Detection of AmpC beta-lactamase in Escherichia coli: comparison of three phenotypic confirmation assays and genetic analysis

Peter-Getzlaff, S ; Polsfuss, S ; Poledica, M ; Hombach, M ; Giger, J ; Böttger, E C ; Zbinden, R ; Bloemberg, G V

DOI: https://doi.org/10.1128/JCM.00091-11

JCM Accepts, published online ahead of print on 8 June 2011
J. Clin. Microbiol. doi:10.1128/JCM.00091-11

Detection of AmpC beta-lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis

S. Peter-Getzlaff[1], S. Polsfuss[1], M. Poledica[1,2], M. Hombach[1], J. Giger[1], E. C. Böttger[1], R. Zbinden[1] and G.V. Bloemberg[1*]

Affiliations:

[1] University of Zurich, Institute of Medical Microbiology, Gloriastrasse 30/32, CH-8006 Zurich, Switzerland

[2] Present address: Unilabs Zurich, Dufourstrasse 90, CH-8034 Zurich, Switzerland

*** Corresponding author:**

Phone 41 44 634 2660 Fax 41 44 634 4906

Email bloemberg@imm.uzh.ch

Mailing address: University of Zurich, Institute of Medical Microbiology, Gloriastrasse 30/32, CH 8006 Zurich, Switzerland

Running title: Detection of AmpC beta-lactamases in *Escherichia coli*

Keywords: AmpC beta-lactamase , *ampC* promoter, *Escherichia coli*

Abstract

Two mechanisms account for AmpC activity in *E. coli:* mutations in the *ampC* promoter and attenuator regions resulting in *ampC* overexpression and/or acquisition of plasmid-encoded *ampC* genes. In this study we analyzed 51 clinical *E. coli* isolates with reduced susceptibility to amoxicillin/clavulanic acid, piperacillin/tazobactam or third generation cephalosporins for the presence of AmpC production. Three phenotypic AmpC confirmation assays (cefoxitin/cloxacillin disk diffusion test, cefoxitin/EDTA disk diffusion test, AmpC ETest) were compared for the detection of AmpC activity. All 51 isolates were genetically characterized by mutation analysis of the chromosomal *ampC* promoter/attenuator region and by PCR detection of plasmid-encoded *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, plasmid-encoded *ampC* genes were detected in 8/21 isolates. 1/21 strains contained both, *ampC* promoter mutations and a plasmid-encoded *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 third generation cephalosporines, e.g. ceftriaxone, cetazidime and cefotaxime, was found in 9 of the 21 AmpC positive strains. When considering the elevated zone diameter breakpoints of the 2010 CLSI guidelines 2/21 AmpC positive strains were categorized susceptible to third generation cephalosporines.

Introduction

The prevalence of multidrug resistant Gram negative bacteria has continuously increased over the past 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, ceftazidime), cephamycins (e.g. cofexitin, cefotetan) and monobactams (15). Cloxacillin and 3-aminophenylboronic acid inhibit AmpC beta-lactamases (2, 15, 36), while AmpC beta-lactamase activity is not affected by the ESBL inhibitor clavulanic acid. In Gram negative bacteria AmpC beta-lactamase production is chromosomally- or plasmid-mediated. Chromosomal *ampC* genes are constitutively expressed at a low level. Some *Enterobaceriaceae* such as *Enterobacter* spp., *Citrobacter* spp. and *Serratia* spp.,, encode an inducible *ampC*. 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 (15). The regulation of chromosomal *ampC* expression in *E. coli* differs considerably from that in other *Enterobacteriaceae*. *E. coli* lacks *ampR* and thus *ampC* expression is not inducible (14). In *E. coli*, *ampC* is constitutively expressed at a low level (16). Various mutations in the *ampC* promoter/attenuator region of *E. coli* have been identified that result in constitutive overexpression (7, 8, 18, 22, 24, 34, 39, 40). In addition to chromosomal *ampC*, *E. coli* may carry plasmids encoding *ampC* (pAmpC), transferred via horizontal gene transfer and derived from the chromosomal *ampC*'s of other *Enterobacteriaceae* spp. (15). Plasmid based *ampC* genes are in most of the cases constitutively expressed. However, some plasmid-encoded *ampCs*, such as DHA-1, are inducible by β-lactams, with expression similarly regulated as inducible chromosomal *ampC*s. All plasmid-encoded *ampC* s 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 third generation cephalosporins when initially tested (27, 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 outcome of patients with bloodstream infection caused by plasmid-mediated AmpC producing *Klebsiella pneumoniae* and show high rates of treatment failure when cephalosporins were administered (27).

Different phenotypic AmpC confirmation tests have been reported in literature (15). A recently described disk diffusion test is based on the comparison of the zone diameters around a cefoxitin disk with 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 when investigating 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 specificity of 98% when testing 140 isolates of *Klebsiella* spp., *Proteus mirabilis* and *Salmonella* sp. (4). In this

study we aimed to evaluate and compare the diagnostic performance 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 a suspicion of AmpC production. Molecular analyses were used to asses the specificity of the phenotypic assays and to characterize the genetic basis for AmpC (over)production in these strains.

