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Detection of AmpC Beta-Lactamase in *Escherichia coli*: Comparison of Three Phenotypic Confirmation Assays and Genetic Analysis▽†

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Two mechanisms account for AmpC activity in *Escherichia coli*, namely, mutations in the *ampC* promoter and attenuator regions resulting in *ampC* overexpression and acquisition of plasmid-carried *ampC* genes. In this study, we analyzed 51 clinical *E. coli* isolates with reduced susceptibility to amoxicillin-clavulanic acid, piperacillin-tazobactam, or extended-spectrum cephalosporins for the presence of AmpC production. Three phenotypic AmpC confirmation assays (cefoxitin-cloxacillin disk diffusion test, cefoxitin-EDTA disk diffusion test, and AmpC Etest) were compared for the detection of AmpC activity. All 51 isolates were characterized genetically by mutational analysis of the chromosomal *ampC* promoter/attenuator region and by PCR detection of plasmid-carried *ampC* genes. Altogether, 21/51 (41%) *E. coli* isolates were considered true AmpC producers. AmpC activity due to chromosomal *ampC* promoter/attenuator mutations was found in 12/21 strains, and plasmid-carried *ampC* genes were detected in 8/21 isolates. One strain contained both *ampC* promoter mutations and a plasmid-carried *ampC* gene. All three phenotypic tests were able to detect the majority (>90%) of AmpC-positive strains correctly. Cefoxitin resistance was found to be a discriminative parameter, detecting 20/21 AmpC-producing strains. Susceptibility to extended-spectrum cephalosporins, e.g., ceftriaxone, ceftazidime, and cefotaxime, was found in 9 of the 21 AmpC-positive strains. Considering the elevated zone diameter breakpoints of the 2010 CLSI guidelines, 2/21 AmpC-positive strains were categorized as susceptible to extended-spectrum cephalosporins.

The prevalence of multidrug-resistant Gram-negative bacteria has increased continuously over the past few years, and bacterial strains producing AmpC beta-lactamases and/or extended-spectrum beta-lactamases (ESBLs) are of particular concern. AmpC beta-lactamases can confer resistance to aminopenicillins, cephalosporins, oxyimino-cephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g., cefoxitin and cefotetan), and monobactams (16). Cloxacillin and 3-aminophenylboronic acid inhibit AmpC beta-lactamases (2, 16, 36), while AmpC beta-lactamase activity is not affected by the ESBL inhibitor clavulanic acid. In Gram-negative bacteria, AmpC beta-lactamase production is chromosome or plasmid mediated. Chromosomal *ampC* genes are expressed constitutively at a low level. Some *Enterobacteriaceae*, such as *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp., carry an inducible *ampC* gene. In these cases, the gene is strongly induced by β-lactams, such as cefoxitin and imipenem, with expression mediated by the regulator AmpR. Mutations in the repressor gene *ampD* may lead to overproduction of AmpC beta-lactamases (16). The regulation of chromosomal *ampC* expression in *Escherichia coli* differs considerably from that in other *Enterobacteriaceae*. *E. coli* lacks *ampR*, and thus *ampC* expression is not inducible (15). In *E. coli*, *ampC* is expressed constitutively at a low level (17). Various mutations in the *ampC* promoter/attenuator region of *E. coli* have been identified that result in constitutive overexpression (7, 8, 13, 22, 24, 34, 39, 40). In addition to chromosomal *ampC*, *E. coli* may contain plasmids carrying *ampC* (pAmpC), transferred via horizontal gene transfer and derived from the chromosomal *ampC* genes of other *Enterobacteriaceae* spp. (16). Plasmid-based *ampC* genes are expressed constitutively in most cases. However, some plasmid-carried *ampC* genes, such as the DHA-1 gene, are inducible by β-lactams, with expression regulated similarly to that of inducible chromosomal *ampC* genes. All plasmid-carried *ampC* genes are considered to be of significant clinical relevance (23, 27). AmpC overproduction in addition to porin mutations of the outer membrane can reduce susceptibility to carbapenems, in particular in plasmid-mediated AmpC producers (19, 26).

AmpC producers may appear susceptible to extended-spectrum cephalosporins when initially tested (27, 37, 38, 40), and standardized procedures for the detection and identification of AmpC beta-lactamase-producing strains have not been established thus far. However, proper recognition of AmpC-overproducing *E. coli* strains is important for clinical management, as administration of beta-lactam antibiotics frequently results in therapeutic failure. For example, a recent study described the isolation of AmpC-overproducing *E. coli* strains from patients who did not respond to oxyimino-cephalosporin therapy (34). Another study analyzed the clinical outcomes of patients with bloodstream infection caused by plasmid-mediated AmpC-producing *Klebsiella pneumoniae* and showed high rates of treatment failure when cephalosporins were administered (27).

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Different phenotypic AmpC confirmation tests have been reported in the literature (16). A recently described disk diffusion test is based on comparison of the inhibition zone diameters around a cefoxitin disk and a cefoxitin disk supplemented with the inhibitor cloxacillin. The test was shown to have a sensitivity and a specificity of 95% for the detection of plasmidic AmpC in 127 strains of *E. coli*, *Klebsiella* spp., and *Proteus* spp. (36). Another AmpC confirmation test is based on antagonism phenomena, using a cefoxitin-susceptible indicator strain. This test was evaluated for the detection of plasmid AmpC production in species lacking chromosomal *ampC* (4). Reportedly, the test had a sensitivity of 100% and a specificity of 98% with 140 isolates of *Klebsiella* spp., *Proteus mirabilis*, and *Salmonella* sp. (4). In this study, we aimed to evaluate and compare the diagnostic performances of the two disk diffusion tests and a commercially available assay (Etest; AB bioMérieux, Sweden) as a confirmation test for the detection of AmpC activity in clinical *E. coli* isolates with suspicion of AmpC production. Molecular analyses were used to assess the specificity of the phenotypic assays and to characterize the genetic basis for AmpC (over)production in these strains.

