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Molecular Survey of β-Lactamases Conferring Resistance to Newer β-Lactams in *Enterobacteriaceae* Isolates from Polish Hospitals▽

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The first national survey of resistance to newer β-lactams in nosocomial populations of *Enterobacteriaceae* in Poland was performed. The study covered all nonrepetitive enterobacterial isolates cultured from specimens from inpatients in 13 regional secondary-care hospitals from November 2003 to January 2004. Among 2,388 isolates, the predominant species was *Escherichia coli* (59.6%), followed by *Proteus mirabilis* (14.5%) and *Klebsiella* spp. (8.5%). The frequency of extended-spectrum β-lactamases (ESBLs) was very high, with ESBLs present in 11.1% of all isolates and 40.4% of *Klebsiella pneumoniae* isolates, the latter value greatly exceeding that for *E. coli* (2.5%). The contribution of outbreak isolates was significant, resulting, for example, in a particularly high rate of ESBL producers among *Serratia marcescens* isolates (70.8%). The pool of ESBL types was overwhelmingly dominated (81.7%) by CTX-M-like β-lactamases CTX-M-3 (80.6%) and CTX-M-15, with SHV types (17.5%; SHV-2, SHV-5, and SHV-12) and sporadic TEM-like enzymes (0.7%; TEM-19 and TEM-48) being the next most frequent. Acquired AmpC-type cephalosporinases were observed exclusively in *P. mirabilis*, in 20.5% of the isolates of this species (compared with the frequency of ESBL producers of 11.5% of *P. mirabilis* isolates). All these cephalosporinases (CMY-12, CMY-15, and a novel variant, CMY-38) originated from *Citrobacter freundii*. Four isolates of *E. coli* (two isolates), *K. pneumoniae* (one isolate), and *P. mirabilis* (one isolate) produced class A inhibitor-resistant β-lactamases (TEM-30, TEM-32, TEM-37, and SHV-49), being the first of such producers identified in Poland. The survey documented both specific and more global characteristics of the epidemiology of the β-lactamase-mediated resistance in enterobacteria from Polish hospitals and demonstrated that the ESBL frequency has reached an alarming level.

The dynamic spread of resistance to newer β-lactam antibiotics in gram-negative bacteria has alerted researchers to the epidemiologic problem of β-lactam resistance in recent years; in large part, this resistance results from the production of several kinds of β-lactamases encoded by mobile genes. Among these, class A extended-spectrum β-lactamases (ESBLs) predominate in the family *Enterobacteriaceae*, hydrolyzing penicillins, cephalosporins (except cephamycins), and monobactams and being inhibited by β-lactam inhibitors (8, 26). The prevalence of acquired class C cephalosporinases (AmpCs) is lower than that of ESBLs (4, 31, 35), but the overall role of AmpCs is strengthened by the mutational overexpression of their natural versions in such pathogens as *Enterobacter* spp. and *Citrobacter freundii* (4, 26). AmpC-type enzymes compromise penicillins, cephalosporins (although very weakly in the case of the zwitterionie compounds), and monobactams and are poorly inhibited by site-directed inhibitors (4, 26). Less problematic are acquired class A inhibitor-resistant β-lactamases, conferring resistance to penicillins and their combinations with β-lactam inhibitors (10, 33).

Owing to many difficulties, large-scale molecular surveys of the enterobacterial resistance to newer β-lactams are rare and they are often limited to selected species (e.g., *Klebsiella pneumoniae* or *Escherichia coli*), isolates from specific hospital wards or specimen types (e.g., isolates from intensive care units [ICUs] or invasive isolates), or ESBL producers only (12, 15, 22, 24, 32). Therefore, it is not easy to compare the data from different studies, although it is clear that there are significant variations, e.g., in ESBL frequencies and types from country to country (22, 24, 32). A comprehensive recent survey of ESBLs in Italy showed the prevalence of the ESBL producers to be 7.4% among all enterobacterial isolates from inpatients and 3.5% among those from outpatients (28). In a new study in France, the prevalence of ESBL producers was estimated to be 1.7%, without a distinction between isolates from patients with nosocomial and community-acquired infections (17). Over time, various surveys have shown the predominance of TEM or SHV ESBLs in different countries (8), but recently, a new trend has become clear, that is, the rapidly growing contribution of CTX-M-type enzymes (7, 27).

Here, we report the results of the first systemic survey of

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FIG. 1. Geographic locations of the study centers.

enterobacteria resistant to newer β-lactams in Poland performed so far. The study aimed at determining the frequencies of clinical isolates with ESBLs, acquired AmpCs, and class A inhibitor-resistant β-lactamases and revealing basic aspects of the molecular epidemiologies of these organisms in Polish hospitals.

