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Effects of Phenotype and Genotype on Methods for Detection of Extended-Spectrum-β-Lactamase-Producing Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Norway▽

Ståle Tofteland,[1,2]* Bjørg Haldorsen,[1] Kristin H. Dahl,[1] Gunnar S. Simonsen,[1,5] Martin Steinbakk,[3] Timothy R. Walsh,[4] Arnfinn Sundsfjord,[1,5]* and the Norwegian ESBL Study Group

Reference Centre for Detection of Antimicrobial Resistance (K-res), Department of Microbiology and Infection Control, University Hospital of North Norway, and Department of Microbiology and Virology, Institute of Medical Biology, Faculty of Medicine, University of Tromsø, Tromsø, Norway[1]; Department of Microbiology, Sørlandet Hospital, Kristiansand, Norway[2]; Department of Microbiology, Akershus University Hospital, Lørenskog, Norway[3]; Department of Medical Microbiology, Cardiff University, Cardiff, United Kingdom[4]; and Norwegian Institute of Public Health, Oslo, Norway[5]

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Consecutive clinical isolates of *Escherichia coli* ($n = 87$) and *Klebsiella pneumoniae* ($n = 25$) with reduced susceptibilities to oxyimino-cephalosporins (MICs > 1 mg/liter) from 18 Norwegian laboratories during March through October 2003 were examined for $bla_{TEM/SHV/CTX-M}$ extended-spectrum-β-lactamase (ESBL) genes, oxyimino-cephalosporin MIC profiles, ESBL phenotypes (determined by the ESBL Etest and the combined disk and double-disk synergy [DDS] methods), and susceptibility to non-β-lactam antibiotics. Multidrug-resistant CTX-M-15-like ($n = 23$) and CTX-M-9-like ($n = 15$) ESBLs dominated among the 50 ESBL-positive *E. coli* isolates. SHV-5-like ($n = 9$) and SHV-2-like ($n = 4$) ESBLs were the most prevalent in 19 ESBL-positive *K. pneumoniae* isolates. Discrepant ESBL phenotype test results were observed for one major (CTX-M-9) and several minor (TEM-128 and SHV-2/-28) ESBL groups and in SHV-1/-11-hyperproducing isolates. Negative or borderline ESBL results were observed when low-MIC oxyimino-cephalosporin substrates were used to detect clavulanic acid (CLA) synergy. CLA synergy was detected by the ESBL Etest and the DDS method but not by the combined disk method in SHV-1/-11-hyperproducing strains. The DDS method revealed unexplained CLA synergy in combination with aztreonam and cefpirome in three *E. coli* strains. The relatively high proportion of ESBL-producing *E. coli* organisms with a low ceftazidime MIC in Norway emphasizes that cefpodoxime alone or both cefotaxime and ceftazidime should be used as substrates for ESBL detection.

Systemic infections with extended-spectrum-β-lactamase (ESBL)-producing *Enterobacteriaceae* are associated with severe adverse clinical outcomes (7, 12, 25). It is thus essential for a diagnostic microbiology laboratory to have updated methods for the detection of ESBL-producing strains, taking into account the local epidemiology of ESBL genotypes and their various expression profiles. As very little is known about ESBL genotypes in Norway, we designed a study for the detection and characterization of ESBL production in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* with reduced susceptibilities to oxyimino-cephalosporins from routine diagnostic samples. More specifically, we examined (i) the abilities of different phenotypic methods to detect ESBL-producing strains in relation to MICs of oxyimino-cephalosporins, (ii) the molecular basis for ESBL production by typing of the most prevalent β-lactamase genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) and the relationships between MIC profiles for oxyimino-cephalosporins and different *bla* groups, and (iii) the occurrence of multiple-antibiotic resistance.

(The results of this study were presented in part at the European Congress of Clinical Microbiology and Infectious Diseases, Prague, Czech Republic, 2004.)

