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Countrywide Spread of CTX-M-3 Extended-Spectrum β-Lactamase-Producing Microorganisms of the Family *Enterobacteriaceae* in Poland

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Eighty-four clinical isolates of the family *Enterobacteriaceae*, recovered from 1998 to 2000 in 15 hospitals in 10 Polish cities, were analyzed. All the isolates produced β-lactamases with pIs of 8.4 and 5.4, and the pI 8.4 enzymes were demonstrated to hydrolyze cefotaxime but not ceftazidime in the in vitro bioassay. PCR analysis and DNA sequencing have revealed that in all cases the pI 8.4 β-lactamase was probably the CTX-M-3 extended-spectrum β-lactamase (ESBL) variant, which was originally identified in 1996 in Praski Hospital in Warsaw. In the majority of isolates, $bla_{CTX\text{-}M\text{-}3}$ genes resided within large conjugative plasmids with similar fingerprints, which, in the context of the high degree of diversity of the randomly amplified polymorphic DNA types of the isolates, suggested that horizontal transfer of plasmids was likely the main mechanism of CTX-M-3 spread. The dissemination of plasmids was probably preceded by the center-to-center transmission of several strains, as indicated by the identification by pulsed-field gel electrophoresis of closely related or possibly related *Klebsiella pneumoniae*, *Escherichia coli*, and *Citrobacter freundii* isolates in five different hospitals. CTX-M-3-producing organisms revealed a very high degree of diversity in β-lactam resistance levels and patterns. This was attributed to several factors, such as the production of other β-lactamases including additional ESBLs, possible quantitative variations in CTX-M-3 expression, segregation of AmpC derepressed mutants, and permeability alterations.

The wide geographic spread of some extended-spectrum β-lactamase (ESBL) variants has been a known epidemiological phenomenon since the second half of the 1980s. It may be mediated by the transmission of ESBL-producing strains between hospitals, which is followed by their clonal expansion and/or the horizontal transfer of plasmids carrying the ESBL gene. The intense clonal spread of an ESBL-producing organism was well documented in France by the identification of a *Klebsiella pneumoniae* strain expressing SHV-4 in 14 hospitals by the end of 1988 (1, 11) and of an *Enterobacter aerogenes* clone with TEM-24 in 21 hospitals by the end of 1997 (9). On the other hand, the transfer of plasmids among nonrelated strains was considered the major cause of the dissemination of the TEM-3 enzyme in 29 French hospitals in the 1980s (32, 33). The same ESBL variant may also appear independently in different institutions as a result of convergent evolution (18, 37).

The CTX-M family of β-lactamases groups evolutionarily related ESBLs with a much higher level of activity against cefotaxime than ceftazidime; and their similarity to some species-specific β-lactamases, like those of *Klebsiella oxytoca* and *Citrobacter diversus*, has been known for years (3, 6, 40). The recent finding of the 99% homology between the CTX-M-2 enzyme and the β-lactamase of *Kluyvera ascorbata* has indicated the origins of at least a fraction of the CTX-M variants (29). Whereas only three enzymes of this family (CTX-M-1/MEN-1, CTX-M-2, and Toho-1), found in clinical isolates from Germany, Italy, Argentina, Paraguay, Israel, and Japan, were described between 1990 and 1995 (3, 4, 5, 19), in recent years the list has been increasing very quickly. Eighteen new variants (CTX-M-3, CTX-M-3-like, CTX-M-3-4 to CTX-M-3-17, Toho-2, and Toho-3) have been reported since 1998 in a growing number of countries, including Poland, Latvia, Russia, Greece, Brazil, Spain, Kenya, and China (8, 10, 14, 15, 17, 21, 23, 29, 35; Y. Ishii, M. Galleni, L. Ma, K. Yamaguchi, and J. M. Frère, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1474, p. 98, 2000; www.lahey.org/studies/webt.htm). There are hospitals in which CTX-M β-lactamases are the most prevalent ESBLs (35, 42) and hospitals in which outbreaks caused by CTX-M producers have occurred (10, 30). Nevertheless, it seems that in the majority of countries the isolation of CTX-M-producing strains or outbreaks caused by these organisms remain sporadic.

This work presents the results of a detailed analysis of CTX-M-3-producing isolates of the family *Enterobacteriaceae* that were collected from 15 hospitals in Poland. Data obtained in the study have revealed a complex epidemiology of these organisms on a countrywide scale.

MATERIALS AND METHODS

Clinical isolates. Eighty-four clinical isolates of the family *Enterobacteriaceae* were collected between 1998 and 2000 from 15 hospitals of various types located in 10 different cities of Poland. The isolates belonged to seven species including *K. pneumoniae* ($n = 36$), *K. oxytoca* ($n = 3$), *Escherichia coli* ($n = 18$), *Enterobacter cloacae* ($n = 8$), *Citrobacter freundii* ($n = 11$), *Serratia marcescens* ($n = 7$), and *Morganella morganii* ($n = 1$). Table 1 shows the numbers of isolates recovered in particular centers, the time periods of their identification, and general characteristics of the centers.

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TABLE 1. Centers included in the study, periods of isolation of CTX-M-3-producing organisms, and numbers of isolates recovered in particular institutions

Center[a]	Location	Type of center	Time of collection (mo.yr)	No. of isolates						
				Total	*K. pneumoniae*	*K. oxytoca*	*E. coli*	*E. cloacae*	*C. freundii*	*S. marcescens*	*M. morganii*
BB	Bielsko-Biała	Secondary hospital	11.99–04.00	10	5	2	3				
CZ	Częstochowa	Secondary hospital	09.99–01.00	14	8		1	1	1	3	
GD	Gdańsk	Secondary hospital	04.98	1			1				
KA	Kartuzy	Primary hospital	04.98	1				1			
KR	Cracow	Tertiary teaching hospital	03.98–05.98	2	1		1				
SU	Suwałki	Secondary hospital	08.99–03.00	12	7		4		1		
SZ	Szczecin	Tertiary teaching hospital	03.98–06.98	5	4		1				
WA I	Warsaw	Tertiary specialist hospital	09.98–10.98	9	1		1	2	4	1	
WA II	Warsaw	Tertiary specialist hospital	10.99	2[c]	2						
WA III	Warsaw	Tertiary specialist hospital	10.99–04.00	3			2		1		
WA IV	Warsaw	Outpatients clinic[b]	11.99–02.00	3	1			1		1	
WA V	Warsaw	Tertiary teaching hospital	03.98–05.98	7	1		2		3	1	
WA VI	Warsaw	Tertiary specialist hospital	03.98–05.98	2	2						
WO	Wołomin	Primary hospital	10.99–06.00	8	3		1	1	1	1	1
WR	Wrocław	Tertiary teaching hospital	03.98–05.98	5	1	1	1	2			

[a] Tertiary specialist hospitals WA I, WA II, WA III, and WA VI are reference centers that specialize in hematology, oncology, rheumatology, and pediatrics, respectively.
[b] Isolates identified in the outpatient clinic of WA IV were collected from patients who had been hospitalized shortly before strain identification.
[c] Two *K. pneumoniae* isolates were collected from the same patient.