MATERIALS AND METHODS

Study design. Thirteen hospitals located all over Poland were involved in the study (Fig. 1). The hospitals were of similar sizes and had similar profiles, being ca. 600-bed, regional, secondary-care medical centers with all major types of wards. All nonrepetitive clinical isolates of the family *Enterobacteriaceae* recovered from inpatients with infections between November 2003 and January 2004 were considered. Selected isolates were collected and sent to the National Medicines Institute (NMI) in Warsaw, together with basic clinical information (the date of isolation, the species, the specimen type, the patient's age and sex, and the ward in which the patient was hospitalized). The exceptions were ampicillin-susceptible *E. coli*, *Proteus mirabilis*, and *Salmonella* spp., for which only numbers of isolates were recorded daily by laboratories and provided for statistical purposes.

Clinical isolates. Of the 1,435 samples received by the NMI, 19 samples were excluded because of the lack of viable cells or heavy contamination; the remaining isolates were subjected to phenotypic analyses for β-lactam resistance (see below). All isolates with potentially interesting or unusual phenotypes, as well as a set of randomly selected isolates (452 isolates altogether), were reidentified with the ATB ID32E test (bioMérieux, Charbonnieres-les-Bains, France). Nine further isolates, classified as non-*Enterobacteriaceae* isolates, were excluded at this stage.

Finally, the survey covered 2,388 enterobacterial isolates in total, including 981 ampicillin-susceptible *E. coli*, *P. mirabilis*, and *Salmonella* spp. isolates and 1,407 isolates qualified for the study at the NMI. The distribution of major taxa (Table 1) showed the high prevalence of *E. coli* (59.6%), followed by *P. mirabilis* (14.5%), *Klebsiella* spp. (8.5%), and *Enterobacter* spp. (6.6%). Most of the isolates sent to the NMI were derived from patients in surgical wards, general medicine wards, and ICUs; the most common specimens were urine and those indicative of lower respiratory tract and surgical-site infections (data not shown).

Phenotypic analysis. All isolates sent to the NMI were analyzed for the presence of ESBLs by the double-disk synergy test (DDST) (23) with disks containing cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), and amoxicillin and clavulanate (20 and 10 μg, respectively). The distance between disks with cephalosporins and disks with clavulanate was 20 mm (center to center). Additionally, each isolate was examined with disks containing cefoxitin (30 μg), imipenem (10 μg), and meropenem (10 μg).

In the second round of testing, all isolates with reduced susceptibilities to cefotaxime and ceftazidime without synergism with clavulanate (regardless of the result with cefepime) and without susceptibility to cefoxitin at the same time were analyzed by the DDST in the presence of 250 μg of cloxacillin (Polfa Tarchomin, Warsaw, Poland)/ml. Such isolates were classified as possible AmpC hyperproducers when cloxacillin augmented their inhibition zones. They were also judged to be ESBL producers when the DDST showed a positive result (often correlating with synergism between cefepime and clavulanate in the first round). On the other hand, each DDST-negative isolate susceptible to cefotaxime, ceftazidime, cefepime, and cefoxitin but nonsusceptible to amoxicillin with clavulanate was tested further with a disk containing cephalothin (30 μg). Cephalothin susceptibility in such isolates was interpreted to be suggestive of the production of a class A inhibitor-resistant β-lactamase (10).

Antibiotic disks used were from BD (Sparks, MD), bioMérieux, or Oxoid (Basingstoke, United Kingdom). Susceptibility results were interpreted according to the guidelines of the CLSI (11). *E. coli* ATCC 25922 and ATCC 35218 and *K. pneumoniae* ATCC 700603 were used as controls.

IEF analysis of β-lactamases and bioassay. All isolates identified as potentially having target resistance phenotypes were subjected to β-lactamase profiling by isoelectric focusing (IEF) as described previously (3) by using a model 111 mini IEF cell (Bio-Rad, Hercules, CA). β-Lactamases were visualized with 0.5 mM nitrocefin (Oxoid) and with 0.5 mM nitrocefin plus 0.3 mM cloxacillin. *K. pneumoniae* isolates possibly having SHV ESBLs with a pI of 7.6 were tested further by a bioassay (3) with 2 μg of cefotaxime (Polfa Tarchomin)/ml.