Methods

Clinical isolates.

51 *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 over a period of two years from July 2006 until July 2008. The strains were isolated from urine (n=12), blood culture (n=12), respiratory specimens (n=8), perianal swabs (n=4), wound swabs (n=4), inguinal swabs (n=3), abscesses (n=2), tissue (n=2), vaginal swab (n=1), gastric aspirate (n=1), cerebrospinal fluid (CSF) (n=1) and unknown origin (n=1).

Antibiotic susceptibility testing.

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

Phenotypic AmpC and ESBL activity testings

The AmpC ETest (AB bioMérieux, Sweden) for cefotetan susceptibility was performed according 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 which are ≥ 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 Müller-Hinton agar. The diameters of the cefoxitin inhibition zones with and without cloxacillin were compared; if the difference in inhibition was ≥ 4 mm for the cefoxitin/cloxacillin disk 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 Müller-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) containing each 20 µl of a 1:1 mixture of saline and 100X Tris-EDTA solution. Colonies of the test strain were applied to disk A, colonies of the cefoxitin susceptible *E. coli* strain ATCC 25922 (as 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 induction of the DHA plasmid-encoded AmpC, a disk approximation assay using imipenem as an inducer and ceftazidime, cefoxitin, ceftriaxone and piperacillin/tazobactam as substrate antibiotics was used (12).

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

corresponding third generation cephalosporin alone was considered indicative for ESBL production.

Beta-lactamase hydrolysis assays

For phenotypic detection of beta-lactamase activity the chromogenic nitrocefin (Calbiochem, San Diego, USA) was used (35). 3-aminophenylboronic acid (Sigma-Aldrich Chemie, GmbH, Zug, Switzerland) was used as specific AmpC inhibitor (2) and clavulanic acid (Sigma-Aldrich Chemie, GmbH, Zug, Switzerland) was used as inhibitor of Ambler class A β-lactamases (e.g. ESBL, TEM-1) (5). A bacterial suspension McFarland standard 0.5 in 0.45% NaCl was prepared from overnight cultures. *E. coli* strain DH5α was used as negative control strain. Reaction mixtures consisted of 50 µl bacterial cell suspension, 25 µl nitrocefin (0.5 mg/ml 10 mM phosphate buffer pH 6.8), 25 µl 3-aminophenylboronic acid (3.6 mg/ml 10 mM phosphate buffer pH 6.8) and/or 25 µl potassium clavulanate (2.2 mg/ml 10 mM phosphate buffer pH 6.8). In case 3-aminophenylboronic acid and/or clavulanic acid were not added, the end volume of 125 µl was reached by adding 10 mM phosphate buffer pH 6.8. Reaction mixtures were incubated in micro titerplates at 37 ^{o}C. The nitrocefin hydrolyis product was detected by quantifying the absorption after 8 hours at OD_{492nm} using a titerplate photospectrometer (Biochrom Asys Expert Plus Microplate Reader, Biochrom Ltd, Cambridge, UK)

***ampC* promoter/attenuator sequencing.** DNA was extracted from colonies grown on agar medium using the InstaGene Matrix (Bio-Rad, Switzerland) following the manufacturer's instructions. For the *ampC* promoter/attenuator mutation analysis a 271-bp fragment was amplified using primers AB1(5'-GATCGTTCTGCCGCTGTG-3') and ampC2 (5'-

GGGCAGCAAATGTGGAGCAA-3') (11). PCR amplicons were purified with the QIAquick PCR purification kit (Qiagen, Switzerland) followed by cycle sequencing using the BigDye reagent kit (Applied Biosystems, Switzerland). Sequence analysis was performed on an ABI Prisma 3100 DNA sequencer (Applied Biosystems, Switzerland) following standard protocols. Sequences were analyzed and edited using Lasergene 7 MegAlign software (DNASTAR Inc., USA). The *ampC* promoter/attenuator sequences were compared to the *ampC* wild-type sequence of *E.coli* strain ATCC 25922.

Molecular detection of plasmid-encoded *ampC* beta-lactamase genes.

A multiplex PCR was used for the detection of plasmid-encoded *ampC* beta-lactamase genes (29). This assay is able to detect the six plasmid-encoded *ampC* genes 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 (National Center for Biotechnology Information (GenBank),http://www.ncbi.nlm.nih.gov/).

Detection of ESBL and *Klebsiella* carbapenamase (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., USA). The TEM beta-lactamases sequences were compared to wt *E. coli* AF427133.1 TEM-1 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.,* 2004. For detection of a *Klebsiella* carbapenamase (KPC) gene 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, AmpC cefoxitin/cloxacillin disk test) validated by genetic analysis (presence of plasmid-encoded *ampC* genes, *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 β-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 %) of the *E. coli* isolates all three phenotypic tests gave a positive result for AmpC production (Table 1). The ratios of 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 %) of the *E. coli* strains negative results were obtained in all three phenotypic assays (Table 2). In these strains the ratio of the AmpC ETest ranged from 1 to 4 and the differences in zone diameters of the cefoxitin/cloxacillin disk test measured 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 positives and 28/28 negatives).