Except for the pair of *K. pneumoniae* isolates recovered in center WA II (WA II isolates 8932 and 8933), all isolates were collected from different patients who were hospitalized in diverse types of wards, mostly in intensive care units (28 patients), neonatal wards (10 patients), and internal medicine wards (10 patients). Eleven isolates were cultured in centers WA I and WA II, which are hospitals that specialize in the treatment of patients with hematological and/or oncological disorders. The majority of the isolates (47 isolates; 56.0%) were identified in urine samples, followed by those recovered from bronchial exudates (7 isolates), blood (6 isolates), and infected postoperative wound swabs (5 isolates). Species identification was performed with the ATB ID32E test (bioMérieux, Charbonnieres-les-Bains, France), and the isolates were tested for ESBL production by the double-disk synergy (DDS) test (20) with disks containing cefotaxime, ceftazidime, and amoxicillin with clavulanate (Oxoid, Basingstoke, United Kingdom). The disks were placed 20 mm apart (from center to center); for selected isolates the test was repeated with the distance reduced to 15 mm.

Antimicrobial susceptibility testing. The MICs of various antibiotics were determined by the agar dilution method in accordance with National Committee for Clinical Laboratory Standards guidelines (27). The following compounds were used: ampicillin and cefotaxime (Polfa, Tarchomin, Poland), aztreonam (Bristol-Myers Squibb, New Brunswick, N.J.), cefoxitin (Sigma Chemical Company, St. Louis, Mo.), ceftazidime (Glaxo Wellcome, Stevenage, United Kingdom), lithium clavulanate (SmithKline Beecham Pharmaceuticals, Betchworth, United Kingdom), imipenem (Merck Sharp & Dohme Research, Rahway, N.J.), piperacillin (Wyeth Ayerst Laboratories, Pearl River, N.Y.), and tazobactam (Lederle Laboratories, Pearl River, N.Y.). In all β-lactam–inhibitor combinations the constant concentrations of clavulanate and tazobactam were 2 and 4 μg/ml, respectively. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains.

Conjugative transfer of β-lactamase genes (mating). Bacterial conjugation was performed as described previously (17) with *E. coli* A15 R^- recipient strains resistant to rifampin or nalidixic acid. Transconjugants were selected on MacConkey agar (bioMérieux) plates supplemented with cefotaxime (2 μg/ml; Polfa) and rifampin (128 μg/ml; Polfa) or nalidixic acid (64 μg/ml; Sigma Chemical Company).

IEF of β-lactamases and bioassay for cephalosporin-hydrolyzing activities. Isoelectric focusing (IEF) of the β-lactamases was carried out with bacterial sonicates by the modified procedure described previously (5, 26) in a Mini IEF Cell (model 111; Bio-Rad, Hercules, Calif.). After IEF, the cefotaxime- and ceftazidime-hydrolyzing activities of particular β-lactamases were detected by the bioassay approach, as described by Bauernfeind et al. (5). Each of the two cephalosporins was used at a concentration of 2 μg/ml.

PCR detection of bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes. Total DNAs of the isolates or their transconjugants were purified with a Genomic DNA Prep Plus kit (A & A Biotechnology, Gdańsk, Poland). The complete coding sequences of the bla_{CTX-M} genes were amplified with primers P1C and P2D, and PCRs were run as reported previously (17). Primers SHV-A and SHV-C (16, 30) were used for partial amplification of the genes encoding SHV β-lactamases, whereas the entire bla_{TEM} genes were amplified with primers TEM-A and TEM-B (24). PCR conditions were identical to those described previously (16, 30).

Sequencing of bla_{CTX-M} genes. The PCR products encompassing the complete coding regions of the bla_{CTX-M} genes were sequenced (36) by using consecutive primers specific for the $bla_{CTX-M-1}$ gene (6) in a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, Calif.). Sequencing of both DNA strands was performed with the following primers: P1C (5′-TTAATTCGTCTCTTCCAGA-3′), A (5′-GAAAAGTGAAAGCGAACCGA-3′), P1A (5′-GGCGATCCGCGTGATACCAC-3′), P2D (5′-CAGCGCTTTTGCCGTCTAAG-3′), P2A (5′-GTGGTATCACGCGGATCGCC-3′), and P2B (5′-CGCTGATTTAACAGATTCGG-3′) (6).

RAPD typing. The genomic DNAs of the isolates were used in the randomly amplified polymorphic DNA (RAPD) analysis with primers RAPD-7 and RAPD-1283 (34). PCRs were run as described previously (16). A representative set of clinical isolates identified as CTX-M producers in 1996 and 1997 in Praski Hospital in Warsaw (center WA VII) was analyzed in the study. This set included *K. pneumoniae* WA VII 9, WA VII 10, WA VII 18, and WA VII 179; *K. oxytoca* WA VII 16; *E. coli* WA VII 6, WA VII 13, and WA VII 279; *E. cloacae* WA VII 186 and WA VII 272; *C. freundii* WA VII 21; and *S. marcescens* WA VII 11, WA VII 178, WA VII 275, and WA VII 278 (30).

PFGE typing. Pulsed-field gel electrophoresis (PFGE) analysis was performed with a CHEF DRII PFGE system (Bio-Rad) by the procedure described by Struelens et al. (38). Results were interpreted in accordance with the criteria proposed by Tenover et al. (39). Three CTX-M-producing isolates identified in 1996 and 1997 in Praski Hospital in Warsaw, *K. pneumoniae* WA VII 10, *E. coli* WA VII 13, and *C. freundii* WA VII 21 (30), were included in the study.