TABLE 1. Distribution of bacterial species among all isolates and among ESBL producers and prevalence of ESBLs among the species

Species	Total no. of isolates (% of all isolates)	No. of ESBL producers	Prevalence (%) of isolates among all ESBL producers	Prevalence (%) of ESBL producers among isolates of the species	No. of CTX-M producers[a]	No. of SHV producers[a]	No. of TEM producers
S. marcescens	96 (4.0)	68	25.8	70.8	55	13	
K. pneumoniae	141 (5.9)	57	21.6	40.4	35[b]	26[b]	
P. mirabilis	347 (14.5)	40	15.2	11.5	40		
E. coli	1,423 (59.6)	35	13.3	2.5	31	3	1
Enterobacter cloacae	127 (5.3)	24	9.1	18.9	22	2	
K. oxytoca	62 (2.6)	15	5.7	24.2	15		
C. freundii	44 (1.8)	13	4.9	29.5	11	2	
Other *Enterobacter* spp.[c]	31 (1.3)	9	3.4	29.0	8	1	
Others[d]	117 (4.9)	3	1.1	2.6	2		1
All	2,388	264		11.1	219	47	2

[a] For the calculation of the percentages, isolates with both CTX-M- and SHV-type ESBLs were counted twice, among the CTX-M producers and among the SHV producers.
[b] Four *K. pneumoniae* isolates produced two ESBLs (CTX-M and SHV types).
[c] Identified by the ATB ID32E test as *E. aerogenes*, *E. amnigenus*, or *E. asburiae*.
[d] *Citrobacter koseri*, *Morganella morganii*, *Proteus vulgaris*, *Providencia rettgeri*, *Providencia stuartii*, *Raoultella planticola*, *Raoultella terrigena*, *Salmonella* spp., and *Serratia fonticola*.

TABLE 2. Distribution of ESBL producers among the participant centers

ESBL type	No. of ESBL-producing isolates[a] from center no.:												
	1 (n = 122)	2 (n = 282)	3 (n = 245)	4 (n = 119)	5 (n = 110)	6 (n = 178)	7 (n = 125)	8 (n = 158)	9 (n = 107)	10 (n = 155)	11 (n = 283)	12 (n = 236)	13 (n = 268)
CTX-M	6	25	21	5	10	20	6	10	5	5	70	34	2
SHV	2	1	6	2	4			3	2	2	25		
TEM										1	1		
Total no. (%)	8 (6.6)	26 (9.2)	27 (11.0)	5[b] (4.2)	13[b] (11.8)	20 (11.2)	6 (4.8)	12[b] (7.6)	7 (6.5)	8 (5.2)	96 (33.9)	34 (14.4)	2 (0.7)

[a] The total number of isolates from each center is indicated in parentheses.
[b] Two isolates from center 4, one isolate from center 5, and one isolate from center 8 with two ESBLs (CTX-M and SHV types) were identified.

Mating. For all *K. pneumoniae* isolates with SHV-type ESBLs, resistance transfer experiments were performed with *E. coli* A15 isolates resistant to rifampin or nalidixic acid as recipients, as described previously (18). Transconjugants were selected on MacConkey agar (Oxoid) with 2 μg of ceftazidime (GlaxoSmithKline, Stevenage, United Kingdom)/ml or 20 μg of cephalothin (Sigma, St. Louis, MO)/ml and 128 μg of rifampin (Polfa Tarchomin)/ml or 64 μg of nalidixic acid (Sigma)/ml.

PCR detection of bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes. Bacterial DNA was purified with the genomic DNA prep plus kit (A & A Biotechnology, Gdynia, Poland). Genes coding for CTX-M β-lactamases of the CTX-M-1 group (7) (bla_{CTX-M}) were amplified using primers P1C and P2D (19). Primers SHV-A and SHV-B were used for the partial amplification of genes encoding SHV β-lactamases (bla_{SHV}) (18). For *K. pneumoniae* isolates with SHV ESBLs, the PCRs were performed with DNA of the corresponding *E. coli* transconjugants. Genes coding for TEM β-lactamases (bla_{TEM}) were amplified with primers TEM-A and TEM-B (18, 29).

PCR detection of *ampC*-like genes. The *C. freundii ampC*-like genes (bla_{CMY}) were amplified with primers CF-1 and CF-2 (9, 25). Multiplex PCR for other types of mobile *ampC* genes was performed according to the method of Pérez-Pérez and Hanson (34).

Sequencing of β-lactamase genes. Sequences of the PCR products with complete bla_{CTX-M}, bla_{TEM}, and bla_{CMY} genes were determined with consecutive primers as described previously (2, 18, 25, 29). The entire coding regions of bla_{SHV} genes were amplified in two overlapping fragments, with primers SHV-E (5′-ATGCGTTATATTCGC-3′) and SHV-B for the 5′ part and SHV-A and SHV-H (16) for the 3′ part. For SHV-type ESBL genes of *K. pneumoniae* isolates, DNA of *E. coli* transconjugants was used in the PCRs. Subsequently, SHV-E-SHV-B amplicons (783 bp) were sequenced with primers SHV-E, SHV-F, SHV-G, SHV-A, and SHV-B, whereas SHV-A-SHV-H amplicons (375 bp) were sequenced with primers SHV-A, SHV-B, and SHV-H (16, 18). Prior to sequencing, PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and the analysis was carried out using an ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA). β-Lactamase sequences were identified using the database available at http://www.lahey.org/studies/webt.asp.

