-53, and one novel type, TEM-63. All the isolates had multiple and diverse plasmids. These complex and diverse patterns of ESBL production and strain epidemiology are far removed from the concept of an ESBL outbreak and suggest a situation in which ESBL production has become endemic and in which evolution is generating a wide range of enzyme combinations. This complexity and diversity complicates patient management and the design of antibiotic use policies.

β-Lactams are prescribed more often than any other antibiotics. This heavy usage has selected for resistance, which is most often caused by β-lactamases (17). Recent concerns have centered on extended-spectrum β-lactamases (ESBLs), which are an increasing problem in members of the family *Enterobacteriaceae* in general and especially in *Klebsiella* spp.

Most ESBLs are variants of the classical TEM and SHV β-lactamases, but with one or more amino acid substitutions (22, 25; G. A. Jacoby and K. Bush, Amino acid sequences for TEM, SHV, and OXA extended-spectrum and inhibitor-resistant β-lactamases [http://www.lahey.org/studies/webt.htm]). These changes alter the catalytic center, permitting hydrolysis of oxyimino-aminothiazolyl cephalosporins. ESBLs have been reported worldwide, but most studies have examined producers collected in Europe, North America, and Southeast Asia (17, 25), and only a few (2) have examined bacteria collected in Africa (20). To redress this situation, we investigated β-lactamase types, including ESBLs, in nosocomial *Klebsiella pneumoniae* isolates collected at a major teaching hospital in Durban, Kwazulu-Natal, South Africa.

Although the primary purpose of the study was identification of the types of enzymes produced in South African isolates, the major finding was the remarkable diversity and complexity of β-lactamase and strain types among the small number of isolates examined.

* Corresponding author. Mailing address: Antibiotic Resistance Monitoring and Reference Laboratory, Central Public Health Laboratory, 61 Colindale Ave., London NW9 5HT, United Kingdom. Phone: 0208-200-4400. Fax: 0208-200-7449. E-mail: DLivermore@phls.nhs.uk.

MATERIALS AND METHODS

Bacterial cultures. The 25 *K. pneumoniae* isolates investigated were from clinical material collected at the King Edward VIII Hospital in Durban (Table 1). This government-run institution is one of the largest tertiary-care hospitals in southern Africa, with 2,000 beds. Twelve isolates were collected during September and October 1994, and 13 were collected during June and July 1996. These organisms were selected solely because they were reported to be resistant to one or more oxyimino-aminothiazolyl cephalosporins by the diagnostic laboratory. The species of the organisms were verified by tests with the API 20E system (bioMerieux, Lyons, France). Isolates 117, 118, 122, and 202 were collected from a single patient (patient I) within 7 days in 1994; isolates 4183, 4265, 4744, and 8143 were collected from patient II within 1 month in 1996, and isolates 4175 and 4291 were collected from patient III within 6 days in 1996. Reference producers of SHV and TEM enzymes were described previously (18). *Escherichia coli* NCTC 50192 served as a source of plasmid markers (16).

Susceptibility testing. Susceptibility tests and ESBL detection were performed on Mueller-Hinton agar by Etests, which were used according to the manufacturer's directions (AB Biodisk, Solna, Sweden).

Strain typing. Total DNA was extracted from the isolates, restricted with *Xba*I (Promega, Madison, Wis.), and fingerprinted by pulsed-field gel electrophoresis (PFGE). Methods were as described previously (6).

β-Lactamase typing. Isoelectric focusing was performed on ultrasonic extracts by a protocol described elsewhere (19). Genes for TEM and SHV β-lactamases were detected by hybridization. Briefly, total DNA was extracted from the isolates as described previously (11) and was restricted with *Sal*I for detection of bla_{SHV} and variously (see Results) with *Bam*HI, *Hin*dIII, or *Hin*cII for detection

of bla_{TEM}. Restriction conditions were those suggested by the enzyme supplier (Promega). The restricted DNA was electrophoresed on 0.9% agarose gels, which were Southern blotted (24) and then hybridized with gene probes for bla_{SHV} or bla_{TEM}. These probes were generated from reference strains by PCR with primers 5′-ATGAGTATTCAACATTTCCGTG (positions 1 to 22, as numbered from the start of the enzyme-coding region) and 5′-TTACCAATGCTTA ATCAGTGAG (positions 861 to 840) for bla_{TEM} and with 5′-ATGCGTTATA TTCGCCTGTG (positions 1 to 20) and 5′-GTTAGCGTTGCCAGTGCTCG (positions 865 to 846) for bla_{SHV}. PCR conditions for bla_{TEM} comprised a thermal ramp to 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; those for bla_{SHV} comprised 95°C for 3 min and then 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min, followed by 5 min at 72°C. Hybridization was performed at 65°C under the conditions described by Sambrook et al. (24) with bla_{SHV} and bla_{TEM} gene probes generated from reference strains by PCR with the primers described above and labeled with digoxygenin (DIG DNA Labeling and Detection Kit; Boehringer Mannheim, Mannheim, Germany). The sizes of the restriction fragments were estimated by comparison with a 1-kb DNA ladder (Gibco BRL, Paisley, Scotland).