MATERIALS AND METHODS

Study design. Consecutive nonduplicate isolates of *E. coli* and *K. pneumoniae* with reduced susceptibilities to oxyimino-cephalosporins (MIC > 1 mg/liter) were collected in 18 of 24 Norwegian diagnostic microbiology laboratories covering >90% of the Norwegian population from March through October 2003. Initial antimicrobial susceptibility testing was performed in each laboratory using agar disk diffusion systems from AB Biodisk (Solna, Sweden) or Rosco tablets (Taastrup, Denmark) on paper disk method agar (AB Biodisk) and/or the automated systems Vitek2 (bioMérieux, Marcy l'Etoile, France) and the MAST multipoint system (Mast Diagnostics, Merseyside, United Kingdom), in agreement with breakpoints from the Norwegian Working Group on Antibiotics (NWGA) (16). All laboratories used either cefotaxime (CTX), ceftazidime (CAZ), or cefpodoxime (CPD) alone or two of these substrates in various combinations to screen for reduced susceptibility to oxyimino-cephalosporins. Isolates expressing reduced susceptibilities to oxyimino-cephalosporins were submitted to the Reference Centre for Detection of Antimicrobial Resistance, Tromsø, Norway, with a registration form containing information on sex, age, inpatient and outpatient status, hospital department, and specimen type. Final bacterial identification was performed at the Reference Centre using the Vitek2 ID-GNB system (bioMérieux) or API ID32E (bioMérieux) and/or 16S rRNA gene sequence typing in cases of low discrimination. Strains confirmed as *E. coli* or *K. pneumoniae* were included in the study.

Antimicrobial susceptibility testing. *E. coli* ($n = 89$) and *Klebsiella pneumoniae* ($n = 27$) isolates with reduced susceptibilities to oxyimino-cephalosporins in the

* Corresponding author. Mailing address for Ståle Tofteland: Department of Microbiology, Sørlandet Hospital, Serviceboks 416, 4604 Kristiansand, Norway. Phone: 47 38073309. Fax: 47 38074173. E-mail: staale.tofteland@sshf.no. Mailing address for Arnfinn Sundsfjord: Department of Microbiology and Virology, Institute of Medical Biology, Faculty of Medicine, University of Tromsø, 9038 Tromsø, Norway. Phone: 47 906161118. Fax: 47 77645350. E-mail: arnfinn.sundsfjord@fagmed.uit.no.

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TABLE 1. PCR primers used in this study

Amplicon	Primer sequence (5′ to 3′)	Size of amplicon (bp)	Annealing temp (°C)	Reference or source
16S rRNA gene	AGA GTT TGA TCM TGG CTC AG ACG GHT ACC TTG TTA CGA CTT	~1,500	55	27a
bla_{TEM}	ATG AGT ATT CAA CAT TTC CG CCA ATG CTT AAT CAG TGA GG	858	50	This study
bla_{SHV}	ATG CGT TAT ATT CGC CTG TG AGC GTT GCC AGT GCT CGA TC	862	58	This study
bla_{CTX-M}	SCS ATG TGC AGY ACC AGT AA ACC AGA AYV AGC GGB GC	585	58	This study

initial testing were examined at the Reference Centre using the following panel of Etest β-lactams (AB Biodisk) according to the manufacturer's instructions: ampicillin, amoxicillin-clavulanic acid (CLA), piperacillin, piperacillin-tazobactam, cefoxitin, CPD, CTX, CAZ, cefepime (FEP), aztreonam, imipenem, and meropenem. Vitek2 ASTN023 was used to determine susceptibility to non-β-lactam antibiotics. Interpretations were in accordance with NWGA guidelines. Breakpoints for cefpodoxime have not been established by the NWGA, and a MIC of >1 mg/liter was thus defined as indicative of reduced susceptibility.

Phenotypic detection of ESBL production. Phenotypic tests were performed on the same day from the same subculture with ampicillin selection (100 mg/liter). ESBL production on isolates expressing a reduced susceptibility (MIC > 1 mg/liter) to an oxyimino-cephalosporin (cefpodoxime and/or cefotaxime and/or ceftazidime) was examined using (i) CTX-CLA, CAZ-CLA, and FEP-CLA ESBL Etests and (ii) disks containing cefpodoxime, ceftazidime, or cefotaxime with and without CLA (called the combined disk method) (Oxoid, Basingstoke, United Kingdom). An ESBL phenotype was defined by reduced susceptibility (MIC > 1 mg/liter) to an oxyimino-cephalosporin (cefpodoxime and/or cefotaxime and/or ceftazidime) and a significant increase in susceptibility to oxyimino-cephalosporins tested in combination with CLA by the Etest and/or the combined disk method. For Etest analyses, ESBL production was defined as a ≥8-fold decrease in the MIC of cefotaxime, ceftazidime, or cefepime in the presence of CLA or the presence of so-called phantom or deformity zones. In the combined disk method, ESBL production was defined as an increase of ≥5 mm in the zone around CLA disks compared to the zones of corresponding disks without CLA. In comparison, a modified version of the Jarlier double-disk synergy (DDS) method (10) for detecting CLA synergy was used. Aztreonam (30 μg), cefpodoxime (10 μg), ceftazidime (30 μg), cefotaxime (5 μg), and cefpirome (30 μg) disks (Oxoid) were placed around an amoxicillin (20 μg)-clavulanic acid (10 μg) disk at a distance of 25 to 30 mm center to center. A clearly visible extension of the edge of the inhibition zone of any disk towards the amoxicillin-clavulanic acid disk was interpreted as positive for CLA synergy.