RAPD typing. Typing by the randomly amplified polymorphic DNA (RAPD) method was carried out separately with two primers, RAPD-7 and RAPD-1283 (36), as reported previously (18).

Nucleotide sequence accession number. The nucleotide sequence of the bla_{CMY-38} gene coding region has been deposited in the GenBank database under the accession number AM931008.

RESULTS

ESBL frequency. Results of the phenotypic analysis, confirmed by the biochemical and molecular data, revealed the presence of ESBLs in 264 isolates in total, which corresponded to 11.1% of all 2,388 *Enterobacteriaceae* isolates (Table 1). The prevalence of ESBL producers among isolates from the particular centers varied from 0.7% for center 13 to 33.9% for center 11 (Table 2). Among the major species, the frequency of ESBL producers varied from 2.5% of *E. coli* isolates to 40.4% of *K. pneumoniae* isolates, but the highest value (70.8%) was observed for *Serratia marcescens* (Table 1).

In the cases with reports of clinical data (n = 261), the ESBL-producing organisms were recovered mainly from urine (48.7%) and specimens from patients with lower respiratory tract and surgical-site infections (15.7% each). Invasive isolates accounted for 5.4% of ESBL producers. The main types of wards in which the affected patients were hospitalized included surgical wards (34.4%), ICUs (21.4%), and general medicine wards (20.6%). More ESBL producers were derived from males than females (58.5 and 41.5%, respectively); most of the patients (53.3% of the 225 for whom reports were available) were between 19 and 64 years old.

ESBL types. The IEF analysis, followed by specific PCRs, allowed for the identification of ESBL families in the study isolates. Later, for each hospital, ESBL genes from isolate representatives of each species, RAPD type, and β-lactamase profile were subjected to sequencing.

ESBLs with six different pIs, 8.9 (ESBLs from 3 isolates), 8.4 (ESBLs from 216 isolates), 8.2 (ESBLs from 44 isolates), 7.6 (ESBLs from 3 isolates), and 6.0 and 5.4 (ESBLs from 1 isolate each), were identified (Table 3). Considering that four *K. pneumoniae* isolates produced both pI 8.4 and 8.2 β-lactamases, 268 ESBLs were found in total. The pI 8.9 and the pI 8.4 enzymes were identified as CTX-M-15 and CTX-M-3, respectively. The pI 8.2 β-lactamases were SHV-5 or, sporadically, SHV-12, whereas the pI 7.6 ESBLs were SHV-2. The pI 6.0 and 5.4 enzymes corresponded to TEM-48 and TEM-19, respectively. The analysis demonstrated a high-level predominance of CTX-M β-lactamases (81.7% of all ESBLs, including CTX-M-3 at 80.6%), followed by SHV- and TEM-type enzymes (17.5 and 0.7%, respectively). CTX-M β-lactamases were prevalent among isolates from all centers, being the only ESBL types in isolates from four of the centers (Tables 2 and 3).

Clonal outbreaks of infections with ESBL producers. All ESBL-producing isolates were typed by the RAPD method. In the first round of typing, all species groups of more than one isolate from each center were analyzed separately. The analysis revealed that outbreak isolates (forming clusters of two or more isolates with identical RAPD patterns) significantly contributed to populations of ESBL producers from several centers. From center 11, two types of *S. marcescens* corresponded to 42 of 46 ESBL producers of that species (30 isolates with CTX-M-3 and 12 with SHV-5), and one type of *K. pneumoniae* comprised 10 of 21 ESBL-producing isolates. From center 2, almost all ESBL-producing *E. coli* isolates (11 of 12 isolates) represented a clonal outbreak group. Overall, among the ma-

TABLE 3. ESBL, AmpC, and inhibitor-resistant β-lactamase types identified and their occurrence in isolates from the participant centers

Enzyme type	pI	No. of enzymes	No. of sequences[a]	Sequence variant	Presence in isolate(s) from center no.[b]												
					1	2	3	4	5	6	7	8	9	10	11	12	13
ESBL	8.9	3	3	CTX-M-15						+					+		+
ESBL	8.4	216	110	CTX-M-3	+	+	+	+	+	+	+	+	+	+	+	+	+
ESBL	8.2	44	20	SHV-5 (n = 18)	+	+		+	+			+	+	+	+		
ESBL	8.2			SHV-12 (n = 2)										+	+		
ESBL	7.6	3	3	SHV-2			+										
ESBL	6.0	1	1	TEM-19											+		
ESBL	5.4	1	1	TEM-48										+			
AmpC	~9.0	71	24	CMY-12 (n = 4)					+		+				+		+
AmpC	~9.0			CMY-15 (n = 19)	+	+	+	+	+		+	+	+	+	+	+	+
AmpC	~9.0			CMY-38 (n = 1)								+					
Inhibitor-resistant β-lactamase	7.6	1	1	SHV-49			+										
Inhibitor-resistant β-lactamase	5.4	1	1	TEM-32			+										
Inhibitor-resistant β-lactamase	5.2	2	2	TEM-30												+	
Inhibitor-resistant β-lactamase	5.2			TEM-37			+										

