Detection of AmpC β Lactamases in Gram-negative Bacteria

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ABSTRACT

AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many Enterobacteriaceae and a few other organisms, where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor/β-lactam combinations. The increase in antibiotic resistance among Gram-negative bacteria is a notable example of how bacteria can procure, maintain and express new genetic information that can confer resistance to one or several antibiotics. Detection of organisms producing these enzymes can be difficult, because their presence does not always produce a resistant phenotype on conventional disc diffusion or automated susceptibility testing methods. These enzymes are often associated with potentially fatal laboratory reports of false susceptibility to β-lactams phenotypically. With the world-wide increase in the occurrence, types and rate of dissemination of these enzymes, their early detection is critical. AmpC β-lactamases show tremendous variation in geographic distribution. Thus, their accurate detection and characterization are important from epidemiological, clinical, laboratory, and infection control point of view. This document describes the methods for detection for AmpC β-lactamases, which can be adopted by routine diagnostic laboratories.

Key words: AmpC β-lactamases, disk approximation test, gram-negative bacteria, three-dimensional extract test

INTRODUCTION

Drug resistance poses a therapeutic problem not only in the hospital settings, but also in the community as most of the bacteria have acquired resistance to multiple antibiotics.[1,2] The various mechanisms of drug resistance in Gram-negative bacteria include extended spectrum beta-lactamases (ESBL) production, AmpC β-lactamase production, efflux mechanism and porin deficiency. In the clinical laboratory settings, the commonly detected enzymes causing resistance are AmpC β-lactamases and ESBLs. Clinical relevance of AmpC β-lactamases lies in the fact that they confer resistance to both narrow and broad spectrum cephalosporins, beta-lactam/beta-lactamase inhibitor combinations and aztreonam.[3]

AmpC β-lactamases can be chromosomally or plasmid mediated. The plasmid mediated AmpC β-lactamases hydrolyze all β-lactam antibiotics except ceftime and carbapenems. The plasmid-mediated AmpC genes are derived from inducible chromosomal genes that have been mobilized among various organisms. The commonly reported genotypes are ACC, FOX, MOX, DHA, CMY, CIT and EBC.[4-6] These mobilized plasmid mediated enzymes confer a resistance pattern similar to the overproduction of chromosomal AmpC β-lactamases, which also involve all β-lactam antibiotics except for carbapenems and ceftime.[7]

Detection of AmpC is important to improve the clinical management of patients suffering from infections and would also provide us with sound epidemiological data. However, there are no Clinical and Laboratory Standards Institute guidelines for detection of AmpC mediated resistance in Gram-negative clinical isolates and hence, it usually poses a problem due to misleading results, especially so in phenotypic tests.[8]

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Personnel qualifications

The test performer should be having a diploma in laboratory technologies and preferably university graduate in biological sciences with sufficient experience.

Education and training

Personnel are required to be knowledgeable of the procedures in the microbiology laboratory. The laboratory staff should confirm that they can properly perform the procedures before commencing work. The details are given in Table 1.

PROCEDURE

Techniques to identify AmpC β-lactamase-producing isolates are available but are still evolving and are not yet optimized for the clinical laboratory, which leads to the underestimation of these resistance mechanisms. Carbapenems can usually be used to treat infections due to AmpC-producing bacteria, but carbapenem resistance can arise in some organisms by mutations that reduce influx (outer membrane porin loss) or enhance efflux (efflux pump activation).

REQUIREMENTS

The list of all the requirements are given in Table 2.

Isolates

Gram-negative bacterial isolates recovered from clinical samples.

Preparation of reagents and chemicals

1. 300 μg phenylboronic acid
   - Weigh 150 mg phenylboronic acid and dissolve in 10 ml sterile distilled water
   - Use it as 15 μg/μl stock
   - Store at 4°C

2. 0.5 M ethylenediaminetetraacetic acid (EDTA) buffer
   - Weigh 18.6 g EDTA and add distilled water to 90 ml
   - Adjust pH to 8.0
   - Add distilled water and make final volume 100 ml
   - Store at 4°C