DNA analyses. Bacterial DNA extraction was performed in a QIAGEN model M48 BioRobot (QIAGEN, Hilden, Germany) using a MagAttract DNA mini M48 kit (QIAGEN). 16S rRNA gene PCR-positive DNA extracts were screened for the presence of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} by consensus PCRs in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) using Applied Biosystems standard PCR mixtures with GeneAmp PCR buffer and *Taq* DNA polymerase. PCR information is given in Table 1. Bidirectional sequencing was performed using a BigDye v. 3.1 cycle sequencing kit and a model 3100 genetic analyzer (Applied Biosystems). Editing and alignment of DNA sequences were performed using the SeqMan II software package (DNAStar, Inc., Madison, WI).

Isoelectric focusing of β-lactamases. Analytic isoelectric focusing (IEF) of sonicated crude cell extracts was performed in precast Ampholine PAGplate polyacrylamide gels with a pH range of 3.5 to 9.5 (GE Healthcare, St. Giles, United Kingdom) using a Multiphor II apparatus (GE Healthcare). β-Lactamase activity was detected with nitrocefin (0.5 g/liter). The β-lactamases TEM-1 (isoelectric point [pI] 5.4) and SHV-1 (pI 7.6) and IEF protein standards of pI 4.45 to 9.6 (Bio-Rad Laboratories) were used for pI comparisons.

Quality control strains. *E. coli* J62 (bla_{TEM-3}), *E. coli* ($bla_{CTX-M-3}$), *Kluyvera georgiana* (bla_{KLUG-1}), *K. pneumoniae* ILT-2 ($bla_{CTX-M-14}$), and *K. pneumoniae* ILT-3 ($bla_{CTX-M-19}$), kindly provided by David Livermore and Laurent Poirel, as well as *K. pneumoniae* ATCC 700603 (bla_{SHV-18}) and *E. coli* ATCC 25922, were used.

RESULTS

ESBL detection and *bla* genotyping in *E. coli*. An ESBL phenotype was recognized in 52/87 (60%) isolates expressing reduced susceptibility to an expanded-spectrum cephalosporin by Etest. Fifty isolates were ESBL positive by the combined disk method. Fifty-two isolates were positive by the ESBL Etests and included those positive by the combined disk method.

Fifty of the 52 (96%) ESBL phenotype-positive *E. coli* isolates carried the bla_{CTX-M}, bla_{SHV}, or bla_{TEM} ESBL gene. The PCR results are summarized in Table 2. bla_{CTX-M} was detected in 45 isolates (90%). CTX-M sequence grouping and typing performed according to the method of Bonnet (3) revealed the CTX-M-1 group (n = 29), the CTX-M-9 group (n = 15), and the CTX-M-2 group (n = 1). Within the CTX-M-1 group, $bla_{CTX-M-15/28}$ (n = 23) was the most prevalent genotype. Sequence typing within the CTX-M-9 group (n = 15) revealed $bla_{CTX-M-9/9a}$ (n = 4), $bla_{CTX-M-16}$ (n = 1), and various indistinguishable $bla_{CTX-M-9}$ genogroup ($bla_{TOHO2/3/CTX-M-14/17/18/21/24}$) types ($n$ = 10). bla_{SHV} and bla_{TEM} were detected in 4 and 34 isolates, respectively. bla_{SHV} typing revealed bla_{SHV-1} (n = 2) and $bla_{SHV-ESBL}$ (n = 2). The 34 bla_{TEM} genes were de-

TABLE 2. Distribution of ESBL genes in 50 *E. coli* and 19 *K. pneumoniae* isolates

Organism	No. of isolates with *bla* type:									
	CTX-M-1	CTX-M-3	CTX-M-15/28	CTX-M-2	CTX-M-9-like[a]	SHV-5/12	SHV-28	SHV-2/2a	TEM-52	TEM-128
E. coli	4	2	23	1	15	2			1	2
K. pneumoniae			2		1	9	2	4	1	

[a] Includes one CTX-M-16 isolate.