Sequencing of TEM and SHV genes. DNA fragments corresponding in size to those carrying β-lactamase genes were excised from 0.9% agarose gels that had been electrophoresed overnight at 46 V. The TEM and SHV genes were then amplified by PCR as described above. Only *Bam*HI-restricted DNA was used as a source of bla_{TEM} genes. The products were cleaned with either a QIAquick Gel Extraction or a PCR Purification kit (Qiagen, Crawley, West Sussex, United Kingdom) and were then sequenced with an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA Polymerase FS (Perkin-Elmer, Branchburg, N.J.). A GeneAmp PCR System 2400 (Perkin-Elmer) was used and was operated in accordance with the manufacturer's protocols. The extension products were purified by Ethanol Precipitation Protocol 1 (Perkin-Elmer) and were then loaded onto an ABI Prism 377 sequencer (Perkin-Elmer). The primers for sequencing bla_{TEM} were 5′-TTCTGTGACTGGTGAGTACT (positions 324 to 305), 5′-GAGTAAGTAGTTCGCCAGTT (positions 595 to 576), and 5′-TTACCAATGCTTAATCAGTGAG (positions 861 to 840); those for bla_{SHV} were 5′-ATGCGTTATATTCGCCTGTG (positions 1 to 20), 5′-CG TTTCCCAGCGGTCAAGG (positions 489 to 471), and 5′-GTTAGCGTTGC CAGTGCTCG (positions 865 to 846). All sequences were confirmed by two independent determinations and were analyzed with Sequence Navigator software (Perkin-Elmer). All the sequences were confirmed by two independent PCR experiments.

Plasmid profiles and transfers. Plasmids were extracted and electrophoresed by the method of Kado and Liu (14), with *E. coli* NCTC 50192 (16) used as a source of molecular weight markers.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers for novel β-lactamase genes in GenBank were as follows: bla_{TEM-63}, AF045475; bla_{SHV-19}, AF117743; bla_{SHV-20}, AF117744; bla_{SHV-21}, AF117745; and bla_{SHV-22}, AF117746.

RESULTS

Strain structure and ESBL production. Table 1 summarizes the strain types, plasmid profiles, and β-lactamase characteristics for the isolates, and Table 2 shows the MIC data. Isolates were categorized as putatively ESBL positive on the basis of ratios of the MIC of ceftazidime to the MIC of ceftazidime plus clavulanate of 8 or more. Twenty isolates were ESBL positive on the basis of this criterion, with MIC ratios ranging from 8 to >128. For five isolates MIC ratios were less than 8, and it was inferred that the isolates lacked ESBLs, although two of these (isolates 117 and 118) later proved to have bla_{TEM-53}, which may not have been expressed (see below). Among the organisms collected in 1994, isolates 79, 113, 114, and 122 gave PFGE restriction patterns identical to each other and it was inferred that they belong to an outbreak strain type, designated the type A strain. Isolates with PFGE patterns that differed from that of the type A strain by two or three fragments were categorized as subtypes of this strain. Thus, isolates 10 and 202 were designated subtype A1, isolate 73 was designated subtype A2, and isolates 91 and 117 were designated subtype A3. The other isolates collected in 1994 differed from outbreak strain type A and each other by 10 or more PFGE bands and were designated strain types B to D. Of the organisms collected in 1996, isolate 4291 belonged to type A, and isolate 4495 gave a similar profile and was designated subtype A4, whereas all the other organisms gave profiles unrelated to those seen among isolates collected in 1994. Isolates 4120 and 4448 differed by three fragments by PFGE and were designated types F and F1, respectively; the other isolates collected in 1996 differed from types A to F and from each other by ≥10 bands and were designated types G to L. In 1994, patient I yielded isolates 122 (type A), 202 (subtype A1), 117 (subtype A3), and 118 (type C). Isolates 4175 (type G) and 4291 (type A) were both collected from patient II in 1996 but were unrelated. Isolates 4265, 4744, and 8143 were collected from patient III in 1996, and all belonged to type E; isolate 4183 was from the same patient but belonged to type H.

β-Lactamase identification. Five electrofocusing profiles were found among the isolates collected in 1994 and eight were found among those collected in 1996. The isolates expressed one to five β-lactamases each. Most isolates appeared to produce both TEM and SHV enzymes, as evidenced by isoelectric focusing bands in the regions typical of both enzyme families (pI 5.4 to 6.3 and pI 7.0 to 8.2, respectively). DNA hybridization confirmed this inference, detecting both bla_{TEM} and bla_{SHV} genes in all 25 isolates, including those for which the ceftazidime MIC/ceftazidime plus clavulanate MIC ratios were less than 8. Many isolates carried multiple bla_{TEM} and bla_{SHV} copies, as demonstrated by the hybridization of probes with multiple restriction fragments (Table 1). Among the ESBL producers collected in 1994, 3 had two bla_{TEM} copies and 1 had two bla_{SHV} copies; among 12 ESBL producers collected in 1996, 6 had two bla_{TEM} copies and 5 had three copies; all 12 had two bla_{SHV} copies. In 13 isolates, the multiple gene copies encoded the same β-lactamase; in 7 isolates different enzyme variants were encoded within an isolate. To identify some of the enzymes, DNAs corresponding to individual restriction fragments were excised and sequenced. In the case of the bla_{TEM} variants, sequencing was undertaken only with fragments from *Bam*HI-restricted DNA, not for additional TEM-encoding fragments revealed after digestion with *Hin*dIII or *Hin*cII. The latter fragments may have encoded additional enzyme variants.