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

Strain no. 19 was positive in the AmpC ETest (ratio=32), negative in the AmpC cefoxitin/cloxacillin disk diffusion test (difference in zone diameter 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 the chromosomal *ampC* gene expression (sequence variant 3; Table 3). Strain no. 20 was negative in the AmpC ETest (ratio=1) and positive in both AmpC disk diffusion tests, the difference in zone diameter 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, 16, 40).

Strain no. 21 was negative in the AmpC ETest (ratio=1.5), negative in the AmpC cefoxitin /EDTA disk diffusion test, but positive in the AmpC cefoxitin/cloxacillin disk diffusion test (diameter

difference 4 mm). The *ampC* promoter and attenuator region of strain no. 21 resembles that of the wildtype *E. coli* K12 strain. The multiplex PCR for plasmid-meditated *ampC* genes was positive for DHA. Induction of the DHA gene in strain no. 21 was revealed by a disk approximation assay (12) using imipenem as inducer and ceftazidime, cefoxitin, ceftriaxone and piperacillin/tazobactam as substrate antibiotics. Nitrocefin hydrolysis assays showed that β-lactamase activity of strains no. 19, 20 and 21 was inhibited by the AmpC inhibitor 3-aminophenylboronic acid (Table 4). Phenotypic assays for ESBL detection (DDS assay) revealed that strains no. 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 β-lactamase was detected in strain no. 19 and 21. A corresponding inhibitory effect of the TEM-1 inhibitor clavulanic acid on hydrolysis of nitrocefin was detected in strains no. 19 and 21, whereas strain 20 did not show such an inhibitory effect. A KPC PCR was negative for strains no. 19, 20 and 21. (for a summary of the results see Table 4). Based on our interpretation criteria and the additional β-lactamase analyses strains no. 19, 20 and 21 were considered true AmpC producers (Table 1).

Two strains (no. 22 and 23) were positive in the AmpC cefoxitin /EDTA disk diffusion test and negative in AmpC cefoxitin/cloxacillin disk diffusion test and the AmpC ETest. Both strains were negative for plasmid-encoded *ampC* genes and genetic analysis of the *ampC* promoter/attenuator region did not reveal mutations typically associated with chromosomal *ampC* up regulation. Additional β-lactamase analysis for strains no. 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 strains no. 22 was ESBL positive and no. 23

was ESBL negative, which was confirmed by the identification of a CTXM-1 gene in strain 22. A TEM-1 β-lactamase was detected in strain no. 23. A corresponding inhibitory effect of clavulanic acid on hydrolysis of nitrocefin was detected in both strains. KPC PCR was negative for strains no. 22 and 23 (see Table 4). Based on our interpretation criteria and the additional β-lactamase analyses, strains no. 22 and 23 were considered AmpC negative (Table 2).

In total, 21/51 (41 %) of the *E. coli* strains investigated in this study were considered AmpC producers and 30/51 (59 %) were negative for AmpC production (Table 1 and Table 2). The AmpC ETest detected 19/21 (90.5 %) correctly positive and showed no false positive results. The AmpC cefoxitin/cloxacillin disk test was correctly positive in 20/21 (95.2%) AmpC positive strains and did not result in false positive results. The AmpC cefoxitin /EDTA disk test was correctly positive in 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 (Figure 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, 5, 6 were found in 11 strains with a positive AmpC production phenotype and negative for plasmid-encoded *ampC.* Sequence variant 5 comprises two AmpC positive strains, one positive for plasmid-encoded *ampC* genes and one negative strain for plasmid-encoded *ampC*. Mutations detected in sequence variants 1, 2, 3, 4, 5 and 6 included (i) mutations that created an alternate displaced promoter (variants 1 and 2), (ii) mutations in the wildtype promoter/attenuator (variants 3 and 4) and (iii) mutations that increased the spacer length between the -35 and -10 box (variants 5 and 6) by insertion of 1 or 2 basepairs. 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 phenotypic AmpC positive strains, which were all positive for the presence of plasmid-encoded *ampC* genes. Mutations in these sequence variants were located in the attenuator region, the coding region of 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 no. 14 resembles the wildtype *E. coli* K12 *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-encoded *ampC* genes were detected in 9 of the 21 strains. In 1 strain both chromosomal and plasmid-mediated mechanisms responsible for AmpC production were found., e.g. a two bp insertion in the spacer of the *ampC* chromosomal promoter/attenuator region and a plasmid encoded *ampC* gene (Table 3). The plasmid-encoded *ampC* genes found in the isolates belonged to the CIT family (n=8) and the DHA family (n=1). Sequences of the PCR product showed 100% homology to the bla_{CMY-2} gene for the CIT family isolates and 100% homology to the bla_{DHA-1} gene for the DHA family isolate (data not shown).

