332
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Contemporary Occurrence of Mobile AmpC-Type Beta-Lactamase Resistances in *E. coli*: Report from the SENTRY Antimicrobial Surveillance Program (USA, 2004)

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SENTRY ANTIMICROBIAL SURVEILLANCE

AMENDED ABSTRACT

Background: For detection of extended spectrum ß-lactamases (ESBLs), the CLSI recommends initial MIC screens (≥ 2 µg/ml for cefotaxime [CTAX] or ceftriaxone [CTRI] or ceftazidime [CTAZ] or aztreonam [AZT]) for *E. coli* (EC), *Klebsiella* spp. and *P. mirabilis*, followed by a confirmatory clavulanate (CA) inhibition test. Isolates that fail the CA inhibition test (no inhibition) can produce an AmpC enzyme mobilized via a plasmid. The prevalence of this resistance (R) pattern and the determination of type in EC was assessed by the SENTRY Program (2004) in the USA.
Methods: A total of 1,429 EC were collected from 30 medical centers (MC) in North America and tested by CLSI broth microdilution methods versus 34 agents. ESBL screen-positive strains (65; 4.5%) were confirmed by a disk approximation (DA) CA assay using 4 substrates (CTRI, CTAZ, AZT, cefepime [CPM]), DA-negative strains (22) were typed for AmpC-like enzymes (CMY, FOX, DHA) by PCR and sequencing techniques.
Results: Molecular methods among PCR-tested strains revealed CMY-2 in 13 strains (46.4%; 9 MCs), FOX-5 in 3 strains (11.5%; 2 MCs) and DHA-type in one isolate. PCR screens were negative in the remaining isolates. AmpC-producing EC were cefoxitin-R (MIC, ≥ 32 µg/ml), but CPM susceptible (S; MICs, ≤ 0.12 - 4 µg/ml). CMY-2 strains were detected in these states: NY (4; 2 MCs), IN (2), HI (1), KY (1), MA (1), NE (1) and TX (1). FOX-5 strains were found in 2 different MCs in NY. Seventy percent of MC had AmpC and ESBL strains (epidemic in 2 NY sites) and 1 MC harbored Group 2f carbapenemases (NY; imipenem-R).
Conclusions: CMY-2 was the major mobile AmpC-like enzyme observed among EC, ESBL screen-positive isolates in the USA (upper Midwest, the northeast and HI; not observed in Canada). Clearly CLSI ESBL screens and CA inhibition detect only part of the EC population with elevated ß-lactam MIC values, therefore requiring re-evaluations of Enterobacteriaceae S breakpoints to guide appropriate treatment.

INTRODUCTION

E. coli isolates rank highest among the Gram-negative pathogens causing significant infections worldwide but are generally quite susceptible to ampicillin, other beta-lactams and cephalosporins. *E. coli* carries a chromosomal AmpC gene which expresses at a basal level and thus does not confer resistance to ampicillin or cephalosporins. Mutations in the promoter and/or the attenuator region of this gene (which is also part of fumarase reductase [frdD] gene) results in hyper production of AmpC enzyme. In the recent years some *E. coli* strains carrying "imported" plasmid mediated AmpC genes have been identified. Promoter modifications made during the recombination event which created the plasmid carrying AmpC are responsible for high level expression. Chromosomal AmpC genes are activated by mutational events whereas plasmid mediated AmpC determinants are clinically significant as they have the ability to spread beta-lactam resistance among Gram-negative bacilli.