Plasmid DNA fingerprinting. Plasmid DNA was purified from bacterial cells by the alkaline lysis method (7) with a QIAGEN Plasmid Midi kit (QIAGEN, Hilden, Germany). For the fingerprinting analysis, plasmid DNA was digested with the *Pst*I restriction enzyme (MBI Fermentas, Vilnius, Lithuania). Plasmids specific for the group of CTX-M-producing isolates from 1996 and 1997 in Praski Hospital in Warsaw (30) were included in the study; these plasmids were purified from *K. pneumoniae* WA VII 18, 179, *E. coli* WA VII 279, *S. marcescens* WA VII 12, and transconjugants of isolates *E. coli* WA VII 6 and WA VII 13 and *S. marcescens* WA VII 275 and WA VII 278.

RESULTS

Resistance phenotypes of isolates in DDS test. Seventy-three isolates were identified as putative ESBL producers on the basis of positive results by the DDS test (20). The remain-

TABLE 2. β-Lactamase contents of isolates, mating, β-lactamases in transconjugant strains, oxyiminocephalosporin-hydrolyzing activities of β-lactamases, ESBL genes identified by PCR, and sequencing of $bla_{CTX-M-3}$ genes

Species	Center(s)	No. of isolates	pIs of β-lactamases in clinical isolates[a]	Mating	β-Lactamases in transconjugants[a] (pIs)	ESBL gene(s) identified by PCR	No. of gene sequences[b]
K. pneumoniae	BB, CZ, SU, SZ, WA I, WA II, WO	21	**8.4**, 7.6, 5.4	+	**8.4**, 5.4	CTX-M	8
K. pneumoniae	WA VI, WR	3	**8.4**, 7.6, 5.4	−		CTX-M	3
K. pneumoniae	WA IV	1	**8.4**, 5.4	−		CTX-M	
K. pneumoniae[c]	WA V	1	**8.4, 8.2**, 7.6, 5.4	+	**8.4, 8.2**, 5.4	CTX-M, SHV	
K. pneumoniae[d]	SU	6	**8.4 8.2**, 7.6, 5.4	+	**8.4**, 5.4	CTX-M, SHV	
K. pneumoniae[e]	SZ	1	**8.4 8.2**, 7.6, 5.4	−		CTX-M, SHV	1
K. pneumoniae[f]	CZ	2	**8.4**, 7.6, **6.0**, 5.4	+	**6.0**	CTX-M, TEM	
K. pneumoniae[g]	KR	1	**8.4**, 7.6, **6.0**, 5.4	−		CTX-M	
K. oxytoca	BB, WR	2	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	1
K. oxytoca	BB	1	**8.4**, 5.4	−		CTX-M	
E. coli	BB, CZ, GD, KR, SU, SZ, WA I, WA III, WA V, WO, WR	17	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	9
E. coli	BB	1	**8.4**, 5.4	−		CTX-M	
E. cloacae	WA I, WO, WR	5	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	2
E. cloacae[h]	CZ, WA IV	2	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	
E. cloacae[i]	KA	1	**8.4, 8.0**, 5.4	+	**8.4**, 5.4	CTX-M	
C. freundii	CZ, WA I, WA III, WA V	5	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	1
C. freundii[j]	SU, WA V	2	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	1
C. freundii[k]	WA V	1	**8.4**, 5.4	−		CTX-M	
C. freundii	WA I	2	**8.4**, 7.0, 5.4	+	**8.4**, 5.4	CTX-M	
C. freundii	WO	1	**8.4**, 7.0, 5.4	−		CTX-M	
S. marcescens	CZ	1	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	
S. marcescens	CZ, WA IV, WO	3	9.0,[l] **8.4**, 5.4	−		CTX-M	
S. marcescens[m]	CZ, WA I, WA V	3	**9.0, 8.4**, 5.4	+	**8.4**, 5.4	CTX-M	
M. morganii	WO	1	**8.4**, 5.4	+	**8.4**, 5.4	CTX-M	1

[a] pI values in boldface refer to β-lactamases that were found to hydrolyze only cefotaxime in the bioassay experiment, whereas pI values which are in boldface and underlined represent enzymes that hydrolyzed both cefotaxime and ceftazidime.
[b] Numbers of $bla_{CTX-M-3}$ genes sequenced in particular groups of isolates.
[c] *K. pneumoniae* isolate WA V 7888.
[d] *K. pneumoniae* isolates SU 8406, SU 8815, SU 8912, SU 9148, SU 9161, and SU 972.
[e] *K. pneumoniae* isolate SZ 2941.
[f] *K. pneumoniae* isolates CZ 9455 and CZ 9459.
[g] *K. pneumoniae* isolate KR 3443; this isolate was not tested for the presence of a bla_{TEM} gene.
[h] *E. cloacae* isolates CZ 8320 and WA IV 838.
[i] *E. cloacae* isolate KA 3663.
[j] *C. freundii* isolates SU 9050 and WA V 3324.
[k] *C. freundii* isolate WA V 3570.
[l] The pI ~9.0 β-lactamases of these isolates produced "weak" IEF bands and were negative by the bioassay under the conditions used.
[m] *S. marcescens* isolates CZ 9424, WA I 4172, and WA V 3033.

ing 11 isolates produced DDS test results that were either negative or difficult to interpret, despite the decreased distance between the disks. These were two *K. pneumoniae* isolates (WA II 8933 and WA IV 9172), three *E. cloacae* isolates (isolates CZ 8320, KA 3663, and WA IV 838), three *C. freundii* isolates (isolates SU 9050, WA V 3324, and WA V 3570), and three *S. marcescens* isolates (isolates CZ 9424, WA I 4172, and WA V 3033).