The classical bla_{TEM-1} gene was identified in isolates 97, 122, 571, 4120, 4175, 4183, 4265, 4495, 4634, 4699, 4744, 4824, and 8143; and bla_{TEM-53} was identified in isolates 73, 113, 114, 117, 118, 4291, and 4448. It should be noted that the mature TEM-53 enzyme corresponds to TEM-12, as the mutation that distinguishes these enzymes (Leu21Phe) lies in the signal peptide (15). Curiously, pI 5.2 bands (as predicted for TEM-12/53) were not seen for any of bla_{TEM-53}-positive isolates, suggesting that enzyme expression was minimal. The sequence that determined a novel TEM variant, TEM-63, was found in isolates 10, 79, 91, 202, and 4700; and details of the nucleotide and amino acid changes present in bla_{TEM-63} and its product are given in Table 3.

Detection of pI 7.6 to 8.2 β-lactamases corresponded with the detection of bla_{SHV}. Isolates 4175, 4265, 4291, 4495, 4700, and 4824 had classical SHV-1, whereas isolates 10, 73, 79, 91, 97, 113, 114, 117, 118, 122, 202, and 4448 had a novel variant, SHV-19, with Leu173Phe (Table 3). Isolates 571, 4120, 4291,

TABLE 1. Epidemiology, phenotypes, and β-lactamase genes of *K. pneumoniae* isolates collected in 1994 and 1996

Isolate (yr collected)	Date collected (day/mo)	Ward	Source	Etest MIC ratio[a]	PFGE type	Plasmid size (kb)	β-Lactamase(s)		
							Gene hybridization[b]	pI value(s)	Sequence(s) found
79 (1994)	04/10	ICU	Central venous catheter	16	A	140 87	1 × TEM 1 × SHV	5.6 6.3 7.6	TEM-63 SHV-19
113 (1994)	No data	No data	Blood	>32	A	136 103 80 63	2 × TEM 1 × SHV	 5.6 7.6	TEM-53 SHV-19
114 (1994)	No data	No data	Blood	>32	A	136 103 80 63	2 × TEM 1 × SHV	 5.6 7.6	TEM-53 SHV-19
122[c] (1994)	09/10	ICU	Central venous catheter	<2	A	142 111 88 72 7	1 × TEM 1 × SHV	5.4 7.6	TEM-1 SHV-19
202[c] (1994)	12/10	ICU	Peritoneal fluid	>32	A1	186 111	1 × TEM 1 × SHV	5.6 7.6	TEM-63 SHV-19
10 (1994)	27/09	Surgical	Pus	>32	A1	140 41	2 × TEM 1 × SHV	5.6 6.3 7.6	TEM-63 SHV-19
73 (1994)	02/10	Orthopedic	Pus	8	A2	140 41	1 × TEM 1 × SHV	5.6 6.3 7.6	TEM-53 SHV-19
91 (1994)	No data	No data	Blood culture	>32	A3	136 103 35	1 × TEM 1 × SHV	5.6 7.6	TEM-63 SHV-19
117[c] (1994)	05/10	ICU	Pus (abdomen)	<2	A3	103 80 63 35 30	1 × TEM 1 × SHV	 7.6	TEM-53 SHV-19
118[c] (1994)	05/10	ICU	Pus (abdomen)	<2	C	136 103 80 63	1 × TEM 1 × SHV	 7.6	TEM-53 SHV-19
97 (1994)	No data	No data	Blood	<2	B	136 103	2 × TEM 1 × SHV	 7.6	TEM-1 SHV-19
571 (1994)	No data	No data	Blood	8	D	140 87 71 59 49 41 35	1 × TEM 2 × SHV	5.4 5.6 6.3 6.8 7.6	TEM-1 SHV-2 and -21
4291[e] (1996)	28/06	ICU	Endotracheal aspirate	64	A	139 88 50	3 × TEM 2 × SHV	 5.4 5.6 6.3 6.8 7.6	TEM-53 SHV-1 and -2