[a] The number of sequences determined in the study.
[b] + indicates that a particular β-lactamase was identified.

jor species, outbreak isolates constituted around 65% of *S. marcescens*, 43% of *E. coli*, and 40% of *K. pneumoniae* ESBL producers (data not shown).

In the second round of typing, single representatives of each RAPD type were compared in species groups of isolates from all hospitals together. The analysis has not revealed unambiguous cases of the transfer of ESBL producers between different centers (results not shown).

AmpCs. Phenotypes indicative of high-level constitutive AmpC expression were identified in the natural AmpC producers (*Enterobacter* spp., *C. freundii*, *S. marcescens*, *Morganella morganii*, and *E. coli*, etc.) (4, 26) and only in *P. mirabilis* of the remaining species. In the cases of the natural producers, the multiplex PCR assays yielded only the products which corresponded to a given species (if covered by the assay). Therefore, these isolates were classified as probable AmpC-derepressed mutants or hyperproducers (4, 26).

Seventy-one *P. mirabilis* isolates, recovered from all but one of the centers (Table 3), which constituted 20.5% of all *P. mirabilis* isolates, demonstrated the putative AmpC-mediated phenotype. IEF revealed the presence of β-lactamases with a pI of ~9.0 that stained with nitrocefin but not with nitrocefin and cloxacillin (Table 3). All these isolates produced amplicons corresponding to *C. freundii ampC*-like bla_{CMY} genes, 24 of which, representing isolates from each center, were sequenced. The majority of these encoded CMY-15, a smaller set of genes encoded CMY-12, and one encoded a new variant, CMY-38, which is a derivative of CMY-15 with a single amino acid change, D237N (corresponding to the nucleotide change G709A). Eight isolates from three centers expressed both CMY-type AmpCs and the pI 8.4 CTX-M ESBLs (probably CTX-M-3).

Most of the AmpC-producing *P. mirabilis* isolates were recovered from urine (64.3%) and specimens from patients with surgical-site infections (21.4%). The patients affected were mainly those hospitalized in surgical wards (42.9%), followed by those in general medicine wards (22.9%) and ICUs (21.4%). The majority of these patients were males (71.4%) and were between the ages of 19 and 64 years (54.0%).

Class A inhibitor-resistant β-lactamases. Four isolates from two centers (Table 3) demonstrated phenotypes which could be attributed to the production of class A inhibitor-resistant β-lactamases (10). IEF revealed single β-lactamase bands with pIs of 5.2 or 5.4 for two *E. coli* isolates, 5.2 for *P. mirabilis*, and pI 7.6 for *K. pneumoniae* (Table 3). PCR and sequencing identified the pI 5.2 enzymes as TEM-30 (IRT-2) and TEM-37, whereas the pI 5.4 enzyme was identified as TEM-32 (IRT-3). The *K. pneumoniae* isolate produced SHV-49. Three of these isolates were recovered from postoperative wound specimens, and one was recovered from urine.

DISCUSSION

This study is the first national survey of the presence of newer types of β-lactamases in *Enterobacteriaceae* strains from Polish hospitals. The most important result is the demonstration of the scale of the problem of ESBL producers. With values of 11.1% for all enterobacteria and 40.4% for *K. pneumoniae*, the ESBL producer frequencies determined here are some of the highest ever observed in broad analyses (15, 17, 22, 24, 28). Interestingly, the rate of ESBL producers among *E. coli* isolates, 2.5%, was far behind that among *K. pneumoniae* isolates. The results correlate with data from studies with other designs, like those of the European Antimicrobial Resistance Surveillance System. The surveillance system data for 2006, available at http://www.earss.rivm.nl, indicated that around

40% of invasive *K. pneumoniae* isolates in Poland may be resistant to newer cephalosporins. Considering the probably wide use of empirical therapy, as reflected by low numbers of microbiological examinations in Polish hospitals (17 examinations per bed per year [W. Hryniewicz, P. Grzesiowski, and E. Strzyżewska, unpublished data]), it is possible that resistant isolates are overrepresented in clinical laboratories. Nevertheless, the prevalence of ESBL producers in Poland has certainly reached an alarming level.