MATERIALS AND METHODS

Clinical isolates. Fifty-one *E. coli* clinical strains with reduced susceptibility to amoxicillin-clavulanic acid, piperacillin-tazobactam, or oxyimino-cephalosporins (ceftazidime, cefotaxime, or ceftriaxone) were collected at the Institute of Medical Microbiology, Zurich, Switzerland, over a period of 2 years, from July 2006 until July 2008. The strains were isolated from urines ($n = 12$), blood cultures ($n = 12$), respiratory specimens ($n = 8$), perianal swabs ($n = 4$), wound swabs ($n = 4$), inguinal swabs ($n = 3$), abscesses ($n = 2$), tissue ($n = 2$), a vaginal swab ($n = 1$), a gastric aspirate ($n = 1$), cerebrospinal fluid (CSF) ($n = 1$), and a sample of unknown origin ($n = 1$).

Antibiotic susceptibility testing. Antibiotic susceptibility testing was performed using susceptibility test disks (Becton Dickinson, Germany), and interpretation was done according to 2009 and 2010 CLSI guidelines (9, 10). For cefotetan susceptibility testing, the AmpC Etest strip (AB bioMérieux, Sweden) was used as described below. Susceptibility testing was performed on Mueller-Hinton agar (bioMérieux, France), using overnight cultures at a 0.5 McFarland standard followed by incubation at 35°C for 16 to 18 h.

Phenotypic AmpC and ESBL activity testing. The AmpC Etest (AB bioMérieux, Sweden) for cefotetan susceptibility was performed according to the manufacturer's instructions. The AmpC Etest consists of a strip containing cefotetan on one end and cefotetan-cloxacillin on the other end. Ratios of the MICs of cefotetan and cefotetan-cloxacillin of ≥8 are considered positive for AmpC beta-lactamase production.

The cefoxitin-cloxacillin disk diffusion test was performed as described by Tan et al. (36). The test is based on the inhibitory effect of cloxacillin on AmpC. In brief, 30-μg cefoxitin disks (Becton Dickinson, Germany) were supplemented with 200 μg cloxacillin. The test strain was inoculated on Mueller-Hinton agar. The diameters of the cefoxitin inhibition zones were compared with and without cloxacillin; if the difference in inhibition was ≥4 mm, the strain was considered positive for AmpC production.

The cefoxitin-EDTA disk test was performed as described by Black et al. (4). In brief, a lawn of the cefoxitin-susceptible *E. coli* strain ATCC 25922 was inoculated on a Mueller-Hinton agar plate. A 30-μg cefoxitin disk (Becton Dickinson, Germany) was placed on the bacterial lawn and flanked by two disks (A and B), each containing 20 μl of a 1:1 mixture of saline and 100× Tris-EDTA solution. Colonies of the test strain were applied to disk A, and colonies of the cefoxitin-susceptible *E. coli* strain ATCC 25922 (as a negative control) were applied to disk B. Flattening or indentation of the growth inhibition zone of the cefoxitin disk at the side of disk A containing the test strain indicated the release of AmpC beta-lactamase.

To analyze the induction of plasmid-encoded DHA AmpC, a disk approximation assay was used, with imipenem as an inducer and ceftazidime, cefoxitin, ceftriaxone, and piperacillin-tazobactam as substrate antibiotics (12).

For phenotypic detection of ESBL activity according to CLSI guidelines, a DDS test using ceftazidime and cefotaxime (30 μg) disks, with and without clavulanic acid (10 μg) (Liofilchem, Roseto degli Abruzzi, Italy), was used. The bacterial test strains were inoculated onto Mueller-Hinton agar at a 0.5 McFarland standard, followed by incubation at 35°C for 16 to 18 h. Diameters of inhibition zones were measured with a standard caliper. A difference in inhibition zones of ≥5 mm for at least one extended-spectrum cephalosporin-clavulanic acid combination versus the corresponding extended-spectrum cephalosporin alone was considered indicative of ESBL production.

Beta-lactamase hydrolysis assays. For phenotypic detection of beta-lactamase activity, the chromogenic substrate nitrocefin (Calbiochem, San Diego, CA) was used (35). 3-Aminophenylboronic acid (Sigma-Aldrich Chemie, GmbH, Zug, Switzerland) was used as a specific AmpC inhibitor (2), and clavulanic acid (Sigma-Aldrich Chemie, GmbH, Zug, Switzerland) was used as an inhibitor of Ambler class A beta-lactamases (e.g., ESBL and TEM-1 beta-lactamases) (5). A bacterial suspension at a 0.5 McFarland standard in 0.45% NaCl was prepared from overnight cultures. *E. coli* strain DH5α was used as a negative-control strain. Reaction mixtures consisted of 50 μl bacterial cell suspension, 25 μl nitrocefin (0.5 mg/ml in 10 mM phosphate buffer, pH 6.8), 25 μl 3-aminophenylboronic acid (3.6 mg/ml in 10 mM phosphate buffer, pH 6.8), and/or 25 μl potassium clavulanate (2.2 mg/ml in 10 mM phosphate buffer, pH 6.8). In cases where 3-aminophenylboronic acid and/or clavulanic acid was not added, the end volume of 125 μl was reached by adding 10 mM phosphate buffer, pH 6.8. Reaction mixtures were incubated in microtiter plates at 37°C. The nitrocefin hydrolysis product was detected by quantifying the optical density at 492 nm (OD_{492}) after 8 h, using a titer plate spectrophotometer (Biochrom Asys Expert Plus microplate reader; Biochrom Ltd., Cambridge, United Kingdom).

***ampC* promoter/attenuator sequencing.** DNAs were extracted from colonies grown on agar medium by using an InstaGene matrix (Bio-Rad, Switzerland) following the manufacturer's instructions. For *ampC* promoter/attenuator mutation analysis, a 271-bp fragment was amplified using primers AB1 (5′-GATC GTTCTGCCGCTGTG-3′) and ampC2 (5′-GGGCAGCAAATGTGGAGCAA-3′) (11). PCR amplicons were purified with a QIAquick PCR purification kit (Qiagen, Switzerland) followed by cycle sequencing using a BigDye reagent kit (Applied Biosystems, Switzerland). Sequence analysis was performed on an ABI Prism model 3100 DNA sequencer (Applied Biosystems, Switzerland) following standard protocols. Sequences were analyzed and edited using Lasergene 7 MegAlign software (DNASTAR Inc.). The *ampC* promoter/attenuator sequences were compared to the *ampC* wild-type sequence of *E. coli* strain ATCC 25922.