Continued on following page

TABLE 1—*Continued*

Isolate (yr collected)	Date collected (day/mo)	Ward	Source	Etest MIC ratio[a]	PFGE type	Plasmid size (kb)	β-Lactamase(s)		
							Gene hybridization[b]	pI value(s)	Sequence(s) found
4495 (1996)	08/07	ICU	Endotracheal aspirate	>128	A4	139 110 59	3 × TEM 2 × SHV	5.4 5.6 7.6 8.2	TEM-1 (2)[f] SHV-1 SHV-5
4265[d] (1996)	27/06	ICU	Endotracheal aspirate	>128	E	142	2 × TEM 2 × SHV	5.4 5.6 6.3 7.6 8.2	TEM-1 (2) SHV-1 SHV-5
4744[d] (1996)	18/07	Nursery	Sputum	>128	E	142	2 × TEM 2 × SHV	5.4 6.3 7.6 8.2	TEM-1 (2) SHV-5 (2)
8143[d] (1996)	29/06	ICU	Blood	>128	E	142	2 × TEM 2 × SHV	5.4 5.6 7.6 8.2	TEM-1 (2) SHV-5 and -22
4120 (1996)	20/06	ICU	Endotracheal aspirate	32	F	87 71 59	2 × TEM 2 × SHV	5.4 6.3 6.8 7.6	TEM-1 (2) SHV-2 (2)
4448 (1996)	05/07	Nursery	Pus (drip site)	64	F1	139 110 59 50	3 × TEM 2 × SHV	 5.4 5.6 7.6 8.2	TEM-53 SHV-19 SHV-5
4175[e] (1996)	22/06	ICU	Endotracheal aspirate	4	G	140 87 7 5	1 × TEM 2 × SHV	5.4 6.3 6.8 7.6	TEM-1 SHV-1 (2)
4183[d] (1996)	22/06	ICU	Endotracheal aspirate	>128	H	139 110 50	1 × TEM 2 × SHV	5.4 5.6 6.3 7.6 8.2	TEM-1 SHV-20 SHV-5
4634 (1996)	No data	No data	No data	>64	I	139 110 50 23	3 × TEM 2 × SHV	5.4 6.8 7.6 8.2	TEM-1 SHV-2 (2)
4669 (1996)	17/07	Nursery	Pleural cavity tap	>32	J	139 110 72 59	3 × TEM 2 × SHV	5.4 6.3 7.6 8.2	TEM-1 (3) SHV-2 and -20
4700 (1996)	17/07	Pediatric	Urine	>64	K	186 111	2 × TEM 2 × SHV	5.4 5.6 6.3 7.6	 TEM-63 (2) SHV-1 (2)
4824 (1996)	22/07	ICU	Endotracheal aspirate	8	L	142 59 7	2 × TEM 2 × SHV	5.4 7.6	TEM-1 (2) SHV-1 and -20

[a] Ratio of MIC of ceftazidime to the MIC of ceftazidime plus clavulanate at 4 μg/ml, as determined by the Etest.
[b] Numbers of SHV genes were estimated from Southern blots of whole genomic DNA digested with *Sal*I and hybridized with a SHV-specific probe, and the numbers of TEM genes were estimated from Southern blots of whole genomic DNA digested with *Bam*HI, *Hin*dIII, or *Hin*cII and hybridized with a TEM-specific probe.
[c] Four isolates from patient I.
[d] Four isolates from patient II.
[e] Two isolates from patient III.
[f] Numbers in parentheses indicate the number of copies of the gene encoding the β-lactamase indicated.

TABLE 2. MICs for isolates collected in 1994 and 1996

Isolate	Strain	MIC (μg/ml)[a]										
		PIP	PIP-TAZ	AMOX-CLAV	AMP-SUL	LOT	CXM	CTX	CAZ	CFM	FOX	ATM	IMP
79	A	**>256**	2	8	**16**	8	4	0.5	**128**	1	2	**4**	0.12
113	A	**>256**	4	8	**16**	8	4	0.5	**64**	1	2	**4**	0.25
114	A	**>256**	4	8	**16**	8	4	0.25	**64**	0.5	2	2	0.12
122[b]	A	32	2	4	8	4	2	<0.06	0.25	<0.06	2	<0.06	0.12
202[b]	A1	>256	4	8	**16**	4	4	0.25	**64**	1	2	2	0.12
10	A1	**>256**	4	**16**	**32**	16	4	0.5	**>256**	2	2	16	0.25
73	A2	**>256**	2	4	8	**256**	8	1	1	0.25	2	0.25	0.25
91	A3	**>256**	2	8	8	8	4	0.5	**256**	1	2	8	0.12
117[b]	A3	8	2	2	4	4	4	<0.06	0.12	<0.06	1	<0.06	0.12
97	B	**>256**	2	**16**	**32**	**64**	4	0.5	1	0.1	2	<0.06	0.12
118[b]	C	4	2	2	4	4	2	<0.06	0.25	<0.06	2	<0.06	0.12
571	D	**>256**	4	8	**16**	**>256**	**32**	4	4	2	2	2	0.12
4291[d]	A	**>256**	16	**16**	**32**	**>256**	**32**	8	**16**	4	2	2	0.12
4265[c]	E	**>256**	4	8	**16**	**>256**	**32**	4	**256**	2	2	**>256**	0.12
4744[c]	E	**>256**	2	8	**16**	**>256**	**64**	8	**128**	2	2	**128**	0.12
8143[c]	E	**>256**	4	8	**16**	**>256**	**32**	4	**>256**	2	4	**256**	0.25
4120	F	**>256**	4	8	**16**	**>256**	**16**	8	4	2	2	1	0.25
4448	F1	**>256**	4	**16**	4	**>256**	**32**	4	8	2	1	4	0.12
4175[d]	G	**>256**	4	**16**	**32**	**>256**	**16**	4	4	1	2	1	0.12
4183[c]	H	**>256**	2	8	**16**	**>256**	**16**	8	**32**	2	2	**32**	0.12
4634	I	**>256**	8	8	**16**	**>256**	**256**	**32**	**>256**	4	2	**256**	0.12
4669	J	**>256**	**>256**	**16**	**128**	**>256**	**128**	**16**	**256**	4	2	**256**	0.25
4700	K	**>256**	4	**16**	**128**	**>256**	4	2	**>256**	4	2	**16**	0.25
4824	L	**>256**	**32**	8	**32**	**>256**	**32**	4	**16**	1	8	2	0.12

[a] Abbreviations: AMOX, amoxicillin; AMP, ampicillin; AZT, aztreonam; CAZ, ceftazidime; CFM, cefepime; CLAV, clavulanic acid; CTX, cefotaxime; CXM, cefuroxime; FOX, cefoxitin; IMP, imipenem; LOT, cephalothin; PIP, piperacillin; SUL, sulbactam; TAZ, tazobactam. Boldface numbers indicate resistance.
[b] Four isolates from single patient I.
[c] Four isolates from patient II.
[d] Two isolates from patient III.