β-Lactamase contents of isolates. The β-lactamase contents of the isolates are shown in Table 2. All the isolates were found to produce β-lactamases with a pI of 8.4; these were perpetually accompanied by enzymes with a pI of 5.4. Other β-lactamases of various pI values were also identified in some of the isolates, and these included enzymes with pIs of 8.2 and 6.0 that were expressed by 11 *K. pneumoniae* isolates. The most frequent were pI 8.2 β-lactamases observed in eight isolates from three hospitals (isolates SU 8406, SU 8815, SU 8912, SU 9148, SU 9161, and SU 972, SZ 2941, and WA V 7888), whereas enzymes with a pI of 6.0 were produced by three isolates from two other institutions (isolates CZ 9455, CZ 9459, and KR 3443). Almost all *K. pneumoniae* isolates expressed β-lactamases with a pI of 7.6, and some isolates of other species produced enzymes with pIs of ≈9.0 (multiple *S. marcescens* isolates), 8.0 (*E. cloacae* KA 3663), or 7.0 (*C. freundii* WA I 4185, WA I 4358, and WO 9105). The *S. marcescens* pI ≈9.0 β-lactamases were observed either as "strong" IEF bands (isolates CZ 9424, WA I 4172, and WA V 3033) or as relatively "weak" ones.

Resistance transfer and β-lactamase content of transconjugant strains. The results of the analyses of resistance transfer and the β-lactamase contents of the transconjugant strains are shown in Table 2. Seventy-one isolates of all species identified produced transconjugants that were selected on the cefotaxime-containing medium. For the majority of isolates (56

isolates), conjugation efficiency was high (about 10^{-3} recombinants per donor cell). Almost all transconjugant strains (68 strains) were found to express just β-lactamases with pIs of 8.4 and 5.4, and only one transconjugant (of *K. pneumoniae* WA V 7888), produced the pI 8.2 β-lactamase, in addition to the enzymes with pIs of 8.4 and 5.4. Transconjugants of two *K. pneumoniae* isolates that coexpressed the pI 8.4, 6.0, and 5.4 β-lactamases (*K. pneumoniae* CZ 9455 and CZ 9459) were shown to produce only the pI 6.0 enzyme.

Identification of β-lactamases with oxyiminocephalosporin-hydrolyzing activities. The bioassay experiment was carried out with protein extracts obtained from all clinical isolates and their transconjugants. The results are presented in Table 2. In all transconjugants the pI 8.4 β-lactamase demonstrated cefotaxime-hydrolyzing activity but not ceftazidime-hydrolyzing activity under the conditions used in the assay, whereas the pI 8.2 enzyme (from the transconjugant of *K. pneumoniae* WA V 7888) and the pI 6.0 enzyme (from recombinants of *K. pneumoniae* CZ 9455 and CZ 9459) hydrolyzed the two compounds. The analysis performed with extracts from clinical isolates confirmed these observations in general; however, it has also assigned the activity against both cefotaxime and ceftazidime to some β-lactamases produced by DDS test-negative or problematic *E. cloacae*, *C. freundii*, and *S. marcescens* isolates. These were enzymes with pIs of ≈9.0 (only in *S. marcescens* isolates CZ 9424, WA I 4172, and WA V 3033), 8.4 (*E. cloacae* CZ 8320 and WA IV 838 and *C. freundii* SU 9050, WA V 3324 and WA V 3570), and 8.0 (*E. cloacae* KA 3663).

Detection of ESBL-encoding genes by PCR. The results of the detection of ESBL-encoding genes by PCR are shown in Table 2. All clinical isolates were tested for the presence of genes coding for the CTX-M family of ESBLs with primers that are specific for both the $bla_{\text{CTX-M-1}}$ and the $bla_{\text{CTX-M-3}}$ genes (6, 17). PCR products of the expected size (approximately 1 kb) were obtained for all isolates. Detection of bla_{SHV} genes was performed for all the *K. pneumoniae* isolates which produced, in addition, the pI 8.2 β-lactamase (isolates SU 8406, SU 8815, SU 8912, SU 9148, SU 9161, and SU 972, SZ 2941, and WA V 7888) and for the only transconjugant that did so (a transconjugant of *K. pneumoniae* WA V 7888). The primers used in this analysis are specific for genes encoding SHV β-lactamases, which possess ESBL-type G238S and E240K substitutions (30). PCR products of the expected size of about 200 bp were obtained for the strains analyzed. Total DNAs of the transconjugants expressing the pI 6.0 ESBL (*K. pneumoniae* CZ 9455 and CZ 9459) were used for the detection of bla_{TEM} genes, and amplicons of the expected size of about 1 kb were observed.

Sequencing of $bla_{\text{CTX-M}}$ genes. The complete nucleotide sequence of the $bla_{\text{CTX-M}}$ gene coding region was determined for 27 isolates. These isolates represented different species (12 *K. pneumoniae* isolates, 1 *K. oxytoca* isolate, 9 *E. coli* isolates, 2 *E. cloacae* isolates, 2 *C. freundii* isolates, and 1 *M. morganii* isolate) and different centers (all centers except KA and WA IV). In addition, the MICs for the transconjugants varied within the range of values determined for recombinant strains (see below). The results are listed in Table 2. All isolates analyzed were found to contain $bla_{\text{CTX-M-3}}$ genes with coding regions with identical nucleotide sequences (17).

TABLE 3. Relatedness of *K. pneumoniae, E. coli*, and *C. freundii* isolates recovered in different centers

Isolate[a]	pIs of β-lactamases	RAPD type[b]	PFGE type
K. pneumoniae WA VII 10	8.4, 7.6, 5.4	i	A1
K. pneumoniae WA I 4356	8.4, 7.6, 5.4	i	A2
K. pneumoniae WA V 7888	8.4, 8.2, 7.6, 5.4	h	B1
K. pneumoniae SZ 2941	8.4, 8.2, 7.6, 5.4	h	B2
E. coli WA VII 13	8.4, 5.4	i	a1
E. coli SZ 3624	8.4, 5.4	i	a2
C. freundii WA VII 21	8.4, 5.4	c	α1
C. freundii WA I 4174	8.4, 5.4	c	α2
C. freundii WA I 4181	8.4, 5.4	c	α3
C. freundii WA V 3570	8.4, 5.4	c	α4
C. freundii WA I 4185	8.4, 7.0, 5.4	d	β1
C. freundii WA I 4358	8.4, 7.0, 5.4	d	β2
C. freundii WO 9105	8.4, 7.0, 5.4	d	β3

[a] "WA VII" is the designation for Praski Hospital in Warsaw, and the isolates collected in this center in 1996 and 1997 (30) were used only in the comparative typing in this work.
[b] RAPD types were designated by letters independently for each group of isolates by species.