Susceptibility to amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin and third-generation cephalosporins.

All 21 AmpC positive strains showed reduced susceptibility to amoxicillin/clavulanic acid. 20 of 21 strains were tested resistant, one 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 tested susceptible.

For third-generation cephalosporins the following test results were obtained for the 21 AmpC positive strains applying the 2009 CLSI guideline (9) zone diameter breakpoints: ceftazidime (11 strains susceptible, 3 strains intermediate and 7 strains resistant), cefotaxime (9 strains susceptible, 7 strains intermediate and 5 strains resistant) and ceftriaxone (12 strains susceptible, 4 strains intermediate and 5 strains 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: ceftazidime (susceptible 5 strains, intermediate 6 strains and resistant 10 strains), cefotaxime (susceptible 2 strains, intermediate 7 strains and resistant 12 strains) and ceftriaxone (susceptible 9 strains, intermediate 3 strains and resistant 9 strains) (supplemental material Table 1A). Results for the AmpC negative strains are summarized in supplemental material Table 1B.

By disk diffusion susceptibility testing and according to CLSI guidelines 2009, 17/21 AmpC producing strains (81 %) were resistant to cefoxitin, 3/21 strains were intermediate and 1/21 strain was susceptible (inhibition zone diameter 18 mm) (Table 1). 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 two-year period for putative AmpC production based on reduced susceptibility to amoxicillin/clavulanic acid, piperacillin/tazobactam or oxyimino-cephalosporins (ceftriaxone, ceftazidime, cefotaxime).

Several AmpC confirmation tests have recently been evaluated (4, 36) or became commercially available (AmpC Etest, AB bioMérieux). In this study we compared the performance of three of these tests for accurate identification of AmpC producing *E. coli* strains, i.e. AmpC ETest (AB bioMérieux, Sweden), AmpC cefoxitin /cloxacillin disk test (36), and 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 further analyzed for β-lactamase production by nitrocefin hydrolysis assays, ESBL phenotypic testing, genetic testing for the presence of SHV, TEM, CTX-M β-lactamases and KPC (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 (plasmidic *ampC* n=8, overexpression of chromosomal *ampC* n=12, 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 20/21AmpC 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 as carbapenamases 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 (R > 64 mg/l) was not consistently present in the AmpC positive strains (Table 1), the AmpC Etest was able to detect 19/21 strains correctly positive, 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 (references 7, 16, 17, 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-encoded *ampC* genes. One strain had a two basepair insertion in the *ampC* promoter spacer region (variant 5) and a plasmid-encoded *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 studies conducted in France, Spain and Norway (6, 13, 20). We did not detect any strain that was positive in the genetic analysis and negative in all three phenotypic confirmation tests.

As 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 > 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 criterium of cefoxitin inhibition zone ≤ 18 mm, all AmpC positive strains would have been detected plus an additional 4 false positives. However, the use of cefoxitin as 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 may appear cefoxitin susceptible (1, 15, 32). ACC-1 has first been isolated in Germany and in several other European countries. (1, 25, 31, 32). Recently, AmpC betalactamase 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-lactamases gene were not detected.

Analyzing cefotetan MICs in the AmpC ETest revealed that 2 of the 21 (10%) AmpC positive strains were resistant to cefotetan. 10 of 21 (48%) of the 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 can not recommend cefotetan susceptibility for initial AmpC screening.

We also evaluated whether reduced sensitivity to third generation cephalosporins can be used as screening parameter for AmpC testing. Several studies showed that cephalosporine 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 CLSI 2010 zone diameter breakpoints two strains were susceptible to ceftazidime, cefotaxim and ceftriaxone; another two strains were susceptible to ceftazidime and ceftriaxone, one strain was susceptible to

354 ceftazidime only and five strains were susceptible to ceftriaxone only (supplemental material
355 Table 1A). On the basis of our results we cannot recommend third generation cephalosporins as
356 screening parameters for AmpC .
357 In summary, we demonstrate that after a first screening procedure each of the three
358 phenotypic AmpC tests used in this study was capable to confirm the majority of AmpC beta-
359 lactamase producing *E. coli* strains (>90 %) including plasmid-mediated AmpC beta-lactamases
360 and chromosomal AmpC hyperproduction strains. Each of the three tests is an acceptable
361 phenotypic confirmation tool when AmpC production is suspected in *E. coli*.

Acknowledgements

This study was supported in part by the University of Zurich. The authors have no conflict of interest to disclose.