4634, and 4699 had the SHV-2 enzyme, whereas isolates 4183, 4699, and 4824 had a novel SHV-2 variant, SHV-20, with the Leu173Phe substitution (which is also seen in SHV-19) as well as the Gly238Ser mutation that distinguishes SHV-2 from SHV-1. Isolate 571 had SHV-21, which was identical to SHV-20 except for Leu122Phe. The SHV-5 β-lactamase was found in isolates 4183, 4265, 4448, 4495, 4744, and 8143. Isolate 8143 additionally had SHV-22, an SHV-5 variant with Asn158Lys. The presence of a pI 8.2 band in extracts of isolate 4634 implied that it, too, might have the SHV-5 enzyme, but this was not confirmed by sequencing.

TABLE 3. Nucleotide and amino acid sequence changes of TEM-63, SHV-19, SHV-20, SHV-21, and SHV-22[a]

Enzyme	Nucleotide positions[b]	Nucleotide change	Amino acid position[c]	Amino acid change
TEM-63	555–57	CTT→TTT	21	Leu→Phe
	304–306	GAG→AAG	104	Glu→Lys
	484–486	CGT→AGT	164	Arg→Ser
	538–540	ATG→ACG	182	Met→Thr
SHV-19	505–507	CTT→TTT	173	Leu→Phe
SHV-20	505–507	CTT→TTT	173	Leu→Phe
	700–702	GGC→AGC	238	Gly→Ser
SHV-21	352–354	CTC→TTC	122	Leu→Phe
	505–507	CTT→TTT	173	Leu→Phe
	700–702	GGC→AGC	238	Gly→Ser
SHV-22	460–462	AAC→AAG	158	Asn→Lys
	700–702	GGC→AGC	238	Gly→Ser
	703–705	GAG→AAG	240	Glu→Lys

[a] Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; Ala, alanine; Asn, asparagine; Glu, glutamate; Leu, leucine; Lys, lysine; Phe, phenylalanine; Ser, serine; Gly, glycine.
[b] Nucleotide numbering is in accordance with the AGT codon start of previously reported SHV sequences.
[c] Amino acid numbering is according to Ambler et al. (1).

The novel substitutions in SHV-19, -20, -21, and -22 were remote from those positions (positions 35, 39, 43, 69, 104, 164, 179, 205, 237, 238, 240, 244, 245, 265, and 276) normally associated with ESBL activity in the SHV family (2), and the kinetics of these enzymes were not studied. Several isolates had β-lactamase activities with pIs of 6.3 and 6.8 (Tables 1 and 2). These may have been distinct non-TEM, non-SHV types, but they were not pursued.

β-Lactamases and resistance. The 20 isolates that were phenotypically ESBL positive were resistant to piperacillin (MICs, >256 μg/ml). Eighteen were susceptible to piperacillin-tazobactam (MICs, ≤16 and 4 μg/ml, respectively), 16 were susceptible to amoxicillin-clavulanate (MICs, 8 and 4 μg/ml, respectively), and 7 were susceptible to ampicillin-sulbactam (MICs, 8 and 4 μg/ml, respectively). The MICs of oxyimino-aminothiazolyl cephalosporins were very variable among these 20 ESBL producers; those of ceftazidime were ≥1 μg/ml for all the isolates and ≥4 μg/ml for 19 of 20 isolates; the MICs of cefepime were consistently ≤4 μg/ml but were two- to sixfold higher than those for ESBL nonproducers. Four of the five isolates for which ceftazidime MIC/ceftazidime plus clavulanate MIC ratios were below 8, including those with *bla*$_{\text{TEM-53}}$

(isolates 117 and 118), were fully susceptible to oxyimino-aminothiazolyl cephalosporins (MICs, <1 μg/ml). The exception was isolate 4175, which had low-level resistance to these cephalosporins (MICs 1 to 4 μg/ml) (Table 2). Susceptibility to piperacillin, amoxicillin-clavulanate, and ampicillin-sulbactam was variable among these five isolates (Table 2), but all were susceptible to piperacillin-tazobactam (MICs, ≤4 plus 4 μg/ml, respectively), aztreonam (MICs, ≤1 μg/ml), and cefoxitin (MICs, ≤2 μg/ml).

Plasmid profiles. Nine different plasmid profiles were observed among the 12 isolates collected in 1994 and 11 different plasmid profiles were observed among the 13 isolates collected in 1996 (Table 1). Isolates had one to seven plasmid bands ranging in size from 5 to 186 kb. Some isolates belonging to the same strain had similar plasmid profiles (e.g., isolates 10 and 73, isolates 113 and 114, and isolates 4265, 4744, and 8143), but there was also much variation in the profiles within the strains defined by PFGE.

