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Unreliable Extended-Spectrum β-Lactamase Detection in the Presence of Plasmid-Mediated AmpC in *Escherichia coli* Clinical Isolates[▽]

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The emergence of extended-spectrum β-lactamase (ESBL) and plasmid-mediated AmpC (pAmpC) enzymes in *Escherichia coli* raises concern regarding accurate laboratory detection and interpretation of susceptibility testing results. Twenty-six cefpodoxime ESBL screen-positive, cefoxitin-resistant *E. coli* clinical isolates were subjected to clavulanate ESBL confirmatory testing employing disk augmentation, Etest, and the BD Phoenix NMC/ID-132 panel. Phenotypic pAmpC production was assessed by boronic acid disk augmentation. ESBL and pAmpC genes were detected by gene amplification and sequencing. ESBL genes (SHV and/or CTX-M-type genes) were detected in only 7/26 ESBL screen-positive isolates. Of 23 aminophenylboronic acid screen-positive isolates, pAmpC genes were detected in 20 (CMY-2 or FOX-5 genes). High incidences of false-positive ESBL confirmatory results were observed for both clavulanate disk augmentation (9/19) and BD Phoenix (5/19). All were associated with the presence of pAmpC genes with or without TEM-1. Etest performed poorly, as the majority of interpretations were nondeterminable. In addition, false-negative ESBL confirmatory results were observed in isolates possessing concomitant ESBL and pAmpC genes for Etest (four of five), BD Phoenix (three of five), and disk augmentation (one of five). The results indicate poor performance of currently employed ESBL confirmatory methods in the setting of concomitant pAmpC. Some isolates with pAmpC and ESBL genes fell within the susceptible category to extended-spectrum cephalosporins, raising concern over currently employed breakpoints.

Ambler class A extended-spectrum β-lactamase (ESBL) genes in *Escherichia coli* are well documented. Possible ESBL production has been reported to occur in up to 9% of European *E. coli* isolates (15). In addition, chromosomal Ambler class C AmpC genes have been mobilized and are now being disseminated on plasmids, reminiscent of the early dissemination and evolution of ESBLs (11). Increasingly, reports document the detection of plasmid-mediated AmpC resistance (pAmpC) in *E. coli* (4, 6, 12). Data from the SENTRY antimicrobial surveillance program for North America show that 19/65 ESBL screen-positive *E. coli* isolates harbored pAmpC (5).

The ESBL hydrolytic spectrum includes the oxyimino-cephalosporins and monobactams but not 7-α-methoxy-cephalosporins (cephamycins) and is inhibited by clavulanate, sulbactam, and tazobactam. The broader spectrum of the AmpC enzymes includes the cephamycins, and AmpC enzymes are not inhibited by clavulanate, sulbactam, or tazobactam. The Clinical and Laboratory Standards Institute (CLSI) recommends that antimicrobial susceptibility testing include screening for ESBL production in *E. coli*, employing cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone, followed by phenotypic confirmation with clavulanate (3).

ESBL screening results are reviewed to select for those organisms that need phenotypic ESBL confirmation, as recommended by CLSI, and results are issued with the aim of preventing inappropriate use of cephalosporins or monobactams (3), as treatment with these agents in the setting of ESBL production has been correlated with treatment failure (16). ESBL detection results are additionally employed by microbiologists and infection control practitioners to identify and track nosocomial infection and dissemination of strains or plasmids.

In addition to the CLSI-recommended disk diffusion assay, a number of alternative commercial formats are available. Etest (AB Biodisk, Solna, Sweden) employs immobilized antimicrobial agents on a calibrated test strip, enabling determination of the MIC as read at the microbial growth strip intersect. The addition of clavulanate to the cephalosporin-impregnated strip decreases the MIC, indicative of ESBL production. Automated identification and susceptibility systems that similarly incorporate clavulanate to infer ESBL production are commercially available.