Molecular detection of plasmid-carried *ampC* beta-lactamase genes. A multiplex PCR was used for the detection of plasmid-carried *ampC* beta-lactamase genes (29). This assay is able to detect the six plasmid-carried *ampC* gene families. Resulting PCR amplicons were sequenced with the amplification primers following the protocol described above. The sequences were compared to reference sequences in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

Detection of ESBL and KPC genes. For detection of TEM and SHV beta-lactamase genes, a multiplex PCR was performed as described previously (21). Sequences were analyzed and edited using Lasergene 7 MegAlign software (DNASTAR Inc.). The TEM beta-lactamase sequences were compared to the wild-type *E. coli* TEM-1 sequence (GenBank accession no. AF427133.1) by using the publicly available database at http://www.lahey.org/studies. For the detection of CTX-M beta-lactamase genes, a multiplex PCR was performed as described by Pitout et al. (31). For detection of *Klebsiella* carbapenemase (KPC) genes, a PCR was used as described previously (33).

Interpretation. *E. coli* strains positive for AmpC activity in at least one phenotypic test (AmpC Etest, AmpC cefoxitin-EDTA disk test, or AmpC cefoxitin-cloxacillin disk test) and validated by genetic analysis (presence of plasmid-carried *ampC* genes or *ampC* promoter/attenuator mutations associated with chromosomal *ampC* overexpression) were considered to be true AmpC producers. Strains with discrepant test results were analyzed in further detail for beta-lactamase production, using nitrocefin hydrolysis assays, phenotypic ESBL assays, molecular assays for ESBL and KPC detection, and sequence analysis of detected TEM and SHV genes.

RESULTS

Phenotypic screening for AmpC production. For 18/51 (35%) *E. coli* isolates, all three phenotypic tests gave a positive result for AmpC production (Table 1). The MIC ratios obtained with the AmpC Etest ranged from 8 to 64. In the AmpC cefoxitin-cloxacillin disk test, the differences in zone diameters ranged from 4 mm to 14 mm (Table 1). For 28/51 (55%) *E. coli*

TABLE 1. Characterization of the 21 *E. coli* strains positive for AmpC activity[a]

Strain	AmpC Etest			AmpC cefoxitin-cloxacillin disk test		AmpC cefoxitin-EDTA disk test result	*ampC* plasmid PCR result[d] (class)	*ampC* chromosomal sequence analysis result[e] (promoter variant)	Final AmpC result[f]	Antibiotic resistance disk test result (zone of inhibition [mm])[g]						
	Cefotetan susceptibility (MIC [μg/ml])	MIC ratio[b]	AmpC result	Inhibition zone diam difference (mm)[c]	AmpC result					CAZ	CTX	CRO	FEP	AMC	TZP	FOX
1	S (12)	**24**	**Positive**	**8**	**Positive**	**Positive**	Negative	**Positive (1)**	**Positive**	S (18)	S (23)	S (23)	S (29)	**R (7)**	S (21)	**R (7)**
2	S (4)	**8**	**Positive**	**8**	**Positive**	**Positive**	Negative	**Positive (1)**	**Positive**	S (21)	**I (22)**	S (21)	S (27)	**R (10)**	S (23)	**R (12)**
3	**I (32)**	**43**	**Positive**	**8**	**Positive**	**Positive**	Negative	**Positive (1)**	**Positive**	**R (12)**	**I (19)**	S (21)	S (28)	**R (7)**	**I (20)**	**R (7)**
4	**I (32)**	**64**	**Positive**	**11**	**Positive**	**Positive**	Negative	**Positive (1)**	**Positive**	S (21)	S (24)	S (26)	S (30)	**R (7)**	**I (20)**	**R (13)**
5	**I (32)**	**21**	**Positive**	**5**	**Positive**	**Positive**	Negative	**Positive (1)**	**Positive**	S (20)	**I (19)**	S (21)	S (26)	**I (16)**	S (22)	**R (7)**
6	S (12)	**24**	**Positive**	**5**	**Positive**	**Positive**	Negative	**Positive (1)**	**Positive**	S (18)	S (25)	S (26)	S (28)	**R (7)**	**I (18)**	**I (15)**
7	S (4)	**8**	**Positive**	**8**	**Positive**	**Positive**	Negative	**Positive (3)**	**Positive**	S (28)	S (27)	S (28)	S (32)	**R (11)**	S (26)	**I (17)**
8	S (24)	**48**	**Positive**	**5**	**Positive**	**Positive**	Negative	**Positive (2)**	**Positive**	S (19)	S (23)	S (23)	S (30)	**R (7)**	**I (19)**	S (18)
9	S (16)	**32**	**Positive**	**10**	**Positive**	**Positive**	Negative	**Positive (5)**	**Positive**	S (20)	S (23)	S (25)	S (32)	**R (10)**	S (23)	**R (13)**
10	S (12)	**24**	**Positive**	**6**	**Positive**	**Positive**	Negative	**Positive (6)**	**Positive**	S (19)	S (25)	S (28)	S (29)	**R (12)**	S (21)	**R (14)**
11	**R (>32)**	**8**	**Positive**	**5**	**Positive**	**Positive**	**Positive (CIT)**	Negative (12)	**Positive**	**R (7)**	**R (7)**	**R (7)**	S (24)	**R (7)**	**I (18)**	**R (7)**
12	**R (>32)**	**8**	**Positive**	**4**	**Positive**	**Positive**	**Positive (CIT)**	Negative (12)	**Positive**	**R (7)**	**R (7)**	**R (7)**	S (20)	**R (7)**	**R (14)**	**R (7)**
13	**I (32)**	**8**	**Positive**	**9**	**Positive**	**Positive**	**Positive (CIT)**	Negative (12)	**Positive**	**R (7)**	**R (10)**	**R (7)**	S (25)	**R (7)**	**I (20)**	**R (7)**
14	**I (32)**	**64**	**Positive**	**13**	**Positive**	**Positive**	**Positive (CIT)**	Negative (15)	**Positive**	**I (15)**	**I (15)**	**I (16)**	S (29)	**R (7)**	S (21)	**R (7)**
15	**I (32)**	**64**	**Positive**	**14**	**Positive**	**Positive**	**Positive (CIT)**	Negative (15)	**Positive**	**I (16)**	**I (17)**	**I (16)**	S (25)	**R (10)**	S (25)	**R (7)**
16	**I (32)**	**64**	**Positive**	**10**	**Positive**	**Positive**	**Positive (CIT)**	Negative (11)	**Positive**	**R (7)**	**R (14)**	**R (12)**	S (28)	**R (10)**	S (21)	**R (7)**
17	S (8)	**16**	**Positive**	**10**	**Positive**	**Positive**	**Positive (CIT)**	Negative (11)	**Positive**	**I (17)**	**I (20)**	**I (18)**	S (26)	**R (11)**	S (24)	**R (9)**
18	**I (32)**	**43**	**Positive**	**10**	**Positive**	**Positive**	**Positive (CIT)**	**Positive (5)**	**Positive**	**R (7)**	**I (18)**	**I (15)**	S (28)	**R (7)**	S (22)	**R (7)**
19	**I (32)**	**32**	**Positive**	0	Negative	**Positive**	Negative	**Positive (3)**	**Positive**	S (23)	S (25)	S (26)	S (31)	**R (10)**	S (24)	**R (14)**
20	S (0.5)	1	Negative	**6**	**Positive**	**Positive**	Negative	**Positive (4)**	**Positive**	S (26)	S (28)	S (37)	S (28)	**R (12)**	S (25)	**I (17)**
21	S (0.75)	2	Negative	4	**Positive**	Negative	**Positive (DHA)**	Negative (14)	**Positive**	**R (11)**	**R (13)**	**R (7)**	S (18)	**R (9)**	S (23)	**R (7)**