Typing by RAPD analysis and PFGE. All clinical isolates except for the single *M. morganii* strain were typed by RAPD analysis in conjunction with 15 CTX-M-producing isolates recovered in 1996 and 1997 in Praski Hospital in Warsaw (center WA VII), in which the CTX-M-3 ESBL was originally identified (17). These isolates represented almost all RAPD types of CTX-M producers that had been discerned at the time in the institution (30). In general, a high degree of diversity of RAPD patterns was observed among isolates of all species identified. Including the isolates from Praski Hospital, 19 RAPD types were identified among 40 *K. pneumoniae* isolates, 17 types were identified among 21 *E. coli* isolates, 8 types were identified among 10 *E. cloacae* isolates, and 6 types were identified among 12 *C. freundii* isolates. The analysis discriminated all 4 *K. oxytoca* isolates and 11 *S. marcescens* isolates. At a single-center level, only four clusters of more than two indistinguishable isolates were revealed; and these were clusters of *K. pneumoniae* isolates collected in four hospitals (BB, five isolates; CZ, five isolates; SU, seven isolates; SZ, three isolates). Additionally, 10 pairs of isolates of different species were characterized by identical RAPD patterns within particular centers.

Several cases of the identity of RAPD patterns were found among isolates recovered in different hospitals (Table 3). This was observed for two pairs of *K. pneumoniae* isolates, one pair of *E. coli* isolates, and two clusters of four and three *C. freundii* isolates, respectively. All these isolates were subjected to PFGE typing, and the results of the analysis, shown in Table 3, confirm the observations obtained by RAPD analysis. The *K. pneumoniae* isolates of the two pairs analyzed could be classified as closely related isolates (isolates WA VII 10 and WA I 4356) or possibly related isolates (isolates WA V 7888 and SZ 2941) within two distinct PFGE types (types A and B). This is also the case with the pair of possibly related *E. coli* isolates (isolates WA VII 13 and SZ 3624; PFGE type a). Four *C. freundii* isolates collected in centers WA VII, WA I, and WA V and indistinguishable by RAPD analysis (isolates WA VII 21, WA I 4174, WA I 4181, and WA V 3570) produced similar

PFGE patterns (PFGE type α) and were found to be possibly related. The possible relatedness of three *C. freundii* isolates from centers WA I and WO (isolates WA I 4185, WA I 4358, and WO 9105; PFGE type β) was also confirmed.

Plasmid fingerprinting. Plasmid DNAs specific for 43 clinical isolates of different species, centers, and RAPD types were subjected to a fingerprinting analysis. Plasmids representing all fingerprints identified in 1996 and 1997 in CTX-M-producing isolates from Praski Hospital in Warsaw (designated A1 to A7 and B) (30) were included in the study. In the majority of cases plasmid DNA was purified from transconjugant strains (only if these were available). All the isolates contained high-molecular-weight plasmids, which in many cases were accompanied by various smaller molecules. Altogether, 31 different fingerprints were distinguished among the isolates; however, large plasmids of 41 isolates revealed highly similar restriction patterns that were either identical (variant A1) or similar (variants A8 to A35) to those previously observed in the isolates from Praski Hospital (variants A1 to A7). The only A-type plasmid variant which was present in multiple isolates was plasmid A1, found alone in 12 isolates of various species that were recovered in nine hospitals (BB, CZ, KA, SU, WA I, WA IV, WA V, WO, and WR). Two single *E. coli* isolates from two centers (isolates SU 8350 and BB 1775) contained large plasmids with remarkably different *Pst*I fingerprints (patterns C and D, respectively).

Antimicrobial susceptibilities of clinical isolates. Table 4 shows the ranges of MICs grouped with regard to specific groups of isolates. The MICs of ampicillin and imipenem are not given in Table 4 as they were the same for all isolates (ampicillin MICs, >512 μg/ml; imipenem MICs, 0.125 to 1 μg/ml). The MIC patterns for 60 isolates of all species identified were characteristic for the CTX-M family of ESBL producers (4, 5, 30). The cefotaxime MICs for these isolates were higher by 3 to 8 dilutions than those of ceftazidime, and clavulanate reduced cefotaxime MICs by at least 5 dilutions and usually 8 or more dilutions. Nevertheless, a high degree of quantitative diversity of MICs was observed among these isolates, especially *K. pneumoniae*, and this is well illustrated by the MICs of cefotaxime (MICs, 2 to 512 μg/ml), ceftazidime (MICs, 0.25 to 16 μg/ml), or piperacillin with tazobactam (MICs, 1 to 256 μg/ml). Besides the cefoxitin resistance of the *E. cloacae*, *C. freundii*, and *S. marcescens* isolates, resistance to this drug appeared in sporadic isolates of this group.

The susceptibility patterns of the 24 remaining isolates differed from those typical of CTX-M producers, and they could be classified into four groups, three of which included only *K. pneumoniae*. The first was formed by eight *K. pneumoniae* isolates (isolates SU 8406, SU 8815, SU 8912, SU 9148, SU 9161, SU 972, SZ 2941, and WA V 7888), which, apart from CTX-M-3, also produced SHV ESBLs. The ceftazidime MICs for these isolates (64 to 128 μg/ml) were higher than the cefotaxime MICs (16 to 64 μg/ml). The ceftazidime MICs for the three *K. pneumoniae* isolates (isolates CZ 9455, CZ 9459, and KR 3443) that coexpressed the CTX-M-3 and TEM ESBLs were also elevated (32 to 64 μg/ml), although the cefotaxime MICs for these three isolates (128 to 256 μg/ml) remained higher. The third specific fraction of *K. pneumoniae* isolates consisted of four isolates from three centers (isolates SZ 3733, SZ 3734, WA II 8933, and WA IV 9172); two of these isolates (isolates WA II 8933 and WA IV 9172) were negative by the DDS test. In general, the β-lactam MICs, including the MICs of inhibitor combinations, for these isolates were very high (e.g., cefotaxime MICs, 512 to >512 μg/ml; ceftazidime MICs, 32 to 64 μg/ml; cefoxitin MICs, 32 to 128 μg/ml, cefotaxime plus clavulanate MICs, 128 to 512 μg/ml).