No standardized method is recognized for screening and confirmation of the presence of pAmpC. Boronic acid reversibly binds to and inhibits the action of AmpC enzymes (1), and some laboratories employ boronic acid to detect AmpC enzymes (14, 17).

A concern with the occurrence of ESBL screen-positive, confirmatory testing-negative *E. coli* isolates harboring pAmpC genes was recently raised based on SENTRY Asia-Pacific data; up to 75% of nonconfirming isolates were shown to harbor pAmpC genes (2). The effect of concurrent ESBL and pAmpC gene expression may adversely affect the performance of current ESBL screening and confirmatory testing, as the two enzyme groups have overlapping hydrolysis spectra, except that AmpC enzymes are not inhibited by clavulanate, sulbactam, or tazobactam. We evaluated the performance of standard phenotypic detection

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methods in the setting of the presence of concurrent ESBL and pAmpC genes in *E. coli* clinical isolates.

MATERIALS AND METHODS

Bacterial strains. Twenty-six consecutive clinical isolates of *E. coli*, received at or isolated by the Clinical Microbiology Laboratory, Mayo Clinic, Rochester, MN, as part of routine diagnostic activities from November 2007 through February 2008, were investigated. Selection of isolates was based on positive ESBL screen test results with cefpodoxime MICs of ≥8 μg/ml (3) and resistance to cefoxitin, suggestive of pAmpC production. Routine identification and susceptibility testing employing standard biochemical procedures and agar dilution methods, respectively, were performed. Susceptibility testing procedures were performed and appropriate quality control strains employed, as indicated by CLSI or individual product manufacturers. *E. coli* strains with previously characterized ESBL genes graciously provided by Karen Bush (Johnson & Johnson Pharmaceutical Research & Development LLC, Raritan, NJ) were employed as PCR controls.

Manual phenotypic ESBL detection. ESBL expression was investigated by employing CLSI-recommended clavulanate disk augmentation. Disk diffusion was performed using antimicrobial impregnated disks (BD Diagnostics, Sparks, MD) containing ceftazidime (30 μg) with or without clavulanate (10 μg) and cefotaxime (30 μg) with or without clavulanate (10 μg). An increased zone size in the presence of clavulanate, as measured with digital calipers, of ≥5 mm was considered indicative of positivity for ESBL expression (3). Etest (AB Biodisk) ESBL detection was performed as recommended by the manufacturer. Briefly, a 0.5 McFarland suspension of overnight culture was streaked onto cation-adjusted Mueller-Hinton agar plates (BD Diagnostics). Etest strips containing ceftazidime or ceftazidime-clavulanate (TZL) and cefotaxime or cefotaxime-clavulanate (CTL) were applied and incubated for 18 h. Interpretation was according to the Etest ESBL package insert as follows: positive (cefotaxime level of ≥0.5 μg/ml and ceftazidime/CTL ratio of ≥8, ceftazidime level of ≥1 μg/ml and ceftazidime/TZL ratio of ≥8, or presence of a phantom zone or deformed ellipse), negative (cefotaxime level of <0.5 μg/ml or cefotaxime/CTL ratio of <8, ceftazidime level of <1 μg/ml, and ceftazidime/TZL ratio of <8), and nondeterminable (cefotaxime level of >16, CTL level of >1, ceftazidime level of >32, and TZL level of >4 or one strip ESBL negative and one nondeterminable) (Etest ESBL package insert; AB Biodisk, Solna, Sweden).

Automated ESBL detection. The BD Phoenix NMIC/ID-132 panel (BD Diagnostics), which employs ceftazidime, TZL, ceftriaxone-clavulanate, CTL, cefpodoxime, and cefepime for ESBL detection, was tested with the BD Phoenix (BD Diagnostics), according to the manufacturer's instructions (BD Phoenix NMC/ID-132 package insert; Becton, Dickinson and Company, Sparks, MD).