365 **References**

366 1. **Bauernfeind, A., I. Schneider, R. Jungwirth, H. Sahly, and U. Ullmann.** 1999. A novel
367 type of AmpC beta Lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain
368 causing nosocomial pneumonia. Antimicrob. Agents Chemother. **43**:1924-1931
369
370 2. Beesley, T.**, N. Gascoyne, V. Knott-Hunziker,S. Petursson, S. G. Walex, B. Jaurin, .**
371 **Grundström.** 1983. The inhibition of class C β-lactamases by boronic acids. Biochem. J.
372 **209:**229-233.
373
374 3. **Bell, J. M., M. Chitsaz, J. D. Turnidge, M. Barton, L. J. Walters, and R. N. Jones.** 2007.
375 Prevalence and significance of a negative extended-spectrum β-lactamase (ESBL)
376 confirmation test result after a positive ESBL test result for isolates of *Escherichia coli*
377 and *Klebsiella pneumoniae*: results from the SENTRY Asia-Pacific Surveillance Program. J.
378 Clin. Microbiol. **45**:1478-1482.
379
380 4. **Black, J. A., E. S. Moland, and K. S. Thomson.** 2005. AmpC disk test for detection of
381 plasmid-mediated AmpC beta-lactamases in *Enterobacteriaceae* lacking chromosomal
382 AmpC beta-lactamases. J. Clin. Microbiol. **43:**3110-3113.
383
384 5. **Bradford, P.A.** 2001 Extended-spectrum beta-lactamases in the 21st century:
385 characterization, epidemiology, and detection of this important resistance threat. Clin
386 Microbiol Rev 14:933-51

6. **Briñas L.**, M. Lantero, I. de Diego, M. Alvarez, M. Zarazaga, and C. Torres. 2005. Mechanisms of resistance to expanded-spectrum cephalosporins in *Escherichia coli* isolates recovered in a Spanish hospital. J. Antimicrob. Chemother. **56**:1107-1110.

7. **Caroff, N., E. Espaze, I. Bérard, H. Richet, and A. Reynaud.** 1999. Mutations in the ampC promoter of *Escherichia coli* isolates resistant to oxyiminocephalosporins without extended spectrum beta-lactamase production. FEMS Microbiol. Lett. **173:**459-465.

8. **Caroff, N., E. Espaze, D. Gautreau, H. Richet, and A. Reynaud.** 2000. Analysis of the effects of −42 and −32 *ampC* promoter mutations in clinical isolates of *Escherichia coli* hyperproducing AmpC. J. Antimicrob. Chemother. **45:**783-788.

9. **Clinical and Laboratory Standards Institute. 2009**. Performance standards for antimicrobial susceptibility testing; Nineteenth informational supplement M100-S19, Clinical and Laboratory Standards Institute, Wayne, PA.

10. **Clinical and Laboratory Standards Institute. 2010**. Performance standards for antimicrobial susceptibility testing; Twentieth informational supplement M100-S20, Clinical and Laboratory Standards Institute, Wayne, PA.

11. **Corvec, S., A. Prodhomme, C. Giraudeau, S. Dauvergne, A. Reynaud, and N. Caroff.** 2007. Most *Escherichia coli* strains overproducing chromosomal AmpC beta-lactamase belong to phylogenetic group A. J. Antimicrob. Chemother. **60:**872-876.

12. **Dunne, W. M. Jr, and Hardin D.J.** 2005 Use of several inducer and substrate antibiotic combinations in a disk approximation assay format to screen for AmpC induction in patient isolates of *Pseudomonas aeruginosa*, *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. J Clin Microbiol. **43:**5945-5949.

13. Haldorsen B., B. Aasnaes, K. H. Dahl, A. M. Hanssen, G. S. Simonsen, T. R. Walsh, A. Sundsfjord, and E. W. Lundblad. 2008. The AmpC phenotype in Norwegian clinical isolates of *Escherichia coli* is associated with an acquired IS*Ecp1*-like *ampC* element or hyperproduction of the endogenous AmpC. J. Antimicrob. Chemother. **62:**694-702.

14. **Honoré, N., M. H. Nicolas, and S. T. Cole.** 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. EMBO J. **5:**3709-3714.

15. **Jacoby, G. A.** 2009. AmpC β-lactamases. Clin. Microbiol. Rev. **22:**161-182.

16. **Jaurin, B., T. Grundström, T. Edlund, and S. Normark**. 1981. The *E. coli* β-lactamase attenuator mediates growth rate-dependent regulation. Nature. **290:**221-225.

17. **Jørgensen, R. L., J. B. Nielsen, A. Friis-Møller, H. Fjeldsøe-Nielsen, and K. Schønning.** 2010. Prevalence and molecular characterization of clinical isolates of *Escherichia coli* expressing an AmpC phenotype. J Antimicrob. Chemother. **65**:460-4.

18. **Forward, K. R., B. M. Willey, D. E. Low, A. McGeer, M. A. Kapala, M. M. Kapala, and L. L. Burrows.** 2001. Molecular mechanisms of cefoxitin resistance in *Escherichia coli* from the Toronto area hospitals. Diagn. Microbiol. Infect. Dis. **41:**57-63.