The last group consisted of nine isolates (*E. cloacae* CZ 8320, KA 3663, and WA IV 838; *C. freundii* SU 9050, WA V 3324, and WA V 3570; and *S. marcescens* CZ 9424, WA I 4172, and WA V 3033) that produced negative or unclear results by the DDS test. They were characterized by high MICs of cefotaxime (256 to 512 μg/ml), ceftazidime (16 to 128 μg/ml), and inhibitor combinations (e.g., cefotaxime plus clavulanate MICs, 32 to 256 μg/ml).

Antimicrobial susceptibilities of transconjugants. Results of the analysis of the antimicrobial susceptibilities of the transconjugants are shown in Table 4. Data for ampicillin and imipenem are not included as they were the same for all transconjugants (ampicillin MICs, 512 to >512 μg/ml; imipenem MICs, 0.125 to 0.25 μg/ml). For most of the transconjugants that expressed only CTX-M-3 and the pI 5.4 β-lactamase, the MICs were much lower than those for clinical isolates, but the MICs for the strains were still markedly diverse, with MIC patterns that are characteristic for CTX-M producer strains (4, 5, 30). Cefotaxime MICs (4 to 32 μg/ml) were 3 or more dilutions higher than those of ceftazidime (0.25 to 2 μg/ml), and β-lactamase inhibitors efficiently reduced the MICs of the β-lactams (e.g., cefotaxime plus clavulanate MICs, ≤0.03 to 0.06 μg/ml). Strongly elevated MICs characterized recombinants of two of the four *K. pneumoniae* isolates for which the MICs of β-lactams and β-lactam plus inhibitor combinations were particularly high (*K. pneumoniae* SZ 3733 and SZ 3734). The MICs of oxyimino-β-lactams for these transconjugants were higher (e.g., cefotaxime MICs, 128 μg/ml; ceftazidime MICs, 8 μg/ml); however, the MICs of inhibitor combinations for these transconjugants were not significantly increased.

The only transconjugant which coexpressed CTX-M-3 and the SHV ESBL (a transconjugant of *K. pneumoniae* WA V 7888) was characterized by similar cefotaxime and ceftazidime MICs (cefotaxime MIC, 16 μg/ml; ceftazidime MIC, 8 μg/ml), as was the case for the recombinants of two *K. pneumoniae* isolates (isolates CZ 9455 and CZ 9459) that produced only TEM ESBLs (cefotaxime MICs, 4 to 8 μg/ml; ceftazidime MICs, 4 μg/ml).

DISCUSSION

The 1998 survey of the prevalence of different ESBL types among ESBL producers in seven hospitals in Poland has suggested an important role of CTX-M enzymes on a wider scale (M. Gniadkowski, A. Baraniak, J. Fiett, and W. Hryniewicz, unpublished results). CTX-M β-lactamases (identified by IEF, bioassay, and PCR) were found in 18.8% of 96 isolates collected within a 4-month period, and the isolates were recovered in six different centers. This frequency was below that of SHV-type ESBLs (60.4%); however, it was comparable to the prevalence of TEM enzymes (20.8%). The study reported here was carried out both with the isolates identified during the survey and with new isolates, recovered later in nine other hospitals, which were suspected to be CTX-M producers on

TABLE 4. Resistance phenotypes of the distinguished fractions of CTX-M-3-producing isolates and their transconjugants

Isolate (no. of isolates)	MIC (μg/ml)[a]								
	PIP	TZP	CTX	CTX + CLA	CAZ	CAZ + CLA	ATM	ATM + CLA	FOX
K. pneumoniae[b] (21)	256–>512	1–256	2–512	≤0.03–4	0.25–16	0.06–2	0.5–128	≤0.03–4	2–16
K. oxytoca[b] (3)	512–>512	1–4	16–64	0.06–0.125	2–4	0.125–0.5	4–8	0.06–0.125	4–64
E. coli[b] (18)	256–>512	≤0.5–32	8–128	≤0.03–0.25	0.5–8	0.06–0.5	2–16	≤0.03–0.5	2–32
E. cloacae[b] (5)	>512	2–16	128–256	0.5–4	8	0.5–2	16–32	0.125–1	128–512
C. freundii[b] (8)	512–>512	1–32	64–512	0.125–1	2–16	0.25–1	8–64	0.06–0.5	64–512
S. marcescens[b] (4)	256–>512	2–8	64–256	1–4	1–2	0.25–0.5	8–32	0.25–2	32–64
M. morganii[b] (1)	>512	≤0.5	16	≤0.03	0.25	0.25	1	≤0.03	8
K. pneumoniae CTX-M-3 + SHV[c] (8)	>512	16–256	16–64	0.125–0.5	64–128	1–4	128–256	0.125–0.5	2–64
K. pneumoniae CTX-M-3 + TEM[d] (3)	>512	4–64	128–256	0.125–2	32–64	0.5–2	32–64	0.125–0.5	4–128
K. pneumoniae SZ 3733 and SZ 3734[e] (2)	>512	>512	>512	128	32	4	128	32	32
K. pneumoniae WAII 8933, WA IV 9172[e] (2)	>512	512–>512	512–>512	256–512	32–64	16–32	128–256	64–256	64–128
E. cloacae CTX-M-3 + AmpC derepressed[f] (3)	>512	32–512	256–512	64–256	64–128	64	32–128	16–64	256–512
C. freundii CTX-M-3 + AmpC derepressed[g] (3)	512–>512	32–64	256–512	32–64	64–128	32–128	32–64	16–32	128–512
S. marcescens CTX-M-3 + AmpC derepressed[h] (3)	512–>512	32–512	512	64–256	16	4–8	32–64	8–32	128–256
R$^+$ strains[i] (65)	128–256	≤0.5–2	4–32	≤0.03–0.06	0.25–2	≤0.03–0.25	1–8	≤0.03–0.06	1–4
R$^+$ (*K. pneumoniae* WAV 7888) CTX-M-3 + SHV (1)	256	≤0.5	16	≤0.03	8	0.125	16	0.06	2
R$^+$ (*K. pneumoniae* CZ 9455 and CZ 9459) TEM (2)	64–128	1–2	4–8	≤0.03	4	0.06	2–4	≤0.03	2
R$^+$ (*K. pneumoniae* SZ 3733 and SZ 3734)[j] (2)	>512	1–2	128	0.125	8	0.5	32	0.125	4
R$^+$ (*K. pneumoniae* WA II 8933)[k] (1)	256	2	16	≤0.03	1	0.25	4	≤0.03	2
E. coli A15	≤0.5	1–2	≤0.03	≤0.03	0.06–0.125	≤0.03	≤0.03	≤0.03	1–2