Manual phenotypic pAmpC detection. The presence of pAmpC was investigated as described by Song et al. (14). Briefly, 100 mg/ml aminophenylboronic acid monohydrate (APB) was prepared in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). Three microliters (300 μg) was applied to a standard disk diffusion paper disk containing either TZL (30 μg/10 μg) (TZL-APB) or CTZ (30 μg/10 μg) (CTZ-APB) (BD Diagnostics). An overnight culture was suspended to give a 0.5 McFarland standard and inoculated onto cation-adjusted Mueller-Hinton agar plates (BD Diagnostics) with TZL, TZL-APB, CTL, and CTL-APB disks. Zone sizes were measured with digital calipers after 18 h of incubation. The effect of APB was determined by subtraction of the TZL zone size from the TZL-APB zone size and the CTL zone size from the CTL-APB zone size. An increased zone size of ≥5 mm was considered indicative of the presence of AmpC gene expression (14).

Amplification template preparation. Four or five single colonies were inoculated into in-house-prepared tubes containing glass beads and neutralization buffer. The tubes were heated at 99°C with shaking at 1,400 rpm for 6 min by using an Eppendorf thermomixer (Hamburg, Germany). The lysate was centrifuged and the pellet resuspended in distilled water for amplification.

Amplification and detection. PCR amplification of the ESBL TEM, SHV, and CTX-M genes was performed as previously described (9, 13). Multiplex amplification of pAmpC genes was performed with primers targeting MOX, CMY, LAT, BIL, DHA, ACC, MIR, and FOX-type β-lactamases, as described by F. J. Pérez-Pérez and N. D. Hanson (10) (Table 1). Amplification reactions were performed as described by Y.-J. Park, M. Saladin, and F. J. Pérez-Pérez, employing Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) in an AB 9600 thermocycler (Applied Biosystems, Foster City, CA) (9, 10, 13). Amplified DNA was detected by electrophoresis employing 2% E-gel 96 (Invitrogen), with size estimation by comparison to a 100-bp ladder (Invitrogen) (Table 1).

DNA sequencing and analysis. Amplification reaction mixtures were prepared for sequencing by removal of remaining primers and deoxynucleoside triphosphates by digestion with exonuclease 1 and shrimp alkaline phosphatase (ExoSap-IT; USB Corporation, Cleveland, OH), according to the manufacturer's recommendations. Purified, amplified DNA was subjected to DNA sequencing employing an ABI PRISM BigDye Terminator v1.1 cycle sequencing ready-reaction kit with AmpliTaq DNA polymerase and analyzed by employing ABI PRISM 3730 XL DNA analyzers (Perkin-Elmer Applied Biosystems, Foster City, CA) in the Molecular Biology Core Facility, Mayo Clinic. TEM and SHV DNA sequence data were compared to known types (reference database of ESBL type-specific amino acid mutations; G. Jacoby and K. Bush, Lahey Clinic; http://www.lahey.org/studies/), employing the online ESBL Genotyping Tool (EGT) hosted by the Institute of Infection Medicine, University Medical Center Schleswig-Holstein Campus, Kiel, Germany (accessible through http://www.informatik.uni-kiel.de/~amino/). CTX-M and plasmid-mediated AmpC gene sequences were compared to published sequences by employing the NCBI Basic Local Alignment Search Tool (BLAST).

TABLE 1. PCR primers employed

Target	Primers	Amplification product size (bp)	Reference
ESBL			
TEM	blaTEMF, blaTEMR	1,079	9
SHV	blaSHVR, blaSHVR	868	9
CTX-M1 group	CTX-1F, CTX-1R	864	13
CTX-M2 group	CTX-2F, CTX-2R	866	13
CTX-M9 group	CTX-9F, CTX-9R	785	7
pAmpC			
MOX-1, MOX-2, CMY-1, CMY-8 to -11	MOXMF, MOXMR	520	10
LAT-1 to -4, CMY-2 to -7, BIL-1	CITMF, CITMR	462	10
DHA-1, DHA-2	DHAMF, DHAMR	405	10
ACC	ACCMR, ACCMF	346	10
MIR-1T, ACT-1	EBCMF, EBCMR	302	10
FOX-1 to -5b	FOXMF, FOXMR	190	10