TABLE 3. Evaluation of ESBL Etest performance in relation to ESBL genotypes in 50 *E. coli* and 19 *K. pneumoniae* strains

ESBL Etest result with[a]:			No. of strains with indicated genotype					
			E. coli			*K. pneumoniae*		
CTX	CAZ	FEP	$bla_{\text{CTX-M-15}}$ ($n = 23$)	$bla_{\text{CTX-M-9}}$ ($n = 15$)	Other ($n = 12$)	bla_{SHV} ($n = 15$)	$bla_{\text{CTX-M}}$ ($n = 3$)	Other ($n = 1$)
+	+	+	23	14[f]	11	12	3	1[g]
+	−	+	0	1	1[b]	1[c]	0	0
−	+	+	0	0	0	1[d]	0	0
−	+	−	0	0	0	1[e]	0	0

[a] +, positive ESBL Etest; −, negative ESBL Etest.
[b] $bla_{\text{CTX-M-1}}$ (CAZ/CAZ-CLA ratio, 5:3) (K4-55).
[c] $bla_{\text{SHV-2}}$ (K5-30).
[d] $bla_{\text{SHV-28}}$ (K2-79).
[e] $bla_{\text{SHV-28}}$ (K4-61).
[f] Includes $bla_{\text{CTX-M-16}}$ (K4-70).
[g] $bla_{\text{TEM-52}}$ (K2-31).

tected in 31 $bla_{\text{CTX-M}}$-positive and three bla_{SHV}- and $bla_{\text{CTX-M}}$-negative strains. Sequence typing of the latter showed $bla_{\text{TEM-52}}$ ($n = 1$) and $bla_{\text{TEM-128}}$ ($n = 2$). $bla_{\text{TEM-1}}$ was detected in six randomly selected $bla_{\text{CTX-M}}$-positive isolates. Two *E. coli* isolates were negative for CLA synergy in the combined disk method but positive by CAZ-CLA and FEP-CLA but not CTX-CLA ESBL Etests. Both isolates contained $bla_{\text{SHV-1}}$ and had phenotypic profiles consistent with hyperproduction of SHV-1: moderate increases in ceftazidime MICs (2 to 4 mg/liter), wild-type cefotaxime MICs (0.125 to 0.25 mg/liter), and piperacillin-tazobactam MICs of >256 mg/liter. IEF analysis revealed single β-lactamase pI bands of approximately 7.5, consistent with an SHV-like enzyme in both strains (data not shown). In summary, the overall prevalence of ESBLs in *E. coli* isolates was 50 of 87 isolates (58%).

The accuracy of the DDS method was evaluated in comparison to the results obtained by ESBL Etests, the combined disk method, and *bla* typing. The DDS method revealed CLA synergy with at least one substrate with 55/87 isolates, including all strains that scored positive in the ESBL Etest analysis. The two SHV-1-hyperproducing strains also expressed CLA synergy by the DDS method. Interestingly, three isolates with negative results by both the ESBL Etest and the combined disk method displayed reproducible CLA synergy to aztreonam and cefpirome in the DDS method. The isolates shared common features, including CLA synergy to aztreonam and cefpirome and negative $bla_{\text{CTX-M/SHV/TEM}}$ PCRs. IEF analyses revealed a single β-lactamase band for which the pI was 9.0, consistent with their AmpC profile of moderately elevated cefoxitin MICs (32 to 48 mg/liter) and increased MICs of oxyimino-cephalosporins and aztreonam (cefpodoxime, 24 to 48 mg/liter; cefotaxime, 2 to 4 mg/liter; ceftazidime, 2 to 6 mg/liter; and aztreonam, 2 to 4 mg/liter). In summary, we have no explanation for the CLA synergy observed in 3 out of 55 DDS-positive isolates. These strains were not defined as ESBL positive.

ESBL detection and *bla* genotyping in *K. pneumoniae*. An ESBL phenotype was recognized in 21/25 (84%) *K. pneumoniae* isolates expressing reduced susceptibility to an expanded-spectrum cephalosporin by the Etest. Eighteen isolates were ESBL positive by the combined disk method. Twenty-one isolates were positive by the ESBL Etests and included those positive by the combined disk method.

A total of 19 ESBL genes, namely, $bla_{\text{CTX-M}}$ ($n = 3$), bla_{SHV} ($n = 15$), and bla_{TEM} ($n = 1$), were detected in 19 isolates (Table 2). $bla_{\text{SHV-5}}$, $bla_{\text{SHV-5a}}$, and $bla_{\text{SHV-12}}$ were the most prevalent ESBL genotypes. $bla_{\text{CTX-M}}$ amplicons were typed as being in the $bla_{\text{CTX-M-15/28}}$ ($n = 2$) and $bla_{\text{CTX-M-9}}$ ($n = 1$) genogroups. The three $bla_{\text{CTX-M}}$ strains were also shown to contain $bla_{\text{SHV-11}}$ or $bla_{\text{SHV-14}}$. Both $bla_{\text{SHV-11}}$ and $bla_{\text{SHV-14}}$ have previously been reported to express a non-ESBL phenotype in *K. pneumoniae* (15, 30). The $bla_{\text{TEM-52}}$ ($n = 1$) and $bla_{\text{TEM-1}}$ ($n = 8$) genes were detected in nine strains. Discordant ESBL phenotype results were observed with three *K. pneumoniae* strains. Interestingly, one $bla_{\text{SHV-28}}$ isolate expressed significant CLA synergy in FEP-CLA and CAZ-CLA ESBL Etests but was negative in the combined disk method. The difference in inhibition zones of ceftazidime disks with and without CLA was 3 mm (24 versus 21 mm).