[a] Abbreviations: PIP, piperacillin; TZP, piperacillin with tazobactam; CTX, cefotaxime; CLA, clavulanate; CAZ, ceftazidime; ATM, aztreonam; FOX, cefoxitin.
[b] Isolates of different species, in which CTX-M-3 was considered to be the major mechanism affecting the resistance phenotype.
[c] *K. pneumoniae* isolates SU 8406, SU 8815, SU 8912, SU 9148, SU 9161, SU 972, SZ 2941, and WA V 7888.
[d] *K. pneumoniae* isolates CZ 9455, CZ 9459, and KR 3443.
[e] The resistance phenotypes of these isolates are postulated to be strongly influenced by the decreased permeability and, in the case of *K. pneumoniae* SZ 3733 and SZ 3734, by the higher level of CTX-M-3 expression.
[f] *E. cloacae* isolates CZ 8320, KA 3663 and WA IV 838.
[g] *C. freundii* isolates SU 9050, WA V 3324, and WA V 3570.
[h] *S. marcescens* isolates CZ 9424, WA I 4172, and WA V 3033.
[i] R$^+$, transconjugant. The major fraction of transconjugant strains expressed only CTX-M-3 and the pI 5.4 β-lactamases.
[j] Transconjugants of the isolates, in which a significantly higher level of CTX-M-3 is postulated.
[k] The MICs for the transconjugant of *K. pneumoniae* WA II 8933 are shown separately only for comparison with those for the clinical isolate.

the basis of preliminary phenotype observations and IEF analysis.

Eighty-four clinical isolates of the family *Enterobacteriaceae* collected from 15 medical institutions in Poland were found to produce a CTX-M β-lactamase with a pI of 8.4 that revealed the characteristic cefotaxime-hydrolyzing activity but not ceftazidime-hydrolyzing activity in vitro. The β-lactamase was always expressed together with a pI 5.4 β-lactamase, and genes coding for the two types of enzymes were located on the same plasmids, which in the majority of isolates had conjugative functions. DNA sequencing, performed for 27 isolates, revealed that they all expressed CTX-M-3. The CTX-M-3 β-lactamase was originally identified in three *C. freundii* isolates in mid-1996 in Praski Hospital in Warsaw (17), and the enzyme was probably spread in that institution in 1996 and 1997 (30). In 1998 a CTX-M-3-producing *E. cloacae* strain was recovered in a hospital in Versailles, France (13), and then, in 1999, *E. coli* strains expressing the same enzyme were found in southern Taiwan (42). CTX-M-3 is closely related to the CTX-M-1/MEN-1 enzyme identified in Germany (5; A. Bauernfeind,, H. Grimm, I. Stemplinger, S. Ernst, and R. Jungwirth, Final Program Abstr. 19th Int. Congr. Chemother., Can. J. Infect. Dis. **6**(Suppl. C)**:**470C, abstr. 4210, 1995) and France (in an isolate from an Italian patient) (3), the CTX-M-10 enzyme reported in Spain (29), the CTX-M-12 enzyme from Kenya (21), and the CTX-M-11 enzyme from China and the CTX-M-3-like enzyme

from Japan (GenBank accession numbers AY005110 and AY013478, respectively) (www.lahey.org/studies/webt.htm). These β-lactamases form a distinct branch within the evolutionary tree of CTX-M ESBLs (8; www.lahey.org/studies/webt.htm).

Isolation of CTX-M-3-producing strains of the family *Enterobacteriaceae* in 15 health care centers in 10 different cities of Poland is indicative of the spread of these organisms in the whole country. Six of the centers are located in Warsaw, and this means that, together with Praski Hospital, there are seven separate institutions in the city in which CTX-M-3 has been identified to date. Such a countrywide dissemination of CTX-M producers seems to be very rare and may be compared only to the situation in Argentina, where CTX-M-2 (4, 6) has been reported to be the most frequently occurring ESBL type (M. F. Galas, M. J. Rapoport, F. G. Pasteran, R. G. Melano, A. E. Petroni, P. G. Ceriana, WHONET Group, and A. Rossi, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1474, p. 165, 1999). The typing and plasmid fingerprinting analyses of the isolates have brought important insight into the spread of CTX-M-3 in Poland. The high degree of diversity of the RAPD types of isolates of all species suggested that the clonal dissemination of producer strains, even if important in some of the centers, did not play the predominant role in the overall situation. On the other hand, the location of the $bla_{CTX\text{-}M\text{-}3}$ genes within large conjugative plasmids with identical or similar fingerprints (A-type plasmids) in the majority of the isolates indicated that horizontal plasmid transfer was the main mechanism of CTX-M-3 spread. The observed heterogeneity of A-type plasmids probably reflects the ongoing diversification of the molecules, and it is likely that the A1 variant, which was carried by multiple isolates from nine hospitals, was the ancestor of the whole family. It is noteworthy, however, that the $bla_{CTX\text{-}M\text{-}3}$ genes may also reside in plasmids with clearly distinct fingerprints, which were found in single *E. coli* isolates from centers BB and SU (plasmids C and D, respectively) and from Praski Hospital in 1996 and 1997 (plasmid B) (30).