DISCUSSION

We examined small numbers of *K. pneumoniae* isolates reported to be resistant to one or more oxyimino-aminothiazolyl cephalosporins at King Edward VIII Hospital in Durban, South Africa, in 2-month periods in 1994 and 1996. These organisms proved to be remarkably complex and diverse in their resistance patterns, β-lactamase combinations, and plasmid profiles. Twenty of the isolates had an ESBL phenotype, defined as a ceftazidime MIC-ceftazidime plus clavulanate MIC ratio of 8 or more. Two further isolates gave ceftazidime MIC/ceftazidime plus clavulanate MIC ratios below 8 but carried $bla_{\text{TEM-53}}$, which should give a mature product identical to TEM-12 (7; Jacoby and Bush, http://www.lahey.org/studies/webt./htm). The susceptibilities of the latter two isolates was perhaps explicable by the low level of expression of the TEM-53 gene, a view supported by the absence of electrofocusing bands with the characteristically low pI (pI 5.2) of TEM-12 (Table 1) (7). However, TEM-12 is among the weakest ESBLs (17, 19), and the susceptibilities of these producers may reflect the low level of activity of this enzyme against oxyimino-aminothiazolyl cephalosporins.

The ESBL producers included multiple subvariants of one strain (PFGE type A), smaller clusters of representatives of other strains (e.g., types E and F), and a diverse scatter of single isolates (types B to D and G to L). Even within strains of types A and E, there was scatter in their antibiogram, plasmid profile, and β-lactamase types. Previous work on a multicenter collection of *K. pneumoniae* isolates with ESBLs from European intensive care units (ICUs) likewise revealed mixtures of epidemic and nonepidemic strains in many hospitals and found considerable microdiversity within epidemic strains in terms of their antibiogram, plasmid profile, and, to a lesser extent, β-lactamase subtypes (29). Others, likewise, have described heterogeneity within outbreak strains of ESBL-producing *K. pneumoniae* strains (3, 10, 27). The diversity of ESBL producers and the variations within the ESBL-positive strains were not, therefore, surprising. What was remarkable was the complexity of the present isolates. Specifically (i) three individual patients (patients I, II, and III) each yielded two different strains of klebsiellae over brief periods, (ii) many isolates carried multiple identical or different bla_{TEM} and bla_{SHV} gene variants, and (iii) one new TEM variant and four new SHV variants were found among only 25 isolates.

Patient I yielded isolates 117, 118, 122, and 202 over 7 days in 1994. Among these, isolates 117, 122, and 202 belonged to type A, whereas isolate 118 was of a distinct type. All four isolates had the SHV-19 enzyme but differed in their TEM variants, with TEM-53 (maybe not expressed) detected in isolates 117 and 118, TEM-1 detected in isolate 122, and TEM-63 detected in isolate 202. In 1 week in 1996, patient II yielded isolates 4175 and 4291, which belonged to different strain types. Also in 1996, patient III yielded isolates 4183, 4265, 4744, and 8143 over a period of 1 month: isolate 4183 belonged to a unique PFGE type (type H), whereas the other three isolates were of PFGE type E. The type E isolates from patient III consistently had the SHV-5 β-lactamase but varied in the other SHV-type enzymes present (variously none, SHV-1, and SHV-22) and in whether or not multiple copies of bla_{TEM} and $bla_{\text{SHV-5}}$ were present. Most of the multiple isolates from patients II and III were from endotracheal aspirates, and it is not difficult to envisage colonization of the airways with multiple *Klebsiella* strains. The results for patient I were rather more surprising, insofar as three of the four isolates, including the unique isolate (isolate 118) were from peritoneal fluid or abdominal pus, suggesting a mixed infective population. Sequential or simultaneous isolation of unrelated strains of *E. coli* and *K. pneumoniae* from individual patients has been reported by others (4, 6, 13, 26), and Weller et al. (27) reported that multiple subvariants of a strain could persist in an infective population without any one becoming dominant.

All the isolates had both the bla_{TEM} and bla_{SHV} genes, and 20 isolates had multiple copies of one or both of these genes. Carriage of multiple copies was more frequent among the isolates collected in 1996 (29 bla_{TEM} copies and 26 bla_{SHV} copies among 13 isolates) than among those collected in 1994 (16 bla_{TEM} copies and 13 bla_{SHV} copies among 12 isolates). The greater proportion of isolates with three or more enzymes in 1996 may imply that complexity was increasing with time, but comparison is confounded by the small numbers of isolates, because an epidemic strain (strain A) was more prevalent in 1994, and because we cannot discount ESBL loss during storage. It should also be reemphasized that the present results almost certainly underestimated the prevalences of the bla_{SHV} and bla_{TEM} genes, as the use of different restriction endonucleases increased the number of bla_{TEM}-bearing fragments identified, and the same effect might be anticipated if multiple endonucleases had been used before probing for bla_{SHV}. Moreover, one route to β-lactamase hyperproduction is gene amplification (20), and with restriction enzymes lacking internal sites in the TEM and SHV genes (i.e., *Bam*HI, *Hin*dIII, and *Sal*I), only one hybridizing fragment would be generated regardless of whether a TEM or SHV gene existed alone or was flanked by copies of itself. With *Hin*cII (which has a site internal to bla_{TEM}), each bla_{TEM} copy would yield two hybridizing fragments, whereas two or more linked copies would still yield only three different sizes of hybridizing fragments. The presence of multiple gene copies increases the likelihood of enzyme hyperproduction, potentially increasing resistance to weak substrates and β-lactamase inhibitor combinations (17). Nevertheless, most of the present isolates were susceptible to

piperacillin-tazobactam (23 of 25 isolates) and amoxicillin-clavulanate (17 of 25 isolates). Multiple TEM β-lactamases have previously been found in single isolates (5, 21), as have combinations of TEM and SHV enzymes (8, 12, 28). Simultaneous production of multiple SHV ESBLs by single isolates has rarely been reported previously, and we believe that the present study is the first to record definitively isolates with multiple ESBLs of both the TEM and SHV families, although a similar situation was inferred from electrofocusing data by Yang et al. (28).