19. **Mammeri, H., Guillon, H., Eb, F., and P. Nordmann.** 2010. Phenotypic and biochemical comparison of the carbapenem-hydrolyzing activities of five plasmid-borne AmpC beta-lactamases. Antimicrob. Agents Chemother. **54:**4556-4560.

20. **Mammeri, H., F. Eb, A. Berkani, and P. Nordmann.** 2008. Molecular characterization of AmpC-producing *Escherichia coli* clinical isolates recovered in a French hospital. J. Antimicrob. Chemother. **61:**498-503.

21. **Moland, E.S. , Hanson, N.D. , Black, J.A., Hossain A., Song, W., Thomson, K.S.** 2006 Prevalence of newer beta-lactamases in gram-negative clinical isolates collected in the United States from 2001 to 2002. J. Clin. Microbiol. 44:3318-24.

22. **Mulvey, M. R., E. Bryce, D. A. Boyd, M. Ofner-Agostini, A. M. Land, A. E. Simor, and S. Paton.** 2005. Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. Antimicrob. Agents Chemother. **49:**358-365.

23. **Munier, G. K., C. L. Johnson, J. W. Snyder, E. S. Moland, N. D. Hanson, and K. S. Thomson.** 2010. Positive extended-spectrum β-lactamase (ESBL) screening results may be due to AmpC β-lactamases more often than to ESBLs. J. Clin. Microbiol. **48:**673-674.

24. **Nelson, E. C., and B. G. Elisha.** 1999. Molecular basis of AmpC hyperproduction in clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **43:**957-959.

25. **Ohana, S., V. Leflon, E. Ronco, M. Rottman, D. Guillemot, S. Lortat-Jacob, P. Denys, G. Loubert, M. H. Nicolas-Chanoine, J. L. Gaillard, and C. Lawrence**. 2005. Spread of a *Klebsiella pneumoniae* strain producing a plasmid-mediated ACC-1 AmpC β-lactamase in a teaching hospital admitting disabled patients. Antimicrob. Agents Chemother. **49:**2095-2097.

26. **Oteo, J., A. Delgado-Iribarren, D. Vega, V. Bautista, M. C. Rodriguez, M. Velasco, J. M. Saavedra, M. Perez-Vazquez, S. Garcia-Cobos, L. Martinez-Martinez, and J. Campos**. 2008. Emergence of imipenem resistance in clinical *Escherichia coli* during therapy. Int. J. Antimicrob. Agents **32:**534–537.

27. **Pai, H., C. I. Kang, J. H. Byeon, K. D. Lee, W. B. Park, H. B. Kim, E. C. Kim, M. D. Oh, and K. W. Choe.** 2004. Epidemiology and clinical features of bloodstream infections caused by AmpC-type-beta-lactamase-producing *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **48:**3720-3728.

28. **Papagiannitsis, C. C., L. S. Tzouvelekis, E. Tzelepi, and V. Miriagou.** 2007. Plasmid-encoded ACC-4, an extended-spectrum cephalosporinase variant from *Escherichia coli*. Antimicrob. Agents Chemother. **51:**3763-3767.

29. **Pérez-Pérez, F. J., and N. D. Hanson.** 2002. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. **40:**2153-2162.

30. **Pitout, J.D., Hanson, N.D., Church, D.L., Laupland, K.B.** 2004 Population-based laboratory surveillance for *Escherichia coli*-producing extended-spectrum beta-lactamases: importance of community isolates with *bla*CTX-M genes. Clin Infect Dis 38(12):1736-41.

31. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC type β-lactamases. Antimicrob. Agents Chemother. **46:**1-11.

494 32. **Ruppé, E., P. Bidet, C. Verdet, G. Arlet, and E. Bingen.** 2006. First detection of the
495 Ambler class C 1 AmpC beta-lactamase in *Citrobacter freundii* by a new, simple double-
496 disk synergy test. J. Clin. Microbiol. **44:**4204-4207.
497
498 33. **Schechner V, Straus-Robinson K, Schwartz D, Pfeffer I, Tarabeia J, Moskovich R,**
499 **Chmelnitsky I, Schwaber MJ, Carmeli Y, Navon-Venezia S.** 2009 Evaluation of PCR-based
500 testing for surveillance of KPC-producing carbapenem-resistant members of the
501 *Enterobacteriaceae* family. J Clin Microbiol. **47**:3261-5**.**
502
503 34. **Siu, L. K., P. L. Lu, J. Y. Chen, F. M. Lin, and S. C. Chang.** 2003. High-level expression of
504 *ampC* β-lactamase due to insertion of nucleotides between −10 and −35 promoter
505 sequences in *Escherichia coli* clinical isolates: cases not responsive to extended-
506 spectrum-cephalosporin treatment. Antimicrob. Agents Chemother. **47:**2138-2144.
507
508 35. **Sutton, L.**D., D.J. Biedenbach, A. Yenn, and R.N. Jones. 1995. Development,
509 characterization and initial evaluations of S1. A new Chromogenic cephalosporin for β-
510 lactamase detection. Diagn. Microbiol. Infect. Dis. **21**:1-8.
511
512 36. **Tan, T.YTA.,L. S. Y. Ng, J. He, T. H. Koh, and L. Y. Hsu.** 2009. Evaluation of screening
513 methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae*,
514 and *Proteus mirabilis*. Antimicrob Agents Chemother. **53**:146–149.
515