A similar phenotypic profile was observed for one $bla_{\text{SHV-1}}$ isolate and one $bla_{\text{SHV-11}}$ isolate expressing phenotypes analogous to those of the two SHV-1-hyperproducing *E. coli* strains described above. They were consequently regarded as SHV-1 and SHV-11 hyperproducers. In summary, the overall prevalence of ESBL-positive strains in the *K. pneumoniae* collection was 19/25 (76%). The lack of chromosomally encoded AmpC β-lactamases in the genus *Klebsiella* may explain the relatively higher occurrence of ESBL production in *K. pneumoniae* than in *E. coli* isolates (50/87; 58%) with reduced susceptibilities to oxyimino-cephalosporins (18, 26).

All 25 *K. pneumoniae* isolates with reduced susceptibilities to oxyimino-cephalosporins were examined by the DDS test. CLA synergy was observed in 21 strains that were identical to the ESBL Etest-positive strains. The putative SHV-1- and SHV-11-hyperproducing strains also expressed CLA synergy in the DDS test.

Performance of ESBL tests. The performance of ESBL Etests and the combined disk method in the detection of ESBL production in *E. coli* and *K. pneumoniae* is summarized in Tables 3 and 4. Significant CLA synergy was easily detected by all substrates in both methods for the most prevalent ESBL genogroups: the $bla_{\text{CTX-M-15/28}}$ genotypes and $bla_{\text{SHV-5/12}}$ genotypes in *E. coli* and *K. pneumoniae*, respectively. However, discordant ESBL test results were observed in one major and several minor ESBL genotypes.

TABLE 4. Evaluation of the performance of the combined disk method in relation to ESBL genotype in 50 *E. coli* and 19 *K. pneumoniae* strains

Combined disk method results with[a]:			No. of strains with indicated genotype					
			E. coli			*K. pneumoniae*		
CTX	CAZ	CPD	$bla_{\text{CTX-M-15}}$ (n = 23)	$bla_{\text{CTX-M-9}}$ (n = 15)	Other (n = 12)	bla_{SHV} (n = 15)	$bla_{\text{CTX-M}}$ (n = 3)	Other (n = 1)
+	+	+	22	1[e]	6	12	3	1[f]
+	−	+	1	14	6[b]	2[c]	0	0
−	−	−	0	0	0	1[d]	0	0

[a] +, positive combined disk test; −, negative combined disk test.
[b] $bla_{\text{TEM-128}}$ (n = 2; K4-71 and K5-25), $bla_{\text{CTX-3/32/22}}$ (K5-58), $bla_{\text{CTX-3/22}}$ (K8-8) (n = 2), and $bla_{\text{CTX-M-1}}$ (n = 2).
[c] $bla_{\text{SHV-2}}$ (K5-30) and $bla_{\text{SHV-2a}}$ (K2-36).
[d] $bla_{\text{SHV-28}}$ (K2-79). The difference between the ceftazidime disk diameter with clavulanate and the one without was 3 mm (24 versus 21 mm).
[e] $bla_{\text{CTX-M-16}}$ (K4-70).
[f] $bla_{\text{TEM-52}}$ (K2-31).

$bla_{\text{CTX-M-9}}$ genogroup *E. coli* strains, except for a single CTX-M-16 isolate, scored negative in the ceftazidime combined disk method (14/15; 93%), in contrast to the results of the CAZ-CLA ESBL Etest, by which most of these strains (14/15; 93%) scored positive. However, a CAZ/CAZ-CLA MIC ratio of ≥8 was observed only for the CTX-M-16 strain, whereas the other 13 CTX-M-9-positive strains scored positive by the CAZ-CLA ESBL Etest by the appearance of deformity in the ellipse only (the "eagle effect"). The single $bla_{\text{CTX-M-9}}$ genogroup *K. pneumoniae* strain scored positive for ESBL production by both methods using all three oxyimino-cephalosporin substrates.