A novel TEM β-lactamase, TEM-63, was found in five isolates. It had four substitutions compared with the sequence of TEM-1: Leu21Phe, which lies in the signal peptide (9); Glu104Lys, which occurs in many TEM ESBLs (15); Arg164Ser, which widens the binding cavity to accommodate the bulky side chains of oxyimino-aminothiazolyl cephalosporins (15); and Met182Thr. Met182Thr also occurs in several inhibitor-resistant TEM β-lactamases (15) and in the TEM-43 ESBL (28); it augments rather than causes inhibitor resistance (15), and the present TEM-63 producers were susceptible to the inhibitor combinations tested (Table 2). TEM-63 has also been described from *K. pneumoniae*, *E. coli*, and *Proteus mirabilis* strains isolated in Durban, Johannesburg, and Cape Town, South Africa (13), and this enzyme, which has not been found elsewhere, may be locally prevalent in South Africa.

Besides TEM-63, four new bla_{SHV} variants were found and numbered SHV-19 to SHV-22. SHV-19 was found in 12 isolates, including 9 of 11 representatives of type A; it was distinguished from SHV-1 by Leu173Phe, a conservative substitution remote from any site associated with ESBL activity but lying within the omega loop. This mutation site and the lack of resistance to oxyimino-aminothiazolyl cephalosporins in organisms with SHV-19 plus TEM-1 (isolates 97 and 122) argue against the SHV-19 enzyme being an ESBL. SHV-20 likewise had Leu173Phe but also had the Gly238Ser substitution, which is almost universal in SHV ESBLs and which is the sole feature that distinguishes the SHV-2 β-lactamase from SHV-1 (15). SHV-20 therefore has the same relationship to SHV-2 that SHV-19 has to SHV-1. SHV-21 had both the substitutions present in SHV-20 and Leu122Phe, another conservative substitution remote from any site known to be associated with ESBL activity. The remaining mutant, SHV-22, resembled SHV-5 in having both Gly238Ser and Glu240Lys, but additionally, it had Asn158Lys, which affected another site not known to be associated with ESBL activity.

SHV-19 was predominantly found in isolates of type A but was not exclusive to this lineage, being found also in isolates of types B, C, and F1. Other enzymes with Leu173Phe, specifically, isolates with SHV-20 and SHV-21, were found in nontype A strains, and this mutation is also present in SHV-23, which was found in a *K. pneumoniae* isolate collected at the same hospital in 1990 (unpublished data). It seems that SHV variants with Leu173Phe are something of a local feature at King Edward VIII Hospital; whether they occur elsewhere in South Africa remains unknown.

In summary, the present study showed that ESBL dissemination at King Edward VIII Hospital reflected the evolution and spread of multiple different enzymes and strains. Even klebsiellae (ESBL producing or not) from single patients varied, belonging to different strain types and having different β-lactamases. Many of the ESBL producers had multiple identical or different bla_{TEM} and bla_{SHV} copy numbers. Some of these gene copies encoded TEM-63, a novel ESBL, and others encoded SHV-19 to SHV-22. In this situation of complexity and diversity, the concept of an ESBL outbreak is redundant; such complexity complicates the design of reliable antibiotic use policies, as well as the molecular biology-based investigations of resistance.

ACKNOWLEDGMENTS

Sabiha Y. Essack is grateful to the Wellcome Trust for a Travelling Fellowship (no. 048863/Z/96/Z) and to the Medical Research Council of South Africa. We are indebted to Brigid Duke for technical support.