[a] Strains were analyzed for AmpC production by use of three phenotypic confirmation assays. Genetic analysis of AmpC production was done by multiplex PCR for plasmid *ampC* detection and by sequence analysis of the chromosomal *ampC* promoter/attenuator region. In addition, antimicrobial susceptibility data were generated for each strain. Data for strains with discrepant results in the three phenotypic AmpC confirmation assays are shaded. Positive results and results indicating resistance and intermediate susceptibility are shown in bold. Abbreviations: S, susceptible; R, resistant; I, intermediate; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CRO, ceftriaxone; CTT, cefotetan; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; TZP, piperacillin-tazobactam.

[b] Ratio of cefotetan to cefotetan-cloxacillin MICs, determined by the AmpC Etest (ratios of ≥8 are considered AmpC-positive results).

[c] Differences between diameters of cefoxitin inhibition zones with and without cloxacillin (increases of ≥4 mm for the cefoxitin-cloxacillin disk are considered AmpC-positive results).

[d] Plasmid-mediated *ampC* was detected by multiplex PCR (29). CIT, plasmid-carried *ampC* originating from *Citrobacter freundii*; DHA, plasmid-carried *ampC* originating from *Morganella morganii.*

[e] For *ampC* promoter/attenuator sequence variants, see Fig. 1.

[f] A final assignment for AmpC activity, combining phenotypic and genetic results, was made according to the definition specified in Materials and Methods. A strain was scored positive when at least one phenotypic test was positive and validated by genetic analysis (plasmid-carried *ampC* or *ampC* promoter mutations associated with *ampC* overexpression).

[g] The zone diameter breakpoints of the 2009 CLSI guidelines were applied (9).

TABLE 3. Genetic characterization of 51 *E. coli* isolates by *ampC* promoter region sequence analysis and multiplex PCR for detection of plasmid-mediated *ampC*

Promoter sequence variant[a]	*E. coli* strain no.	No. of strains (n = 51)	No. (%) of AmpC-positive strains	No. of strains carrying plasmid-mediated *ampC*	Position(s) of mutation(s) in *ampC* promoter/attenuator region[b,c]	Localization and function of mutations[c]
1	1, 2, 3, 4, 5, 6	6	6 (100)		**−42**, −18, (−1), (+58), +81	**Alternate displaced promoter (−35 box** and −10 box) and mutations in the AmpC coding region
2	8	1	1 (100)		**−42**, −18, −15, (−1), (+58), +81	**Alternate displaced promoter (−35 box** and −10 box) and mutations in the AmpC coding region
3	7, 19	2	2 (100)		**−32**, +81	**Promoter mutation** and mutation in the AmpC coding region
4	20	1	1 (100)		**−32**, −28, +17	**Promoter mutation,** mutations in the spacer region and in the AmpC coding region
5	9, 18	2	2 (100)	**1**	**INS (−13.1), INS (−13.2)**	**Increased distance between −35 and −10 boxes**
6	10	1	1 (100)		−14, **INS (−13.1)**, +81	**Increased distance between −35 and −10 boxes,** promoter mutation, and mutation in the *ampC* coding region
7	36, 37, 38, 39, 40, 41, 48, 49	8	None		−28	Mutation in the spacer region
8	23, 35	2	None		−28, (+58)	Mutation in the spacer region
9	46	1	None		−28, +17	Mutation in the spacer region and mutation in the attenuator
10	22, 33, 34	3	None		−28, +34, (+58)	Mutation in the spacer, attenuator mutation, and mutation in the *ampC* coding region
11	16, 17, 30, 31, 32	5	2 (40)	**2**	−18, (−1), (+58), +81	Alternate displaced promoter (−10 box only) and mutation in the *ampC* coding region
12	11, 12, 13, 24	4	3 (75)	**3**	+22, +26, +27, +32, +70, +81	Attenuator mutations and mutations in the *ampC* coding region
13	47	1	None		(+58), +63	Mutation in the *ampC* coding region
14	21, 42, 43, 45, 50, 51	6	1 (17)	**1**	+81	Mutation in the *ampC* coding region
15	14, 15, 25, 26, 27, 28, 29, 44	8	2 (25)	**2**	+70, +81	Mutations in the *ampC* coding region

[a] All isolates with promoter sequence variants 1, 2, 3, 4, 5, and 6 are considered positive for AmpC activity due to chromosomal overexpression of *ampC*. Promoter variants 11, 12, 14, and 15 are found in phenotypically AmpC-positive and AmpC-negative strains; the AmpC-positive strains all harbor plasmid-encoded AmpC. Promoter sequence variants 7, 8, 9, 10, and 13 are not associated with increased phenotypic AmpC activity.

[b] For detailed sequence analysis, see Fig. 1.

[c] Mutations and mechanisms resulting in overexpression leading to *ampC* upregulation are typed in bold and shaded. INS, insertion of nucleotides. Mutations outside functional promoter elements are displayed in parentheses (4).

strains, negative results were obtained in all three phenotypic assays (Table 2). For these strains, the MIC ratios obtained with the AmpC Etest ranged from 1 to 4, and the differences in zone diameters measured in the cefoxitin-cloxacillin disk test were 0 mm (n = 22), 1 mm (n = 4), 2 mm (n = 1), and 3 mm (n = 1) (Table 2). Molecular testing confirmed the results of the concordant phenotypic testing (18/18 positive results and 28/28 negative results).