One of the major factors in the prevalence is the intense clonal spread of ESBL-producing strains. Typing performed in this study revealed that outbreak isolates significantly contributed to ESBL producer subpopulations, including those of *K. pneumoniae* and *E. coli* isolates. An extreme case was illustrated by *S. marcescens* isolates from center 11. This hospital recorded 51.0% of all *S. marcescens* isolates identified in the study, 93.9% of which were ESBL positive. Of the ESBL producers, 91.3% belonged to two disseminated clones with different ESBL types. Therefore, outbreaks occurring in the area of center 11 were mainly responsible for the high prevalence of *S. marcescens* isolates among ESBL-producing organisms in the study. At the same time, the results of typing indicated common shortcomings in infection control systems in the hospitals.

The identification of ESBL types revealed an overwhelming occurrence of CTX-M β-lactamases, owing almost exclusively to CTX-M-3. SHV types still constituted a significant group, but TEM-like enzymes appeared as ephemeras. In 1998, a limited study on ESBL types in six hospitals revealed the predominance of SHVs (60.4%) and comparable frequencies of TEMs and CTX-Ms (20.1 and 18.8%, respectively) (M. Gniadkowski, A. Baraniak, J. Fiett, and W. Hryniewicz, unpublished results). Although the data from that study and the present study are not fully comparable, the rapid increase in the prevalence of CTX-M producers is unquestionable. It corresponds well to a large number of recent observations of such producers in many countries (7, 27); however, this report shows an extreme view of ESBL producer epidemiology in the "CTX-M era." In fact, in Poland, CTX-M producers have been circulating at least from the mid-1990s (19), and already by 2000 their quick spread had been observed (2). The IncL/M transmissible plasmids with the $bla_{\text{CTX-M-3}}$ gene are in large part responsible for the predominance of CTX-M-3 (20). CTX-M-15, prevalent in other countries (27), appears sporadically in Poland owing to a mutation in mobile $bla_{\text{CTX-M-3}}$ genes (1). The survey results sum up the data from the earlier fragmentary studies.

Another important set of data reported here concerns the acquired AmpC-type cephalosporinases. Although in global terms, the producers of such enzymes are less frequent than those of ESBLs, their role in some countries seems to be increasing (12, 30, 31, 37). In most cases, acquired AmpCs have been found in *K. pneumoniae* and *E. coli*, encoded by plasmid-carried genes (4, 35). In contrast, in Poland, acquired AmpCs have been identified so far only in clonally related *P. mirabilis* isolates with chromosomal bla_{CMY} genes originating from *C. freundii* (25). Consistently, all the AmpC-producing *P. mirabilis* isolates investigated here expressed the *C. freundii*-related cephalosporinases, mainly CMY-15 (25). It is noteworthy that the prevalence of these isolates, 20.5% of all *P. mirabilis* isolates in the study, much surpassed the prevalence of ESBL producers among isolates of this species (11.5%). Considering that *P. mirabilis* was the second most prevalent species, it is clear that resistance associated with acquired AmpCs has become a serious problem in Poland. It is also possible that CMY-producing *P. mirabilis* strains will soon be of more general importance, as indicated by recent data from Italy (13, 30).

The screening methodology used in this work also allowed the detection of isolates with the class A inhibitor-resistant β-lactamases (10, 33), namely, four isolates of *E. coli* (two isolates), *P. mirabilis* (one isolate), and *K. pneumoniae* (one isolate) producing TEM-30, TEM-32, TEM-37, and SHV-49 (5, 6, 14, 21). This is the first report of such enzymes in Poland, although it should be noticed that no procedures for their detection have been implemented in microbiology laboratories. Considering the low number of isolates from only two centers, one may assume that these β-lactamases are not widely spread in nosocomial populations of *Enterobacteriaceae* in Poland. It is, however, impossible to assess this problem in the community, where such isolates have been observed more often than in the hospital setting in other countries (10, 33).

This paper reports one of the more detailed molecular surveys of β-lactamases in nosocomial enterobacteria on a countrywide scale. Covering all enterobacterial species and various resistance types, it addressed not only the problem of ESBLs but also that of other enzymes compromising newer β-lactams. The results showed one of the highest rates of ESBL frequency ever reported at the national level and demonstrated how far the spread of CTX-Ms may change the ESBL epidemiology. The study also revealed the problem of AmpC-producing *P. mirabilis*, which may become of broader geographic significance in the future.