REFERENCES

1. **Ambler, R. P., A. W. F. Coulson, J.-M. Frère, J.-M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for class A β-lactamases. Biochem. J. **276:**269–272.
2. **Arlet, G., M. Rouveau, and A. Philippon.** 1997. Substitution of alanine for aspartate at position 179 in the SHV-6 extended-spectrum β-lactamase. FEMS Microbiol. Lett. **152:**163–167.
3. **Bermudes, H., C. Arpin, F. Jude, Z. El-Harrif, C. Bébéar, and C. Quentin.** 1997. Molecular epidemiology of an outbreak due to extended-spectrum beta-lactamase-producing enterobacteria in a French hospital. Eur. J. Clin. Microbiol. Infect. Dis. **16:**523–529.
4. **Blackwood, R. A., C. K. Rode, C. L. Pierson, and C. A. Bloch.** 1997. Pulsed-field gel electrophoresis genomic fingerprinting of hospital *Escherichia coli* bacteraemia isolates. J. Med. Microbiol. **46:**506–510.
5. **Bradford, P. A., C. E. Cherubin, V. Idemyor, B. A. Rasmussen, and K. Bush.** 1994. Multiply resistant *Klebsiella pneumoniae* strains from two Chicago hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing β-lactamases in a single isolate. Antimicrob. Agents Chemother. **38:**761–766.
6. **Branger, C., A. L. Lesimple, B. Bruneau, P. Berry, and N. Lambert-Zechovsky.** 1998. Long-term investigation of the clonal dissemination of *Klebsiella pneumoniae* isolates producing extended-spectrum β-lactamases in a university hospital. J. Med. Microbiol. **47:**201–209.
7. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
8. **Gazouli, M., M. E. Kaufmann, E. Tzelepi, H. Dimopoulou, O. Paniara, and L. S. Tzouvelekis.** 1997. Study of an outbreak of cefoxitin-resistant *Klebsiella pneumoniae* in a general hospital. J. Clin. Microbiol. **35:**508–510.
9. **Gniadkowski, M., I. Schneider, R. Jungwirth, W. Hryniewicz, W., and A. Bauernfeind.** 1998. Ceftazidime-resistant *Enterobacteriaceae* isolates from three Polish hospitals: identification of three novel TEM- and SHV-5-type extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **42:**514–520.
10. **Gori, A., F. Espinasse, A. Deplano, C. Nonhoff, M. H. Nicolas, and M. J. Struelens.** 1996. Comparison of pulsed-field gel electrophoresis and randomly amplified DNA polymorphism analysis for typing extended-spectrum-β-lactamase-producing *Klebsiella pneumoniae*. J. Clin. Microbiol. **34:**2448–2453.
11. **Hall, L. M. C., D. M. Livermore, D. Gur, M. Akova, and H. E. Akalin.** 1993. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**1637–1644.
12. **Hanson, N. D., K. S. Thomson, E. S. Moland, C. C. Sanders, G. Berthold, and R. G. Penn.** 1999. Molecular characterization of a multiply resistant *Klebsiella pneumoniae* encoding ESBLs and a plasmid-mediated AmpC. J. Antimicrob. Chemother. **44:**377–380.
13. **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.
14. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
15. **Knox, J. R.** 1995. Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificities, and three-dimensional structure. Antimicrob. Agents Chemother. **39:**2593–2601.
16. **Leung, M., K. Shannon, and G. French.** 1997. Rarity of transferable β-lactamase production by *Klebsiella* species. J. Antimicrob. Chemother. **39:**737–745.
17. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
18. **Livermore, D. M., and J. E. Corkill.** 1992. Effects of CO_2 and pH on inhibition of TEM-1 and other β-lactamases by penicillanic acid sulfones.

Antimicrob. Agents Chemother. **36:**1870–1876.
19. **Livermore, D. M., and J. D. Williams.** 1996. Mode of action and mechanisms of bacterial resistance, p. 502–578. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. The Williams & Wilkins Co., Baltimore, Md.
20. **Martinez, J. L., and F. Baquero.** 1990. Epidemiology of antibiotic-inactivating enzymes and DNA probes: the problem of quantity. J. Antimicrob. Chemother. **26:**301–305.
21. **Medeiros, A. A., and J. Crellin.** 1997. Comparative susceptibility of clinical isolates producing extended spectrum β-lactamases to ceftibuten: effect of large inocula. Pediatr. Infect. Dis. J. **16:**S49–S55.
22. **Payne, D. J., and S. G. B. Amyes.** 1991. Transferable resistance to extended-spectrum β-lactams: a major threat or a minor inconvenience? J. Antimicrob. Chemother. **27:**255–261.
23. **Pitout, J. D. D., K. S. Thomson, N. D. Hanson, A. F. Ehrhardt, E. S. Moland, and C. C. Sanders.** 1998. β-Lactamases responsible for resistance to expanded-spectrum cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* isolates recovered in South Africa. Antimicrob. Agents Chemother. **42:**1350–1354.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Sirot, D.** 1995. Extended-spectrum plasmid mediated β-lactamases. J. Antimicrob. Chemother. **36**(Suppl. A)**:**19–34.
26. **Soulier, A., F. Barbut, J. M. Ollivier, J. C. Petit, and A. Lienhart.** 1995. Decreased transmission of Enterobacteriaceae with extended-spectrum β-lactamases in an intensive care unit by a nursing reorganization. J. Hosp. Infect. **31:**89–97.
27. **Weller, T. M. A., F. M. MacKenzie, and K. J. Forbes.** 1997. Molecular epidemiology of a large outbreak of multi-resistant *Klebsiella pneumoniae*. J. Med. Microbiol. **46:**921–926.
28. **Yang, Y., N. Bachech, P. A. Bradford, B. D. Jett, D. F. Sahm, and K. Bush.** 1998. Ceftazidime-resistant *Klebsiella pneumoniae* and *Escherichia coli* isolates producing TEM-10 and TEM-43 β-lactamases from St. Louis, Missouri. Antimicrob. Agents Chemother. **42:**1671–1676.
29. **Yuan, M., H. Aucken, L. M. C. Hall, T. Pitt, and D. M. Livermore.** 1997. Epidemiological typing of klebsiellae with extended-spectrum β-lactamases from European intensive care units. J. Antimicrob. Chemother. **41:**527–539.