Discrepant test results were obtained for 5/51 (10%) isolates, i.e., strains 19, 20, 21, 22, and 23. The analysis of the discrepant test results and their resolution are given in detail below.

Strain 19 was positive in the AmpC Etest (MIC ratio = 32), negative in the AmpC cefoxitin-cloxacillin disk diffusion test (difference in zone diameters, 0 mm), and positive in the AmpC cefoxitin-EDTA disk diffusion test. In this strain, the *ampC* promoter/attenuator sequence showed mutations associated with upregulation of chromosomal *ampC* gene expression (sequence variant 3) (Table 3). Strain 20 was negative in the AmpC Etest (MIC ratio = 1) and positive in both AmpC disk diffusion tests; the difference in zone diameters for the AmpC cefoxitin-cloxacillin disk diffusion test was 6 mm. Genetic analysis showed promoter/attenuator mutations associated with chromosomal *ampC* overexpression (variant 4) (Table 3) (7, 17, 40).

Strain 21 was negative in the AmpC Etest (MIC ratio = 1.5), negative in the AmpC cefoxitin-EDTA disk diffusion test, and positive in the AmpC cefoxitin-cloxacillin disk diffusion test (diameter difference, 4 mm). The *ampC* promoter and attenuator region of strain 21 resembles that of the wild-type *E. coli* K-12 strain. The multiplex PCR for plasmid-mediated *ampC* genes was positive for the DHA gene. Induction of the DHA gene in strain 21 was revealed by a disk approximation assay (12) using imipenem as the inducer and ceftazidime, cefoxitin, ceftriaxone, and piperacillin-tazobactam as substrate antibiotics. Nitrocefin hydrolysis assays showed that the beta-lactamase activity of strains 19, 20, and 21 was inhibited by the AmpC inhibitor 3-aminophenylboronic acid (Table 4). Phenotypic assays for ESBL detection (DDS assays) revealed that strains 19 and 20 were ESBL negative and strain 21 was ESBL positive, which was confirmed by the identification of a CTXM-1 gene in strain 21. A non-ESBL TEM-1 beta-lactamase was detected in strains 19 and 21. A corresponding inhibitory effect of the

TABLE 2. Characterization of the 30 *E. coli* strains negative for AmpC activity[a]

Strain	AmpC Etest			AmpC cefoxitin-cloxacillin disk test		AmpC cefoxitin-EDTA disk test result	Plasmid *ampC* PCR result[d]	*ampC* chromosomal sequence analysis result[e] (promoter variant)	Final AmpC result[f]	Antibiotic resistance disk test result (zone of inhibition [mm])[g]						
	Cefotetan susceptibility (MIC [μg/ml])	MIC ratio[b]	AmpC result	Inhibition zone diam difference (mm)[c]	AmpC result					CAZ	CTX	CRO	FEP	AMC	TZP	FOX
22	S (2)	1	Negative	0	Negative	**Positive**	Negative	Negative (10)	Negative	**R (14)**	**R (7)**	**R (7)**	**I (15)**	**R (13)**	S (23)	**I (16)**
23	S (3)	2	Negative	0	Negative	**Positive**	Negative	Negative (8)	Negative	S (30)	S (26)	S (25)	S (29)	**I (16)**	S (22)	**R (12)**
24	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (12)	Negative	S (28)	S (25)	S (29)	S (28)	**I (16)**	S (22)	S (25)
25	S (0.75)	1	Negative	0	Negative	Negative	Negative	Negative (15)	Negative	S (23)	S (28)	S (23)	S (24)	**R (10)**	**R (9)**	S (24)
26	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (15)	Negative	S (20)	S (28)	S (27)	S (28)	**R (10)**	**I (18)**	S (20)
27	S (2)	1	Negative	0	Negative	Negative	Negative	Negative (15)	Negative	S (22)	S (29)	S (26)	S (27)	**R (7)**	**R (7)**	S (20)
28	S (1)	1	Negative	3	Negative	Negative	Negative	Negative (15)	Negative	S (22)	S (25)	S (26)	S (25)	**I (17)**	S (23)	S (27)
29	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (15)	Negative	S (29)	S (28)	S (28)	S (33)	**I (14)**	S (21)	S (24)
30	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (11)	Negative	S (26)	S (28)	S (26)	S (26)	**R (10)**	**I (18)**	S (23)
31	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (11)	Negative	S (28)	S (25)	S (25)	S (24)	**R (7)**	**R (14)**	S (25)
32	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (11)	Negative	S (26)	S (32)	S (28)	S (30)	**R (12)**	**R (14)**	S (30)
33	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (10)	Negative	S (27)	S (23)	S (25)	S (26)	**R (10)**	**R (15)**	S (21)
34	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (10)	Negative	S (28)	S (25)	S (28)	S (28)	**I (15)**	S (23)	S (21)
35	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (8)	Negative	S (26)	**R (7)**	**R (7)**	S (23)	S (19)	**R (7)**	S (24)
36	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	S (20)	**R (7)**	**R (7)**	**I (17)**	**R (10)**	**R (14)**	S (23)
37	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	S (25)	**I (19)**	**I (14)**	S (25)	**I (15)**	**I (20)**	S (24)
38	**I (32)**	1	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	**R (7)**	**R (7)**	**R (7)**	**R (7)**	**R (7)**	**R (7)**	S (18)
39	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	S (24)	**I (19)**	**R (7)**	S (25)	**R (11)**	**I (18)**	S (23)
40	S (0.75)	1	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	S (26)	**I (20)**	**R (7)**	S (26)	**R (13)**	S (22)	S (25)
41	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	S (25)	**R (7)**	**R (7)**	**R (13)**	**I (14)**	**R (7)**	S (28)
42	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (14)	Negative	S (25)	S (25)	S (28)	S (25)	**I (15)**	S (23)	S (22)
43	S (0.5)	1	Negative	0	Negative	Negative	Negative	Negative (14)	Negative	S (27)	S (28)	S (28)	S (26)	**I (14)**	S (23)	S (25)
44	S (0.5)	1	Negative	1	Negative	Negative	Negative	Negative (15)	Negative	S (29)	S (33)	S (26)	S (31)	**R (13)**	S (22)	S (28)
45	S (0.5)	1	Negative	1	Negative	Negative	Negative	Negative (14)	Negative	S (26)	S (28)	S (27)	S (28)	**I (17)**	S (21)	S (21)
46	S (1.5)	1	Negative	1	Negative	Negative	Negative	Negative (9)	Negative	S (21)	S (27)	S (25)	S (25)	**R (10)**	**R (10)**	S (19)
47	S (0.5)	1	Negative	2	Negative	Negative	Negative	Negative (13)	Negative	S (26)	**I (15)**	**I (15)**	S (22)	**I (17)**	S (25)	S (30)
48	S (0.75)	2	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	S (26)	**I (18)**	**I (16)**	S (24)	**I (16)**	**I (18)**	S (25)
49	S (0.75)	2	Negative	0	Negative	Negative	Negative	Negative (7)	Negative	S (25)	**I (20)**	S (25)	**I (15)**	**R (7)**	**R (10)**	S (24)
50	S (0.75)	2	Negative	0	Negative	Negative	Negative	Negative (14)	Negative	S (25)	S (28)	S (26)	S (25)	**I (17)**	**I (20)**	S (18)
51	S (8)	4	Negative	1	Negative	Negative	Negative	Negative (14)	Negative	S (26)	S (27)	S (26)	S (25)	**R (13)**	**I (18)**	S (21)