The spread of a single type of transferable plasmids within bacterial populations within numerous, geographically separated hospitals has raised the question of the origins of these plasmids in particular centers. The most likely hypothesis is that they were transmitted from one center to another with bacterial strains by means of patient transfer, which is a common practice in Poland and which occurs between all types of hospitals. Recognition of all strains that could constitute a "network of epidemiological links" involving all the centers in the study would be difficult in practice; however, the identification of related organisms in two or more hospitals has supported the hypothesis of strain transmission. The majority of related isolates were collected in three Warsaw centers including Praski Hospital, and all these were linked to each other by a single clone of *C. freundii*, designated PFGE type α. This epidemic strain was originally identified as clonally spread in 1996 and in 1996 and 1997 in Praski Hospital (17, 30), and in the present work it was also found in centers WA I and WA V. Other related isolates were observed in Praski Hospital and centers WA I (*K. pneumoniae*) and SZ (*E. coli*), in hospitals WA V and SZ (*K. pneumoniae*), and in centers WA I and WO (*C. freundii*). Although fragmentary, these data suggest that the transmission of several CTX-M-3-producing strains preceded the dissemination of plasmids with the $bla_{CTX\text{-}M\text{-}3}$ gene in Polish hospitals.

Antimicrobial susceptibility testing has revealed the high degree of diversity of the resistance phenotypes of CTX-M-3-producing strains, which to some extent was also demonstrated by the DDS test. This diversity was mostly manifested in the overall level of β-lactam resistance, the ratio of the rate of resistance to cefotaxime and the rate of resistance to ceftazidime, and the potencies of β-lactamase inhibitors in reducing β-lactam MICs. Several factors, including both species- and strain-specific factors, may explain these observations. One of these was the production of an additional ESBL. Altogether, 11 *K. pneumoniae* isolates from five centers also expressed, apart from CTX-M-3, cefotaxime- and ceftazidime-hydrolyzing β-lactamases of the SHV (pI 8.2) or the TEM (pI 6.0) family. These enzymes were probably responsible for the elevated MICs of ceftazidime (compared to those of cefotaxime) which characterized the isolates. The presence of two different ESBL types in a single clinical isolate seems to be rare, although such combinations have already been reported, for example, in France and Argentina (37; Galas et al., 39th ICAAC; F. Pasteran and M. Galas, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1607, p. 153, 2000). Some other β-lactamases that were observed as separate IEF bands in protein extracts of the clinical isolates, such as those with pIs of 5.4 (probably TEM-1), 7.6 (probably species-specific *K. pneumoniae* enzymes), or 7.0, did not show cefotaxime- or ceftazidime-hydrolyzing activity in the bioassay under the conditions used. Most likely, these enzymes did not affect significantly the oxyimino-β-lactam resistance of the isolates; however, in general, they must have contributed to their β-lactam resistance phenotypes.

The other group of isolates with complex phenotypes consisted of nine isolates of *E. cloacae*, *C. freundii*, and *S. marcescens*, all of which had negative or unclear results by the DDS test. These species are known to frequently segregate mutants with the derepressed expression of specific class C (AmpC) cephalosporinases (22). The phenotypes of such mutants manifest in high MICs of the majority of oxyimino-β-lactams and inhibitor combinations, which were observed for the nine isolates. Their protein extracts revealed both cefotaxime- and ceftazidime-hydrolyzing activities within β-lactamase IEF bands with pIs of ≈9.0, 8.4, or 8.0, which is in accordance with the published data on AmpC enzymes (12). The *E. cloacae* and *C. freundii* isolates, in which the cefotaxime- and ceftazidime-hydrolyzing activities were assigned to the pI 8.4 β-lactamase band, produced transconjugants with only the cefotaxime-hydrolyzing pI 8.4 β-lactamase. This indicated that their AmpC enzymes probably comigrated with CTX-M-3 in the IEF analysis. Such ESBL-producing AmpC derepressed mutants have been reported in several papers to date (2, 28). What is noteworthy is that of the remaining isolates of the AmpC producer species, the putative AmpC β-lactamases were observed as separate IEF bands (weak pI ≈9.0 bands) only in *S. marcescens* isolates. Their presence, however, was clear, as indicated by increased cefoxitin MICs for all these isolates (probably due to the AmpC induction effect), and so they must have contributed to the isolates' resistance levels, too. It is possible that at least in the majority of these isolates the AmpC IEF bands were

masked by CTX-M-3, as was the case for the *E. cloacae* and *C. freundii* AmpC-derepressed mutant isolates.

The high degree of quantitative diversity of the MICs observed for the isolates and transconjugants that produced CTX-M-3 as the only oxyiminocephalosporin-hydrolyzing enzyme suggested that the CTX-M-3 expression level and/or the permeability for β-lactam antibiotics could also vary in the isolates. This was especially explicit in the case of four distinguished highly resistant *K. pneumoniae* isolates from centers SZ, WA II, and WA IV. Among the transconjugants, the β-lactam MICs for recombinants of *K. pneumoniae* SZ 3733 and SZ 3734 were particularly high, which indicated the possibility of higher-level CTX-M-3 expression. However, the clearly elevated MICs of cefoxitin and the inhibitor combinations that were shown for the isolates themselves indicated that another mechanism, probably a permeability-based resistance mechanism, could also exist in these isolates. The only two DDS test-negative *K. pneumoniae* strains, strains WA II 8933 and WA IV 9172, were characterized by an even higher level of resistance to β-lactams (including cefoxitin), and β-lactamase inhibitors had almost no effect against the strains. Since the MICs for the transconjugant of isolate WA II 8933 did not reveal any significant increases, it is likely that these two isolates had strongly decreased permeabilities for β-lactam antibiotics. Numerous reports over recent years have documented the decreased permeabilities for β-lactams in strains of the family *Enterobacteriaceae*, which is usually due to mutational changes in genes coding for outer membrane porins. This mechanism has been found to be sufficient to significantly influence the level of bacterial resistance to β-lactams and β-lactam plus inhibitor combinations, especially when it coexists with various β-lactamases, including ESBLs (25, 31, 41).

The data presented in this work document the very complex epidemiology of the CTX-M-3 β-lactamase in Poland, which is one of the major ESBL types in the country. Such a widespread nature of CTX-M producers seems to be unique in Europe, and in the world it may be compared only with the situation in Argentina. The spread of this β-lactamase may mainly be attributed to the very efficient dissemination of plasmids with the $bla_{\text{CTX-M-3}}$ gene in different hospitals (but to some extent it may also be attributed to clonal spread) which followed the noncontrolled transmission of producer strains among them. CTX-M-3-expressing strains constantly evolve in hospitals, acquiring other mechanisms of resistance to β-lactams, which is most likely driven by the high-level use of newer β-lactam antibiotics.

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