According to the Clinical and Laboratory Standards Institute (CLSI [2005], formerly NCCLS) criteria, isolates with MIC value ≥ 2 µg/ml for ceftazidime, ceftriaxone, cefotaxime or aztreonam are potential extended spectrum beta-lactamase (ESBL) producers. The committee recommends that a confirmatory clavulanic acid inhibition test should be performed on clinical isolates before reporting susceptibility results for cephalosporins and monobactam. The Enterobacteriaceae isolates with an ESBL phenotype but a negative ESBL confirmatory test result are potential candidates for production of an AmpC enzyme, either chromosomally derepressed or mobilized on a plasmid. Many clinical microbiologists are unaware of plasmid-mediated AmpC beta-lactamases because phenotypic detection is difficult at best and these beta-lactamases can be misidentified as ESBLs. Isolates with high MIC values to cefoxitin may also be AmpC producers, but cefoxitin resistance alone may also result from loss of outer membrane porin (OMP).

We evaluated the prevalence of mobile AmpC-type ß-lactamase among *E. coli* isolates collected by the SENTRY Antimicrobial Surveillance Program in the United States (USA) in 2004.

MATERIALS AND METHODS

Bacterial Isolates: In the year 2004 SENTRY Program, a total of 1,429 *E. coli* isolates were processed from 30 medical centers in North America. The isolates were collected consecutively from bloodstream infections, skin and soft tissue infections, infections of pediatric patients in the ICU and pneumonia in hospitalized patients and submitted to the SENTRY Program monitoring laboratory (JMI Laboratories, North Liberty, IA). Only the isolates responsible for significant infection were included in the study. Species identification was confirmed at the monitoring laboratory using standard biochemical tests and Vitek System cards, where necessary.

Susceptibility testing: All *E. coli* isolates were tested for susceptibility against 34 antimicrobial agents by the broth microdilution method as described by CLSI using validated, dry form panels manufactured by Trek Diagnostics (Cleveland, OH). Interpretations on susceptibility to all antimicrobials tested were as per CLSI (2005) criteria. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853 were routinely tested in parallel with clinical isolates for quality assurance.

ESBL testing by disk approximation: A confirmatory test was performed on isolates meeting the CLSI screening criteria for potential ESBL production. In brief, test organisms were grown overnight on blood agar plates. The 0.5 McFarland suspensions made from these cultures were used to inoculate the surface of Mueller-Hinton agar plates. A disk containing amoxicillin/clavulanic acid or clavulanic acid alone (10 µg) was placed in the center of the plate and the disks for ESBL substrates viz. ceftazidime, ceftriaxone, aztreonam and cefepime were placed 15 mm from the disk containing clavulanate. The plates were incubated 16-20 hours at 35°C in an ambient air incubator. Widening of the zone or formation of a "key-hole" or "phantom zone" effect between any or all of the substrate inhibitor combinations was considered a positive ESBL test.

Three dimensional (3D) test for detection of AmpC: Isolates with ESBL phenotype but a negative ESBL confirmatory test were considered potential AmpC producers and screened for production of AmpC by the three dimensional test described by Coudron et al (2000), using cefoxitin as substrate.

Multiplex PCR for characterization of bla_{AmpC} type: The *E. coli* isolates with a positive 3D AmpC test were screened by multiplex PCR as described by Perez-Perez and Hanson (2002). Upon completion of PCR the reaction products were electrophoresed on 2% agarose gel to confirm the presence and size of the amplification product. Tentative enzyme type designations were given based on the size of the PCR amplicon and further confirmed by amplification with specific bla_{AmpC} primers followed by gene sequencing.

Gene sequencing: The PCR products were "cleaned" using QIAGEN PCR purification kit. Sequencing was performed using Sanger based dideoxy sequencing strategy involving the incorporation of fluorescent-dye-labeled terminators into the sequencing reaction products. Sequences obtained were compared to the pre-existing sequences via NCBI BLAST search to determine the type of beta-lactamase.