[a] Strains were analyzed for AmpC production by use of three phenotypic AmpC assays. Genetic analysis of AmpC production was done by multiplex PCR for plasmid *ampC* detection and by sequence analysis of the chromosomal *ampC* promoter/attenuator region. In addition, antimicrobial susceptibility data were generated for each strain. Data for strains with discrepant results in the three phenotypic AmpC confirmation assays are shaded. Positive results and results indicating resistance and intermediate susceptibility are shown in bold. Interpretation of susceptibility results was done according to the 2009 CLSI guidelines. For abbreviations and explanations of the footnotes, see the footnotes in Table 1.

TABLE 4. Characterization of β-lactamase activity in *E. coli* strains ($n = 5$) considered falsely positive or falsely negative in phenotypic screening for AmpC overproduction[d]

E. coli strain[a]	Nitrocefin hydrolysis (OD_{492})[b]							ESBL[c]	
	Without inhibitor	With aminophenylboronic acid	Inhibition	With clavulanic acid	Inhibition	With boronic acid and clavulanic acid	Inhibition	Phenotype	Genetic result
19	0.309 ± 0.006	0.106 ± 0.008	+	0.080 ± 0.010	+	0.041 ± 0.004	+	−	(TEM-1)
20	0.151 ± 0.014	0.006 ± 0.005	+	0.120 ± 0.008	−	0.015 ± 0.006	+	−	−
21	0.344 ± 0.020	0.225 ± 0.002	+	0.083 ± 0.0	+	0.051 ± 0.006	+	+	(TEM-1)/CTXM-1
22	0.777 ± 0.007	0.847 ± 0.015	−	0.332 ± 0.006	+	0.395 ± 0.009	+	+	CTXM-1
23	0.366 ± 0.005	0.314 ± 0.005	−	0.074 ± 0.001	+	0.066 ± 0.007	+	−	(TEM-1)

[a] Strain numbers correspond to those in Tables 1 and 2.
[b] Nitrocefin hydrolysis was tested in the absence and presence of boronic acid (as a specific AmpC inhibitor) and/or clavulanic acid (as a specific ESBL and TEM-1 inhibitor). Presented values are averages and standard deviations for duplicate reactions.
[c] The presence of ESBLs was detected phenotypically by the DDS test as described in Materials and Methods. PCR detection of TEM, SHV, and CTX-M genes was done as described previously (21, 31). Note that TEM-1 is not an ESBL but is able to hydrolyze nitrocefin. Therefore, the detection of TEM-1 is shown in parentheses.
[d] PCR detection of KPC genes was performed as described previously (33), and all strains tested negative.

TEM-1 inhibitor clavulanic acid on hydrolysis of nitrocefin was detected in strains 19 and 21, whereas strain 20 did not show such an inhibitory effect. A KPC PCR was negative for strains 19, 20, and 21 (for a summary of the results, see Table 4). Based on our interpretation criteria and the additional beta-lactamase analyses, strains 19, 20, and 21 were considered true AmpC producers (Table 1).

Two strains (22 and 23) were positive in the AmpC cefoxitin-EDTA disk diffusion test and negative in the AmpC cefoxitin-cloxacillin disk diffusion test and the AmpC Etest. Both strains were negative for plasmid-carried *ampC* genes, and genetic analysis of the *ampC* promoter/attenuator region did not reveal mutations typically associated with chromosomal *ampC* upregulation. Additional beta-lactamase analysis of strains 22 and 23 showed that nitrocefin hydrolysis was not inhibited by the AmpC inhibitor 3-aminophenylboronic acid (Table 4). Phenotypic ESBL testing (DDS assay) revealed that strain 22 was ESBL positive and strain 23 was ESBL negative, which was confirmed by the identification of a CTXM-1 gene in strain 22. A TEM-1 beta-lactamase was detected in strain 23. A corresponding inhibitory effect of clavulanic acid on hydrolysis of nitrocefin was detected in both strains. KPC PCR was negative for strains 22 and 23 (Table 4). Based on our interpretation criteria and the additional beta-lactamase analyses, strains 22 and 23 were considered AmpC negative (Table 2).

In total, 21/51 (41%) *E. coli* strains investigated in this study were considered AmpC producers, and 30/51 (59%) strains were negative for AmpC production (Tables 1 and 2). The AmpC Etest detected 19/21 (90.5%) positive strains and showed no false-positive results. The AmpC cefoxitin-cloxacillin disk test was correctly positive for 20/21 (95.2%) AmpC-positive strains and did not give false-positive results. The AmpC cefoxitin-EDTA disk test was correctly positive for 20/21 (95.2%) strains and gave two false-positive results.