The minor ESBL types with aberrant ESBL test results included six *E. coli* strains within the $bla_{\text{CTX-M-1}}$ (n = 4; $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-3/22}}$) and $bla_{\text{TEM-128}}$ (n = 2) genogroups that were negative by the ceftazidime combined disk test (Table 4). In contrast, all these strains were positive by the CAZ-CLA ESBL Etest (Table 4). The $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-128}}$ strains were positive by deformity in the ellipse only, whereas the two $bla_{\text{CTX-M-3/22}}$ strains had the marginally positive CAZ/CAZ-CLA MIC ratios 12 (K5-58) and 11 (K8-8), respectively. Moreover, one $bla_{\text{SHV-2}}$ strain and two $bla_{\text{SHV-28}}$ *K. pneumoniae* strains scored negative in one or two of the ESBL Etests (Table 3). Corresponding results were obtained by the combined disk method, except with one ESBL $bla_{\text{SHV-28}}$ *K. pneumoniae* strain (K2-79) that scored negative for all substrates (Table 4).

MIC profiles for ESBL-producing strains. The MIC means and ranges for oxyimino-cephalosporins and aztreonam within the different ESBL genogroups are presented in Table 5. CTX-M and SHV ESBL-producing strains generally expressed cefotaximase and ceftazidimase profiles, respectively. The MICs of all substrates for the strains with the most-prevalent ESBL genotypes, $bla_{\text{CTX-M-15/28}}$ in *E. coli* and $bla_{\text{SHV-5/12}}$ in *K. pneumoniae*, were high to moderate.

$bla_{\text{CTX-M-9}}$ genogroup *E. coli* strains expressed low MICs of

TABLE 5. MIC means and ranges for oxyimino-cephalosporins and aztreonam in relation to ESBL genogroups of *E. coli* and *K. pneumoniae*

Species	Genotype (no. of isolates)	MIC (mg/liter)[a]							
		Cefpodoxime		Cefotaxime		Ceftazidime		Aztreonam	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
E. coli	$bla_{\text{CTX-M-9}}$ genogroup[b] (14)	55	24–96	13	8–24	0.55	0.094–1	2	0.75–4
	$bla_{\text{TEM-128}}$ (2)	48	32–64	9	6–12	0.63	0.5–0.75	1	1–1
	$bla_{\text{CTX-M-3}}$ (2)	>256	>256	80	64–96	3	3–3	12	12–12
	$bla_{\text{CTX-M-2}}$ genogroup (1)	>256		64		3		12	
	$bla_{\text{CTX-M-1}}$ (4)	184	96–>256	34	16–64	3.1	1–8	12	6–24
	$bla_{\text{CTX-M-16}}$ (1)	>256		96		6		8	
	$bla_{\text{TEM-52}}$ (1)	48		12		12		2	1
	$bla_{\text{SHV-5/12}}$ (2)	136	16–256	25	1.5–48	136	16–256	140	24–256
	$bla_{\text{CTX-M-15}}$ (23)	253	192–>256	177	24–>256	27	6–128	63	6–>256
K. pneumoniae	$bla_{\text{SHV-28}}$ (2)	6.4	0.75–12	0.59	0.19–1	7	6–8	2	0.75–4
	$bla_{\text{SHV-2/2a}}$ (4)	6	2–8	1.9	1–2	1.2	0.75–1.5	0.2	0.19–0.25
	$bla_{\text{SHV-5/12}}$ (8)[c]	61	12–96	9	1.5–32	98	24–192	93	24–256
	$bla_{\text{SHV-5}}$ (K4-49) (1)	256		48		256		256	
	$bla_{\text{TEM-52}}$ (1)	64		24		48		6	
	$bla_{\text{CTX-M-9}}$ (1)	48		32		0.75		1.5	
	$bla_{\text{CTX-M-15}}$ (2)	>256	>256	192	128–192	16	12–16	32	24–32

[a] The value 256 μg/ml has been used to calculate the mean for strains displaying a MIC of >256 mg/liter. For simplicity, mean values are also given for genotypes represented by only one strain.
[b] The $bla_{\text{CTX-M-9}}$ genogroup but without the single $bla_{\text{CTX-M-16}}$ strain.
[c] One $bla_{\text{SHV-5}}$ isolate (K4-49) displayed an aberrant phenotype. For this isolate, the CPD, CAZ, and aztreonam MICs were 256 mg/liter; the CTX MIC was 64 mg/liter; and the cefoxitin MIC was 12 mg/liter.