RESULTS

- Antimicrobial susceptibility profile of *E. coli* isolates is displayed in Table 1. "Third-generation" cephalosporins were highly active against *E. coli* isolates. Ceftazidime, ceftriaxone and aztreonam showed similar activity (96.0- 97.4% isolates susceptible). In contrast, only 91.2% isolates were susceptible to cefoxitin and the resistance rate was 3.4%. Cefepime activity was slightly greater (98.5% susceptible) among all cephalosporins tested. All the isolates were susceptible to imipenem; only three isolates (0.21%) showed MIC ≥ 0.5 µg/ml. Resistance rate toward ciprofloxacin was very high (14.9%).
- A total of 65 (4.5%) isolates met the CLSI criteria for ESBL production and 36 confirmed as ESBL producers by the disk approximation method.
- Nineteen of the 30 medical centers enrolled in the SENTRY Program (USA, 2004) isolated ESBL-positive *E. coli* while AmpC producers were only found in ten hospitals. No ESBL or AmpC producing *E. coli* were isolated from medical centers in Canada.

Table 1. In vitro activity of selected antimicrobial agents against tested *E. coli* strains isolated from North American medical centers of the SENTRY Program (2004).

Antimicrobial agent (n=1,429)	MIC (µg/ml) 50%	MIC (µg/ml) 90%	Category (%) Susceptible	Category (%) Resistant	% ESBL phenotype (MIC, ≥2 µg/ml)
Ceftazidime	≤1	≤1	96.9	1.5	4.3
Ceftriaxone	≤0.25	≤0.25	97.1	2.1	3.8
Cefepime	≤0.12	≤0.12	98.5	1.3	-
Cefoxitin	4	8	91.2	3.4	-
Aztreonam	≤0.12	0.25	97.4	1.8	4.1
Amoxicillin/clavulanate	8	16	81.3	5.7	-
Piperacillin/tazobactam	2	4	96.4	2.0	-
Ciprofloxacin	≤0.03	>4	85.2	14.9	-
Gentamicin	≤2	4	91.3	8.2	-
Imipenem	≤0.12	≤0.12	100.0	0.0	-

- PCR screens for AmpC genes followed by sequencing revealed CMY-2 in 13 (46.4%) isolates while FOX-5 was found in three (17.8%) isolates. CMY-types other than CMY-2 were not detected. All CMY-2 and FOX-5 producing *E. coli* were resistant to cefoxitin as well as "third-generation" cephalosporins, but all were susceptible to cefepime (MIC range, ≤ 0.12- 4 µg/ml).
- bla_{DHA} primers yielded PCR product with one isolate, which was susceptible to all cephalosporins, with highest MIC values to ceftazidime and cefoxitin (8 µg/ml).
- One isolate was found to produce a KPC-2/3 beta-lactamase. This isolate showed elevated MIC values for imipenem and meropenem, in addition to cephalosporin resistance.
- Geographic distribution of CMY-2 and FOX-5 producing isolates is shown in Table 2. Eight of 17 (47.0%) AmpC producers as well as one KPC-2 producer were isolated from three medical centers in New York City.
- Five *E. coli* isolates showing positive 3D AmpC test did not generate PCR products with any of the primer sets. The potentially novel AmpC enzymes are being characterized.

Table 2. Distribution of AmpC enzyme types detected in *E. coli* strains isolated from various USA medical centers.[a]

Location of medical center (state)	ß-lactamase (no. isolates) CMY-2	FOX-5	DHA-type
Indiana	2	-	-
Nebraska	1	-	-
Wisconsin	1	-	-
Massachusetts	1	-	-
New York	5 (A, B)[b]	3 (A, C)[b]	-
Texas	1	-	-
Kentucky	1	-	-
Hawaii	1	-	-
Utah	-	-	1

a. No strains were detected in Canada.
b. A, B and C are designations for three medical centers in New York.

CONCLUSIONS

- ESBL phenotypes in *E. coli* isolates may be due to production of ESBL or AmpC enzymes.
- CMY-2 was the most prevalent plasmid-mediated AmpC among *E. coli* isolates in the United States.
- CMY-2 as well as FOX-5 is prevalent among beta-lactam-resistant *E. coli* isolates from New York City area medical centers.
- It is important to identify the resistance mechanism for cephalosporin resistance among *E. coli* isolates in order to implement an efficient treatment and epidemiology strategy.

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