516 37. **Thomson, K.S.** 2001. Controversies about extended-spectrum and AmpC beta-
517 lactamases. Emerg. Infect. Dis. **7:**333-336.
518
519 38. **Thomson, K. S.** 2010. Extended-spectrum-β-lactamase, AmpC and Carbapenemase issue.
520 J. Clin. Microbiol. **48:**1019-1025.
521
522 39. **Tracz, D. M., D. A. Boyd, L. Bryden, R. Hizon, S. Giercke, P. Van Caeseele, and M.**
523 **Mulvey.** 2005. Increase in *ampC* promoter strength due to mutations and deletion of the
524 attenuator in a clinical isolate of cefoxitin-resistant *Escherichia coli* as determined by RT-
525 PCR. J. Antimicrob. Chemother. **55**:758-72.
526
527 40. **Tracz, D. M., D. A. Boyd, R. Hizon, E. Bryce, A. McGeer, M. Ofner-Agostini, A. E. Simor,**
528 **S. Paton, and M. R. Mulvey.** 2007. *ampC* gene expression in promoter mutants of
529 cefoxitin-resistant *Escherichia coli* clinical isolates. FEMS Microbiol. Lett. **270:**265-271.
530
531
532
533
534
535

	AmpC ETest			AmpC disk test cefoxitin/cloxacillin		AmpC disk test cefoxitin/EDTA	*ampC* plasmid PCR[c]	*ampC* chrom[d]	final AmpC assignment[e]	Antibiotic resistance disk testing (mm)[f]						
strain no.	cefotetan MIC (µg/ml)	Ratio[a]	AmpC	difference[b] (mm)	AmpC	AmpC	(class)	(promoter variant)		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[g])**	**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[h])**	**negative (14)**	**positive**	**R (11)**	**R (13)**	**R (7)**	S (18)	**R (9)**	S (23)	**R (7)**

Table 1. Characterization of 21 *E. coli* strains positive for AmpC activity.

Strains were analyzed for AmpC production using three phenotypic confirmation AmpC assays. Genetic analysis for AmpC production was done by multiplex PCR for plasmid *ampC* detection and sequence analysis of the chromosomal *ampC* promoter/attenuator region. In addition, for each strain antimicrobial susceptibility data were generated. Strains with discrepant results in the three phenotypic confirmation AmpC assays are assays are highlighted in grey.

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;

a. Ratio of MIC cefotetan to cefotetan/cloxacillin is determined in the AmpC ETest (ratios ≥ 8 are considered AmpC positive).

b. Difference between diameters in the cefoxitin inhibition zones with and without cloxacillin were determined (increase ≥ 4 mm for the cefoxitin/cloxacillin disk is considered AmpC positive).

c. Plasmid-mediated *ampC* was detected by multiplex PCR (29).

d. For *ampC* promoter /attenuator sequence variants see Figure 1.

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

f. Diameter of growth inhibition zone in mm. The zone diameter breakpoints of the 2009 CLSI guidelines were applied (9).

g. CIT: plasmid-encoded *ampC* originating from *Citrobacter freundii*.

h. DHA: plasmid-encoded *ampC* originating from *Morganella morganii*.

555

	AmpC ETest			AmpC disk test cefoxitin/cloxacillin		AmpC disk test cefoxitin/EDTA	plasmid *ampC* PCR[c]	AmpC chrom[d]	final AmpC assignment[e]	Antibiotic resistance disk testing (mm)[f]						
strain no.	cefotetan MIC (µg/ml)	Ratio[a]	AmpC	difference[b] (mm)	AmpC	AmpC		Promoter variant		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)

556 **Table 2**. Characterization of the 30 *E. coli* strains negative for AmpC activity. Interpretation of susceptibility results according

557 to the CLSI guideline 2009.

Strains were analyzed for AmpC production using three phenotypic AmpC assays. Genetic analysis for AmpC production was done by multiplex PCR for plasmid *ampC* detection and sequence analysis of the chromosomal *ampC* promoter/attenuator region. In addition, for each strain antimicrobial susceptibility data were generated. Strains with discrepant results in the three phenotypic confirmation AmpC assays are highlighted in grey.

For abbreviations see legend Table 1.

Footnotes a. to f. see Table 1.