RESULTS

The MIC distribution of the isolates tested is shown in Table 2. Two isolates carrying only ESBL genes yielded typical cephalosporin-resistant MICs; both were correctly detected by Phoenix and disk diffusion; however, Etest results were negative for one and nondeterminable for the other. Among the 17 isolates containing only pAmpC, the MICs for the extended-spectrum cephalosporins varied, with cefepime being more active than the other agents tested. The majority of these isolates fell within the category of susceptibility to these agents. Among the five isolates harboring ESBL and pAmpC concomitantly, some tested within the susceptible category: two of five were susceptible to ceftriaxone, cefotaxime, and ceftazidime and three of five to cefepime and aztreonam (Table 2).

The number of isolates for which ESBL detection failed when Phoenix and disk diffusion were employed is indicated in Table 3. Etest correlation was not further analyzed, due to overall poor ESBL detection. Of the manual ESBL confirmation methods, disk augmentation displayed the overall highest positivity rate (15/26), whereas Etest detected ESBL production in only 2/26 isolates and yielded a high number of nondeterminable results (23/26) (Table 3). The BD Phoenix NMC/ID 132 system reported 9/26 isolates as ESBL producers.

Of the 26 isolates, ESBL PCR results were initially positive for 16, including TEM alone in 9, SHV alone in 1, and CTX-M alone in 2. One isolate each harbored TEM and SHV, SHV and CTX-M, and TEM and CTX-M genes. A single isolate contained TEM, SHV, and CTX-M genes concurrently. Se-

TABLE 2. MIC distributions (as determined by agar dilution) of isolates harboring secondary β-lactamases (as detected by PCR and sequencing, excluding TEM-1)

Gene and drug	No. of isolates with indicated MIC (μg/ml)[a]								
	0.25	0.5	1	2	4	8	16	32	>32
ESBL gene only (n = 2)									
Ceftriaxone									2
Cefotaxime									2
Ceftazidime									2
Cefepime					1				1
Aztreonam									2
pAmpC gene only (n = 17)									
Ceftriaxone					1		5	6	5
Cefotaxime						3	6	4	4
Ceftazidime				1				8	8
Cefepime	2	9	4	1		1			
Aztreonam						4	7	6	
pAmpC plus ESBL gene (n = 5)									
Ceftriaxone						1	[1] (1) 1		[2] 3
Cefotaxime							[1] (1) 2	[1] 1	[1] 2
Ceftazidime			1					[1] (1) 1	[1] 3
Cefepime		[1] (1) 1	1			[1] 1			[1] 2
Aztreonam					2	1			2
Neither pAmpC nor ESBL gene (n = 2)									
Ceftriaxone								2	
Cefotaxime							2		
Ceftazidime								2	
Cefepime			2						
Aztreonam							2		

[a] Values in brackets and parentheses are numbers of isolates containing ESBL not detected with Phoenix and disk augmentation, respectively.

quence analysis revealed that all amplified TEM DNA represented TEM-1 genes and were thus not true ESBL genes. Therefore, true ESBL genes were detected in just seven isolates (Table 3).

APB screening yielded increased zone sizes for 22/26 and 23/26 isolates by employing TZL and CTL, respectively, suggestive of AmpC production; APB screening results were negative for 3 isolates. pAmpC genes were detected in 22/26 isolates, of which 20 harbored CMY-2 and 2 FOX-5 genes. PCR amplification confirmed the presence of pAmpC genes in 20 of the 23 APB-positive isolates. APB failed to identify two isolates, shown to contain CMY-2, and in one of these isolates, concurrent TEM-1, SHV, and CTX-M genes were detected.