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REFERENCES

1. **Baraniak, A., J. Fiett, W. Hryniewicz, P. Nordmann, and M. Gniadkowski.** 2002. Ceftazidime-hydrolyzing CTX-M-15 extended-spectrum β-lactamase (ESBL) in Poland. J. Antimicrob. Chemother. **50:**393–396.
2. **Baraniak, A., J. Fiett, A. Sulikowska, W. Hryniewicz, and M. Gniadkowski.** 2002. Countrywide spread of CTX-M-3 extended-spectrum β-lactamase-producing microorganisms of the family *Enterobacteriaceae* in Poland. Antimicrob. Agents Chemother. **46:**151–159.
3. **Bauernfeind, A., H. Grimm, and S. Schweighart.** 1990. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. Infection **18:**294–298.
4. **Beceiro, A., and G. Bou.** 2004. Class C β-lactamases: an increasing problem worldwide. Rev. Med. Microbiol. **15:**141–152.
5. **Belaaouaj, A., C. Lapoumeroulie, M. M. Canica, G. Vedel, P. Névot, R. Krishnamoorthy, and G. Paul.** 1994. Nucleotide sequences of the genes coding for the TEM-like β-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol. Lett. **120:**75–80.
6. **Blázquez, J., M. R. Baquero, R. Cantón, I. Alos, and F. Baquero.** 1993. Characterization of a new TEM-type β-lactamase resistant to clavulanate, sulbactam, and tazobactam in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **37:**2059–2063.
7. **Bonnet, R.** 2004. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. **48:**1–14.
8. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14:**933–951.
9. **Bret, L., C. Chanal-Claris, D. Sirot, E. B. Chaibi, R. Labia, and J. Sirot.** 1998. Chromosomally encoded AmpC-type β-lactamase in a clinical isolate of *Proteus mirabilis*. Antimicrob. Agents Chemother. **42:**1110–1114.
10. **Chaïbï, E. B., D. Sirot, G. Paul, and R. Labia.** 1999. Inhibitor-resistant TEM β-lactamases: phenotypic, genetic and biochemical characteristics. J. Antimicrob. Chemother. **43:**447–458.
11. **Clinical and Laboratory Standards Institute.** 2007. Performance standards