ceftazidime (mean, 0.55 mg/liter) and aztreonam (mean, 1.8 mg/liter), as described previously (4, 24), except for the CTX-M-16 isolate. Interestingly, some of the minor *bla* genotypes showed clinically significant differences in MIC levels for different substrates. *E. coli* $bla_{\text{TEM-128}}$ strains ($n = 2$) had higher mean MICs for cefpodoxime (48 mg/liter) and cefotaxime (9 mg/liter) than for ceftazidime (0.63 mg/liter) and aztreonam (1 mg/liter). Moreover, seven $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-2}}$ genogroup *E. coli* strains were intermediately susceptible to ceftazidime (MICs, 1 to 8 mg/liter). Interestingly, the two $bla_{\text{SHV-28}}$-positive *K. pneumoniae* strains expressed comparatively low MICs for cefotaxime (0.19 and 1 mg/liter) and cefpodoxime (0.75 and 12 mg/liter). The K2-79 strain, with a cefpodoxime MIC of 0.75 mg/liter, scored negative in the combined disk method.

Coresistance. The majority of ESBL-positive *E. coli* strains (36/50; 70%) expressed resistance to two or more non-β-lactam antibiotics (aminoglycosides [AG], fluoroquinolones [FQ], nitrofurantoin [NIT], and/or trimethoprim-sulfamethoxazole [SXT]) and were defined as multidrug resistant (MDR). Sixteen out of 23 (70%) $bla_{\text{CTX-M-15/28}}$ *E. coli* strains were resistant to three or more non-β-lactam antibiotics, in contrast to 3/15 (20%) of the $bla_{\text{CTX-M-9}}$ strains. Twenty-eight (62%), 30 (67%), 18 (40%), and 33 (73%) out of 45 CTX-M-positive strains were resistant to AG, FQ, NIT, and SXT, respectively. Only one of the bla_{TEM} and bla_{SHV} ESBL-positive *E. coli* strains ($n = 5$) was resistant to more than two non-β-lactam antibiotics, and those strains were all susceptible to FQ. Twelve (63%) of the ESBL-producing *K. pneumoniae* strains expressed MDR. Nine (45%), 3 (15%), 18 (90%), and 4 (20%) *K. pneumoniae* strains were resistant to AG, FQ, NIT, and SXT, respectively.

Epidemiological data. ESBL-producing strains were detected in 16 laboratories. There were no indications of nosocomial outbreaks during the study period. Urinary tract isolates ($n = 42$), specifically, 34 (68%) *E. coli* isolates and 8 (42%) *K. pneumoniae* isolates, were dominant. ESBL-positive blood culture isolates were not detected. Twenty-two (44%) of the *E. coli* isolates were from outpatients; however, hospital contact cannot be ruled out as the means of ESBL-positive strain acquisition. We did not detect any specific $bla_{\text{CTX-M}}$ genogroups in hospitalized or nonhospitalized patients, and MDR phenotypes were detected in both groups. Only two (10%) *K. pneumoniae* isolates were from outpatients.

DISCUSSION

We have examined ESBL genotypes and phenotypes of Norwegian clinical *E. coli* and *K. pneumoniae* isolates collected in a prospective multicenter study during an 8-month period in 2003. The relatively low breakpoint (MIC < 1 mg/liter) for susceptibility to oxyimino-cephalosporins in Norway ensures the detection of clinically relevant ESBL expression. The study design did not allow for any estimate of the prevalence of ESBLs. However, the Norwegian surveillance system for antimicrobial resistance showed a prevalence of ESBL production below 1% in clinical isolates of *E. coli* and *K. pneumoniae* in 2003 (1). We observed that CTX-M was the most common ESBL type in Norwegian *E. coli* (90%) isolates. This is consistent with the emergence of CTX-M-producing *E. coli* strains worldwide (2, 5, 7, 8, 9, 28).

The various genotypes and phenotypic expression patterns detected in this study challenge the sensitivities of ESBL detection methods. The predominant CTX-M-15-like enzymes were easily detected by all methods due to their broad oxyimino-cephalosporin substrate profile (11, 20). However, the ceftazidime combined disk method failed to detect 14 out of 15 CTX-M-9-like enzymes in *E. coli* isolates, in contrast to the CAZ-CLA ESBL Etest results showing the presence of deformed inhibition ellipses. Interestingly, all CTX-M-9-producing *E. coli* strains showed reduced susceptibility to cefpodoxime (mean, 55 mg/liter) and expressed significant CLA synergy in the combined disk test using cefpodoxime as the substrate.

Discrepancies between different detection methods were also observed for some of the minor *E. coli* ESBL genotypes. The two TEM-128 strains had a CTX-M-9-like phenotype and scored negative in the ceftazidime combined disk method. $bla_{\text{TEM-128}}$ has a T-to-G mutation, causing an Asp157→Glu substitution (Ambler numbering). ESBL activity was not observed in a TEM-128 *E. coli* strain recently isolated from food animals in Denmark (17), in contrast to the TEM-128-positive strains in this study expressing cefotaximase activity.