TABLE 3. Correlation between phenotypic methods and gene amplification for detection of ESBLs

ESBL PCR result[a]	No. of samples							
	Total	Phenotypic ESBL method						
		Etest			Phoenix		Disk augmentation	
		+	−	ND[b]	+	−	+	−
+	7	2	0	5	4	3	6	1
−	19	0	1	18	5	14	9	10
Total	26	2	1	23	9	17	15	11

[a] Excluding TEM-1.
[b] ND, nondeterminable.

(The second false-negative isolate did not contain any additional β-lactamase genes.) Eleven of the 22 PCR-confirmed pAmpC isolates carried TEM-1 with or without other ESBLs. Of the 22 pAmpC isolates, 5 harbored a concomitant true ESBL gene. Phenotypic detection of ESBLs in isolates harboring pAmpC carriers was poor for Etest (one of five), Phoenix (two of five), and disk augmentation (four of five).

Correlation of ESBL phenotypic methods and ESBL gene amplification is shown in Table 3. Successful detection of the seven isolates shown to contain ESBL genes was least frequent for Etest (two of seven) and Phoenix (four of seven) and most sensitive for disk augmentation (six of seven). Positive ESBL phenotypic test results in the absence of ESBL amplification were most frequent with disk augmentation (9/19) and Phoenix (5/19). Etest showed no apparent false positives; however, 23/26 isolates yielded nondeterminable results.

Among the nine isolates showing apparent disk augmentation false-positive ESBL results, pAmpC genes were detected in all. These false-positive disk diffusion test results were produced with TZL augmentation but not CTL augmentation (results not shown). Neither Etest nor Phoenix yielded positive ESBL results for eight of these nine false-positive disk diffusion isolates.

Of the five isolates with apparent false-positive Phoenix ESBL results, three contained pAmpC genes and the remaining two had no evidence of secondary β-lactamases by PCR. Of note, one SHV-containing isolate was undetected by all three phenotypic methods.

DISCUSSION

The results of this study indicate that among *E. coli* isolates harboring pAmpC genes, currently employed phenotypic ESBL detection methods perform poorly. Of the 26 cefpodoxime screen-positive isolates, only 7 had detectable ESBL genes. Disk diffusion and Phoenix systems failed to confirm ESBL producers (1/7 and 3/7, respectively) and inaccurately reported ESBL production in many isolates (9/19 and 5/19, respectively) lacking amplifiable ESBL genes. All false-positive results were observed among isolates harboring either pAmpC or pAmpC and TEM-1 genes. Etest reported 23/26 as nondeterminable (a result which is of no value to the clinical laboratory).

The majority of cefoxitin-resistant *E. coli* isolates harbored CMY-2 genes (20/26), with two harboring FOX-5. Both of these pAmpC genes have previously been detected in *E. coli* in the United States and have been implicated in ESBL screen-positive, confirmatory testing-negative reports (5). Phenotypic screening with APB detected 20 of 22 amplification-confirmed pAmpC genes and three apparent false-positive results, lending support to this method of screening. Of concern is the large proportion of pAmpC-containing *E. coli* isolates seen to fall within the susceptible MIC range of extended-spectrum cephalosporins and aztreonam, representing perhaps an inaccurate prediction that these agents will all be active in vivo.

Some of the isolates harboring both pAmpC and ESBL genes would be considered to be susceptible to extended-spectrum cephalosporins and aztreonam with the use of current CLSI guidelines, regardless of additional ESBL detection techniques employed. A practical solution for reliable identification of isolates harboring secondary acquired β-lactamases may be to lower the MIC breakpoint values for the cephalosporins and monobactams, such that breakpoints bisect the species MIC distribution pattern seen for those isolates without acquired genes and those that have acquired extended-spectrum and pAmpC β-lactamases. This would have the additional advantage of circumventing time-consuming laboratory investigations of questionable accuracy and clinical value in a setting of increased diversity and prevalence of secondary acquired β-lactamases. Clinical studies are warranted to inform breakpoint establishment for agents thought to have a limited role in the setting of ESBL or pAmpC gene expression.

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