***ampC* promoter/attenuator mutations and plasmid-encoded AmpC beta-lactamases.** In the 51 *E. coli* strains, 15 different *ampC* promoter/attenuator sequence variants were detected (Fig. 1). For the 21 AmpC-positive strains, 10 different promoter/attenuator sequence variants (1, 2, 3, 4, 5, 6, 11, 12, 14, and 15) were found (Table 3). Promoter/attenuator sequence variants 1, 2, 3, 4, 5, and 6 were found in 11 strains with a positive AmpC production phenotype and a negative result for plasmid-carried *ampC*. Sequence variant 5 comprised two AmpC-positive strains, one positive for plasmid-carried *ampC* genes and one negative for plasmid-carried *ampC*. Mutations detected in sequence variants 1, 2, 3, 4, 5, and 6 in-

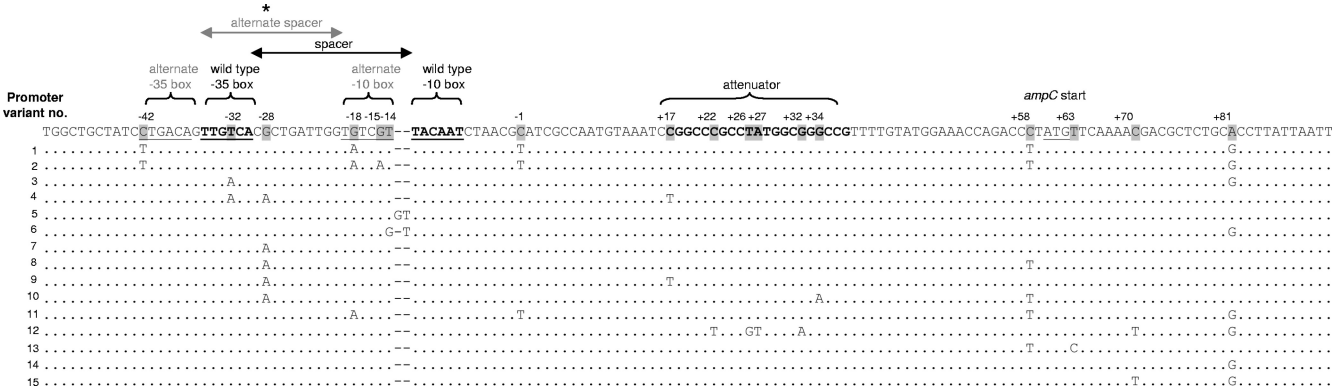

FIG. 1. Alignment of the chromosomal *ampC* promoter, attenuator, and 5′-end regions. For the 51 *E. coli* isolates, 15 different sequence variants were identified. *, chromosomal *ampC* sequence variant classifications and descriptions of functional elements are used as reported in the work of Tracz et al. (40).

cluded (i) mutations that created an alternate displaced promoter (variants 1 and 2), (ii) mutations in the wild-type promoter/attenuator (variants 3 and 4), and (iii) mutations that increased the spacer length between the −35 and −10 boxes (variants 5 and 6) by insertion of 1 or 2 base pairs. The mutations found in these sequence variants are associated with an increase of *ampC* expression (11). Sequence variants 11, 12, 14, and 15 were found in phenotypically AmpC-positive strains which were positive for the presence of plasmid-carried *ampC* genes. Mutations in these sequence variants were located in the attenuator region or coding region for AmpC or resulted in an alternate displaced −10 box. None of these changes has been reported to be associated with significant chromosomal AmpC overproduction (see below). Variant 14 resembled the wild-type *E. coli* K-12 *ampC* promoter/attenuator (Table 3). In total, 13/21 AmpC-positive strains harbored changes in the promoter/attenuator region typically associated with chromosomal AmpC overproduction (32).

Plasmid-carried *ampC* genes were detected in 9 of the 21 strains. In one strain, both chromosome- and plasmid-mediated mechanisms responsible for AmpC production were found, e.g., a 2-bp insertion in the spacer of the *ampC* chromosomal promoter/attenuator region and a plasmid-carried *ampC* gene (Table 3). The plasmid-carried *ampC* genes found in the isolates belonged to the CIT family ($n = 8$) and the DHA family ($n = 1$). Sequences of the PCR products showed 100% homology to the $bla_{\text{CMY-2}}$ gene for the CIT family isolates and 100% homology to the $bla_{\text{DHA-1}}$ gene for the DHA family isolate (data not shown).

Susceptibility to amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoxitin, and extended-spectrum cephalosporins. All 21 AmpC-positive strains showed reduced susceptibility to amoxicillin-clavulanic acid. Twenty of 21 strains tested were resistant, and 1 strain was intermediate. In contrast, only 1/21 strains was resistant to piperacillin-tazobactam, 6/21 isolates showed an intermediate level, and 14/21 strains were susceptible.

For extended-spectrum cephalosporins, the following test results were obtained for the 21 AmpC-positive strains, applying the 2009 CLSI guideline (9) zone diameter breakpoints: for ceftazidime, 11 strains were susceptible, 3 strains were intermediate, and 7 strains were resistant; for cefotaxime, 9 strains were susceptible, 7 strains were intermediate, and 5 strains were resistant; and for ceftriaxone, 12 strains were susceptible, 4 strains were intermediate, and 5 strains were resistant. All AmpC-positive strains were susceptible to cefepime (Table 1). Resistance patterns for AmpC-negative strains are summarized in Table 2. In 2010, the CLSI zone diameter breakpoints for ceftazidime, cefotaxime, and ceftriaxone were elevated (10). Applying these breakpoints resulted in the following interpretation of susceptibility testing for AmpC-positive strains: for ceftazidime, 5 strains were susceptible, 6 strains were intermediate, and 10 strains were resistant; for cefotaxime, 2 strains were susceptible, 7 strains were intermediate, and 12 strains were resistant; and for ceftriaxone, 9 strains were susceptible, 3 strains were intermediate, and 9 strains were resistant (see Table S1A in the supplemental material). Results for the AmpC-negative strains are summarized in Table S1B in the supplemental material.

By disk diffusion susceptibility testing and according to 2009 CLSI guidelines, 17/21 (81%) AmpC-producing strains were resistant to cefoxitin, 3/21 strains were intermediate, and 1/21 strains was susceptible (inhibition zone diameter, 18 mm) (Table 1). A total of 28/30 AmpC-negative strains (93%) were susceptible to cefoxitin, for 1/30 strains an intermediate result was obtained, and 1/30 strains was resistant (Table 2).