SHV-5-like enzymes expressing a ceftazidimase profile predominated among Norwegian clinical *K. pneumoniae* strains (79%). Six isolates containing SHV-28-like ($n = 2$) and SHV-2-like ($n = 4$) enzymes expressed low MICs of cefotaxime and ceftazidime. Accordingly, these strains scored negative in the ESBL confirmation tests when low-MIC substrates were used to detect CLA synergy (27).

SHV-1 hyperproduction in *E. coli* and *K. pneumoniae* has previously been reported to mediate an increased MIC of ceftazidime (MIC > 1 mg/liter) and CLA synergy, suggesting ESBL production (13, 21, 29). The three putative SHV-1-hyperproducing *E. coli* and *K. pneumoniae* strains, as well as the SHV-11-hyperproducing *K. pneumoniae* strain, showed inconsistent results by the ESBL detection methods. They scored positive for ESBL production in FEP-CLA and CAZ-CLA ESBL Etests, in contrast to results by the combined disk method, which were negative for CLA synergy using all three substrates. The basis for this discrepancy is not known, but it could be due to differences in CLA content. The concordance between the ESBL Etest and combined disk method results was otherwise excellent.

The overall high sensitivities of the ESBL Etests, as well as the sensitivity of the combined disk method, for this collection of clinical *E. coli* and *K. pneumoniae* strains were based on the combined use of cefotaxime and ceftazidime or cefpodoxime alone as screening substrates for CLA synergy. All ESBL strains were detected by using both cefotaxime and ceftazidime in combination with CLA. The single SHV-28 *K. pneumoniae* strain that failed in the detection of CLA synergy with cefpodoxime was associated with a low cefpodoxime MIC (0.75 mg/liter).

The detected ESBL genotypes seem to be representative of those circulating in Norway. The dominance of CTX-M ESBLs in *E. coli* and SHV ESBLs in *K. pneumoniae* has been verified in the 2 years following this study. CTX-M-15- and CTX-M-9-like phenotypic patterns confirmed by positive consensus

CTX-M PCRs were observed in 232/282 (82%) clinical ESBL-positive *E. coli* strains submitted to the Reference Centre during 2004 to 2005 (unpublished results). Thus, cefpodoxime alone or the combined use of cefotaxime and ceftazidime could be recommended as screening substrates for ESBL-mediated reduced susceptibility to oxyimino-cephalosporins.

The DDS test is an inexpensive and easy-to-use method for the detection of CLA synergy with various substrates and demonstrated excellent sensitivity when the disks were placed 25 to 30 mm (center to center) apart. However, we detected unexplained CLA synergy with aztreonam and cefpirome in three *E. coli* strains. The clinical significance of these findings is not obvious, and the findings illustrate the problems associated with methods based purely on visual inspection of CLA synergy, without more-objective criteria for interpretation. Moreover, it is well known that disk spacing affects the detection of inhibition. On the other hand, the DDS method represents the maximum flexibility that may enable the detection of β-lactamases with alternative substrate profiles.

Our observations of MDR among Norwegian ESBL-producing *E. coli* strains both in hospitals and in community settings are in accordance with recent studies (6, 22, 23, 28) and indicate the presence of biologically fit, easily transmitted genetic lineages of MDR *E. coli* strains. We did not detect strains with reduced susceptibilities to carbapenems, which consequently seem to be the only reliable therapeutics for systemic infections with these strains.

Interestingly, a substantial proportion of the CTX-M-positive urinary tract *E. coli* isolates were recovered from outpatients representing 15 different laboratories. The widespread appearance of CTX-M-producing clinical isolates of *E. coli* outside hospitals in Norway as well as other countries (6, 14, 19, 22, 28) strongly suggests common reservoirs. The high prevalence of MDR in CTX-M-positive *E. coli* contrasts with the low prevalence of reduced susceptibilities to ciprofloxacin (2.3%), gentamicin (1.3%), and nitrofurantoin (1.6%) in *E. coli* isolates recovered from urinary and blood culture samples in the Norwegian surveillance system in 2003 (1). These observations, combined with the overall low usage of antibiotics in Norway, may suggest that the emergence of MDR CTX-M-positive *E. coli* strains in our country is due to the import of resistant strains rather than local selection (1). It would therefore be of interest to compare the Norwegian strains to international clones in order to elucidate common reservoirs and lines of transmission. The molecular epidemiology of CTX-M-producing Norwegian *E. coli* strains is under investigation.

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