564

promoter sequence variant[a]	*E.coli* strain no.	number. of strains (n=51)	number of AmpC positive strains (%)	number of strains carrying plasmid-mediated *ampC*	a) b) position(s) of mutations in *ampC* promoter/attenuator region	b) localization and function of mutations
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 box**
6	10,16	1	1 (100%)	-	-14, **INS (-13.1**), +81	**Increased distance between -35 and -10 box,** promoter mutation and mutation in the *ampC* coding region
7	36, 37, 38, 39,40, 41,48,	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 coding region *ampC*
11	16, 17, 30, 31, 32	5	2 (40%)	**2**	-18, (-1), (+58), +81	alternate displaced promoter (-10 box only) and mutation in the coding region *ampC*
12	11, 12, 13, 24	4	3 (75%)	**3**	+22, +26, +27, +32, +70, +81	attenuator mutations and mutations in the coding region *ampC*
13	47	1	none	-	(+58), +63	mutation in the coding region *ampC*
14	21, 42, 43, 45, 50, 51,	6	1 (17%)	**1**	+81	mutation in the coding region *ampC*
15	14, 15, 25, 26, 27, 28, 29, 44	8	2 (25%)	**2**	+70, +81	mutations in the coding region *ampC*

565

566 **Table 3** Genetic characterization of the 51 *E.coli* isolates by *ampC* promoter region sequence analysis and multiplex PCR for
567 detection of plasmid-mediated *ampC*. All isolates with promoter sequence variants 1, 2, 3, 4, 5 and 6 are considered positive
568 for AmpC activity due to chromosomal overexpression of *ampC*. Promoter variants 11, 12, 14 and 15 are found in
569 phenotypically AmpC positive and AmpC negative strains; the AmpC positive strains all harboured plasmid-encoded AmpC.
570 Promoter sequence variants 7, 8, 10 and 13 were not associated with increased phenotypic AmpC activity.

571 a. Analysis of *ampC* promoter/attenuator mutations (for detailed sequence analysis see Figure 1).

572 b. Mutations and mechanisms resulting in overexpression leading to *ampC* upregulation are typed in bold and highlighted in

573 grey. Insertion of nucleotides is marked with INS. Mutations outside functional promoter elements are displayed in brackets

574 (4).

575 **Table 4.** Characterization of β-lactamase activity in *E. coli* strains (n=5) considered false positive or false negative in phenotypic
576 screening for AmpC overproduction.

E. coli strain [a]	Nitrocefin hydrolysis ($OD_{nm=492}$)[b]							ESBL [c]		KPC[d]
	Without inhibitor	aminophenylboronic acid		clavulanic acid		boronic acid and clavulanic acid		phenotypic	genetic	
			Inhibition		Inhibition		Inhibition			
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)	-

577 a. Strain numbers correspond to Tables 1 and 2.

578 b. Nitrocefin hydrolysis was tested in absence and presence of boronic acid (as specific AmpC inhibitor) and/or clavulanic acid (as
579 specific ESBL and TEM-1 inhibitor). Presented values are the average of duplicate reactions.

580 c. ESBL were phenotypically detected by the DDS test as described in Material and Methods. PCR detection of TEM, SHV and CTX-
581 M genes were done as described previously (21, 30). **Note**: TEM-1 is **not** an ESBL β-lactamase, but is able to hydrolyse nitrocefin.
582 Therefore the detection of TEM-1 is shown inbrackets
583 d. PCR detection of KPC genes was performed as described previously (33).

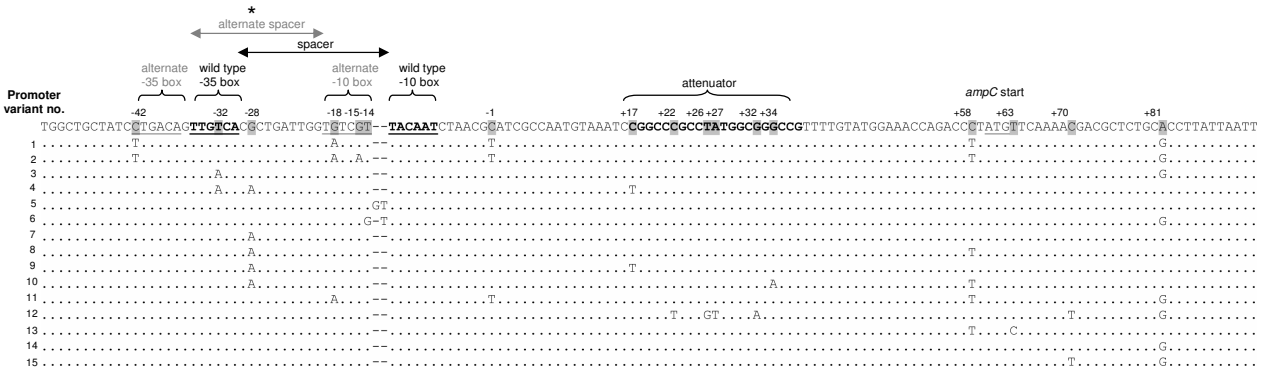

Figure 1 Alignment of the chromosomal *ampC* promotor, attenuator and 5`-end regions. For the 51 *E. coli* isolates, 15 different sequence variants were identified. * The chromosomal *ampC* sequence variant classification and description of functional elements was used according Tracz et al. (40).