for antimicrobial susceptibility testing; 17th informational supplement, M100–S17. CLSI, Wayne, PA.
12. **Coudron, P. E., N. D. Hanson, and M. W. Climo.** 2003. Occurrence of extended-spectrum and AmpC beta-lactamases in bloodstream isolates of *Klebsiella pneumoniae*: isolates harbor plasmid-mediated FOX-5 and ACT-1 AmpC beta-lactamases. J. Clin. Microbiol. **41:**772–777.
13. **D'Andrea, M. M., E. Nucleo, F. Luzzaro, T. Giani, R. Migliavacca, F. Vailati, V. Kroumova, L. Pagani, and G. M. Rossolini.** 2006. CMY-16, a novel acquired AmpC-type β-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from northern Italy. Antimicrob. Agents Chemother. **50:**618–624.
14. **Dubois, V., L. Poirel, C. Arpin, L. Coulange, C. Bebear, P. Nordmann, and C. Quentin.** 2004. SHV-49, a novel inhibitor-resistant β-lactamase in a clinical isolate of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **48:** 4466–4469.
15. **Edelstein, M., M. Pimkin, I. Palagin, I. Edelstein, and L. Stratchounski.** 2003. Prevalence and molecular epidemiology of CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob. Agents Chemother. **47:**3724–3732.
16. **Fiett, J., A. Pałucha, B. Miączyńska, M. Stankiewicz, H. Przondo-Mordarska, W. Hryniewicz, and M. Gniadkowski.** 2000. A novel complex mutant β-lactamase, TEM-68, identified in a *Klebsiella pneumoniae* isolate from an outbreak of extended-spectrum β-lactamase-producing klebsiellae. Antimicrob. Agents Chemother. **44:**1499–1505.
17. **Galas, M., J.-W. Decousser, N. Breton, T. Godard, P. Y. Allouch, P. Pina, and the Collège de Bactériologie Virologie Hygiène (ColBVH) Study Group.** 2008. Nationwide study of the prevalence, characteristics, and molecular epidemiology of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in France. Antimicrob. Agents Chemother. **52:**786–789.
18. **Gniadkowski, M., I. Schneider, R. Jungwirth, W. Hryniewicz, 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.
19. **Gniadkowski, M., I. Schneider, A. Pałucha, R. Jungwirth, B. Mikiewicz, and A. Bauernfeind.** 1998. Cefotaxime-resistant *Enterobacteriaceae* isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing β-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrob. Agents Chemother. **42:**827–832.
20. **Gołębiewski, M., I. Kern-Zdanowicz, M. Zienkiewicz, M. Adamczyk, J. Żylinska, A. Baraniak, M. Gniadkowski, J. Bardowski, and P. Cegłowski.** 2007. Complete nucleotide sequence of the pCTX-M3 plasmid and its involvement in spread of the extended-spectrum β-lactamase (ESBL) gene $bla_{CTX\text{-}M\text{-}3}$. Antimicrob. Agents Chemother. **51:**3789–3795.
21. **Henquell, C., C. Chanal, D. Sirot, R. Labia, and J. Sirot.** 1995. Molecular characterization of nine different types of mutants among 107 inhibitor-resistant TEM β-lactamases from clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **39:**427–430.
22. **Hernández, J. R., L. Martínez-Martínez, R. Cantón, T. M. Coque, A. Pascual, and the Spanish Group for Nosocomial Infections (GEIH).** 2005. Nationwide study of *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. Antimicrob. Agents Chemother. **49:**212–2125.
23. **Jarlier, V., M. Nicolas, G. Fournier, and A. Philippon.** 1988. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. **10:**867–878.
24. **Jeong, S. H., I. K. Bae, J. H. Lee, S. G. Sohn, G. H. Kang, G. J. Jeon, Y. H. Kim, B. C. Jeong, and S. H. Lee.** 2004. Molecular characterization of extended-spectrum beta-lactamases produced by clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a Korean nationwide survey. J. Clin. Microbiol. **42:**2902–2906.
25. **Literacka, E., J. Empel, A. Baraniak, E. Sadowy, W. Hryniewicz, and M. Gniadkowski.** 2004. Four variants of the *Citrobacter freundii* AmpC-type cephalosporinases, including two novel enzymes, CMY-14 and CMY-15, in a *Proteus mirabilis* clone widespread in Poland. Antimicrob. Agents Chemother. **48:**4136–4143.
26. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
27. **Livermore, D. M., R. Cantón, M. Gniadkowski, P. Nordmann, G. M. Rossolini, G. Arlet, J. Ayala, T. M. Coque, I. Kern-Zdanowicz, F. Luzzaro, L. Poirel, and N. Woodford.** 2007. CTX-M: changing the face of ESBLs in Europe. J. Antimicrob. Chemother. **59:**165–174.
28. **Luzzaro, F., M. Mezzatesta, C. Mugnaioli, M. Perilli, S. Stefani, G. Amicosante, G. M. Rossolini, and A. Toniolo.** 2006. Trends in production of extended-spectrum β-lactamases among enterobacteria of medical interest: report of the second Italian nationwide survey. J. Clin. Microbiol. **44:**1659–1664.
29. **Mabilat, C., S. Goussard, W. Sougakoff, R. C. Spencer, and P. Courvalin.** 1990. Direct sequencing of the amplified structural gene and promoter for the extended-broad-spectrum β-lactamase TEM-9 (RHH-1) of *Klebsiella pneumoniae*. Plasmid **23:**27–34.
30. **Migliavacca, R., E. Nucleo, M. M. D'Andrea, M. Spalla, T. Giani, and L. Pagani.** 2007. Acquired AmpC type β-lactamases: an emerging problem in Italian long-term care and rehabilitation facilities. New Microbiol. **30:**295–298.
31. **Molland, E. S., N. D. Hanson, J. A. Black, A. Hossain, W. Song, and K. S. Thomson.** 2006. Prevalence of newer β-lactamases in gram-negative clinical isolates collected in the United States from 2001 to 2002. J. Clin. Microbiol. **44:**3318–3324.
32. **Mulvey, M. R., E. Bryce, D. Boyd, M. Ofner-Agostini, S. Christianson, A. E. Simor, S. Paton, and The Canadian Hospital Epidemiology Committee of The Canadian Nosocomial Infection Surveillance Program, Health Canada.** 2004. Ambler class A extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella* spp. in Canadian hospitals. Antimicrob. Agents Chemother. **48:**1204–1214.
33. **Nicolas-Chanoine, M. H.** 1997. Inhibitor-resistant β-lactamases. J. Antimicrob. Chemother. **40:**1–3.
34. **Pérez-Pérez, F. J., and N. D. Hanson.** 2002. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. **40:**2153–2162.
35. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46:**1–11.
36. **Renders, N., A. van Belkum, A. Barth, W. Goessens, J. Mouton, and H. Verbrugh.** 1996. Typing of *Pseudomonas aeruginosa* strains from patients with cystic fibrosis: phenotyping versus genotyping. Clin. Microbiol. Infect. **1:**261–265.
37. **Yan, J. J., P. R. Hsueh, J. J. Lu, F. Y. Chang, J. M. Shyr, J. H. Wan, Y. C. Liu, Y. C. Chuang, Y. C. Yang, S. M. Tsao, H. H. Wu, L. S. Wang, T. P. Lin, H. M. Wu, H. M. Chen, and J. J. Wu.** 2006. Extended-spectrum β-lactamases and plasmid-mediated AmpC enzymes among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from seven medical centers in Taiwan. Antimicrob. Agents Chemother. **50:**1861–1864.