DISCUSSION

Detection of AmpC beta-lactamases in *E. coli* poses a challenge to microbiological laboratories. For practical reasons, it is not feasible to routinely test all *E. coli* isolates for AmpC production in detail. In our study, we selected 51 *E. coli* clinical isolates collected during a 2-year period for putative AmpC production based on reduced susceptibility to amoxicillin-clavulanic acid, piperacillin-tazobactam, or oxyimino-cephalosporins (ceftriaxone, ceftazidime, and cefotaxime).

Several AmpC confirmation tests have recently been evaluated (4, 36) or become commercially available (AmpC Etest; AB bioMérieux). In this study, we compared the performances of three of these tests for accurate identification of AmpC-producing *E. coli* strains: the AmpC Etest (AB bioMérieux, Sweden), the AmpC cefoxitin-cloxacillin disk test (36), and the AmpC cefoxitin-EDTA disk test (4). AmpC-producing *E. coli* strains were validated by genetic analyses. In addition, strains with discrepant AmpC screening results were analyzed further for beta-lactamase production by nitrocefin hydrolysis assays, ESBL phenotypic testing, and genetic testing for the presence of SHV, TEM, CTX-M, and KPC beta-lactamases (Table 4). The additional test results confirmed the accuracy of our interpretation criteria for AmpC production. In total, 21 of the selected 51 *E. coli* isolates were identified as true AmpC-producing strains (for plasmidic *ampC*, $n = 8$; for overexpression of chromosomal *ampC*, $n = 12$; and for a combination of plasmidic *ampC* and overexpression of chromosomal *ampC*, $n = 1$). We found that the cefoxitin-cloxacillin disk test detected 20/21 AmpC-positive *E. coli* strains (Table 1) and gave 1 false-negative result. The cefoxitin-EDTA disk test (4) detected 20/21 AmpC-positive strains and gave 1 false-negative result and 2 false-positive results. A drawback of the cefoxitin-EDTA disk assay is that carbapenemases may give rise to false-positive results, because carbapenemases are able to inactivate cefoxitin (4), although KPC was not detected in the 2 false-positive strains. The AmpC Etest strip uses cefotetan for AmpC screening. While cefotetan resistance (MIC of >64 mg/liter) was not consistently present in the AmpC-positive strains (Table 1), the AmpC Etest was able to detect 19/21 positive strains, and 2 strains gave a false-negative test result.

The *ampC* promoter/attenuator mutations detected in the 51 *E. coli* isolates (Table 3) included 6 previously described variants associated with *ampC* overexpression (7, 17, 18, 24, 34, 40). Overall, 13/21 (61.9%) positive AmpC strains were associated with chromosomal *ampC* promoter mutations resulting in hyperproduction of AmpC, and 9/21 strains (42.9%) were AmpC positive due to the presence of plasmid-carried *ampC* genes. One strain had a 2-bp insertion in the *ampC* promoter spacer region (variant 5) and a plasmid-carried *ampC* gene; both mechanisms may have contributed to AmpC activity in this strain. The observed ratio of AmpC production due to chromosomal *ampC* upregulation versus plasmid-mediated AmpC is in accordance with the distribution observed in stud-

ies conducted in France, Spain, and Norway (6, 14, 20). We did not detect any strain that was positive in the genetic analysis and negative in all three phenotypic confirmation tests.

Because the cefoxitin- and cefotetan-based AmpC disk assays effectively identify AmpC producers, we decided to evaluate whether cefoxitin and cefotetan susceptibility testing can be used as a screening test for AmpC production. In the group of AmpC-positive strains, 20 of 21 (95%) isolates were resistant or intermediate in the cefoxitin disk test. One strain showed an inhibition zone of 18 mm, which is just within the susceptible range (Table 1). For the AmpC-negative strains, 26 of 30 (87%) strains were susceptible to cefoxitin, with inhibition zones of >18 mm. Two strains scored within the susceptible range, with inhibition zones of 18 mm, one strain was intermediate, and one strain was resistant to cefoxitin (Table 2). Applying a screening criterion of a cefoxitin inhibition zone of ≤18 mm, all AmpC-positive strains would have been detected, plus an additional 4 false-positive results. However, the use of cefoxitin as a screening marker is compromised by isolates producing plasmid-encoded AmpC beta-lactamases of the ACC family. ACC-1 itself is inhibited by cefoxitin, and thus strains carrying it may appear cefoxitin susceptible (1, 16, 32). ACC-1 was first isolated in Germany and in several other European countries (1, 25, 30, 32). Recently, the AmpC beta-lactamase ACC-4 was identified in *E. coli*, conferring increased MICs for oxyimino-cephalosporins, with low MICs for cefoxitin and cefepime (28). In our study, strains with a plasmid ACC beta-lactamase gene were not detected.

Analyzing cefotetan MICs for the AmpC Etest revealed that 2 of the 21 (10%) AmpC-positive strains were resistant to cefotetan. Ten of 21 (48%) AmpC-positive strains were susceptible to cefotetan, and intermediate results were obtained for 9 (42%) isolates (Table 1). On the basis of these results, we cannot recommend cefotetan susceptibility testing for initial AmpC screening.

We also evaluated whether reduced sensitivity to extended-spectrum cephalosporins can be used as a screening parameter for AmpC testing. Several studies showed that cephalosporin susceptibility screening of *E. coli* isolates with the initial purpose of ESBL identification resulted in selection for AmpC-producing strains (3, 23). Nine of the 21 (43%) AmpC-positive strains were susceptible to ceftazidime, cefotaxime, and ceftriaxone *in vitro* according to the CLSI 2009 guidelines. Another two strains were susceptible to ceftazidime and ceftriaxone, and one strain was susceptible to ceftriaxone only (Table 1). Applying the elevated 2010 CLSI zone diameter breakpoints, two strains were susceptible to ceftazidime, cefotaxime, and ceftriaxone, another two strains were susceptible to ceftazidime and ceftriaxone, one strain was susceptible to ceftazidime only, and five strains were susceptible to ceftriaxone only (see Table S1A in the supplemental material). On the basis of our results, we cannot recommend extended-spectrum cephalosporins as screening parameters for AmpC.

In summary, we demonstrate that after a first screening procedure, each of the three phenotypic AmpC tests used in this study was capable of confirming the majority of AmpC beta-lactamase-producing *E. coli* strains (>90%), including those producing plasmid-mediated AmpC beta-lactamases and chromosomal AmpC hyperproduction strains. Each of the three tests is an acceptable phenotypic confirmation tool when AmpC production in *E. coli* is suspected.

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