3. Tris-EDTA (TE) buffer
   - 50X TE (stock solution)

Table 1: Education and training must be given on the following topics

| Potential risks to health                  |
|-------------------------------------------|
| Precautions to be taken to minimize contamination |
| Hygiene requirement                        |
| Wearing and use of protective equipment and clothing |
| Handling of potentially infectious material |
| Laboratory design, including airflow conditions |
| Use of autoclave, incubators (operation, identification of malfunctions, maintenance) |
| Good laboratory practice and good microbiological techniques |
| Organization of work flow and procedure |
| Waste management                           |
| Importance of laboratory results for patients management |
| Training should be given before a staff member takes up his/her post |
| Repeat training periodically, preferably every year |

Table 2: List of all the requirements for performing the test

| Equipments and materials     | Media                  | Reagents and solutions             | Antibiotic disks                  | ATCC strains                  | Other material         |
|------------------------------|------------------------|----------------------------------|-----------------------------------|-------------------------------|------------------------|
| Simple microscope            | Mueller-Hinton agar    | Normal saline                    | Cefoxitin disk (30 μg)            | E. coli ATCC 25922           | Sterile micropipette tips |
| Incubator set at 37°C        | Blood agar             | Tris-EDTA                        | AmpC disk (i.e., filter paper disks containing Tris-EDTA) | E. coli ATCC 11775           | Sterile culture plates  |
| Autoclave                    | MacConkey agar         | Phenylboronic acid               | Imipenem (20 μg)                 | K. pneumoniae ATCC BAA 1144   | Sterile swabs           |
| Water bath                   | Brain heart infusion broth | DNA extraction kits           | Cefoxitin (30 μg)                | E. cloacae ATCC BAA 1143     | Sterile microcentrifuge tube |
| Weighing balance             |                        | Molecular biology grade water    | Amoxicillin-clavulanate (20/10 μg) |                              | PCR tubes (50 μl)       |
| Refrigerator/freezer         |                        | Absolute ethanol                 | Cefazidime (30 μg)               |                              | Sterile 1.5 ml Appendorf |
| Micropipettes (1000 μl, 300 μl, 20 μl and 10 μl) | PCR reagents            | Taq DNA polymerase (5 U/μl)     |                                  | Sterile storage vials     |
| Centrifuge                   |                        | 10X Taq buffer with KCL          |                                  | Forceps                       |
| PCR thermocycler             |                        | Tris-HCl (pH 8.4)                |                                  | Parafilm                     |
| Gel electrophoresis          |                        | 25 mM MgCl₂                     |                                  |                              |
| Gel Doc                      |                        | DNA ladder 100 bp                |                                  |                              |

PCR: Polymerase chain reaction, EDTA: Ethylenediaminetetraacetic acid, DNA: Deoxyribonucleic acid, DNTPS: Deoxynucleotide triphosphate, ATCC: American type culture collection, KCL: Potassium chloride
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- Weigh 121 g Tris base and dissolve in 400 ml distilled water
- Add 50 ml 0.5 M EDTA
- Add distilled water and make final volume 500 ml
- Store at 4°C

4. TE buffer (working)
   - Dilute 50X master stock to 1X with distilled water.

5. Primer reconstitution.
   Primers are often shipped and received in a lyophilized state. First create a master 100X stock (for each primer and then dilute it to a 20X working stock).

Master stock, 100 μM
- 100 μM = X nmoles lyophilized primer + (X × 10 μl molecular grade H₂O)
- To determine the amount of H₂O to add to the lyophilized primer simply multiply the number of nmol of primer in the tube by 10 and that will be the amount of H₂O to add to make a 100 μM primer stock
- Vortex tube and incubate at room temperature for 10 min.

Working stock, 20 μM
- Dilute the primer master stock in a sterile microcentrifuge tube 1:5 with molecular grade H₂O

TEST PROCEDURE

What is unnecessary?

It is unnecessary to detect AmpC production in organisms that produce an inducible chromosomal AmpC β-lactamase because the organism identification is indicative of AmpC production; i.e. 100% isolates of Enterobacter cloaca, Enterobacter aerogenes, Citrobacter freundii, Serratia marcescens, Providencia sp., Morganella morganii, Hafnia alvei, Aeromonas sp., and P. aeruginosa can be assumed to be AmpC producers. Detection of an AmpC β-lactamase in Klebsiella sp., Citrobacter koseri or Proteus mirabilis is confirmatory for plasmid-mediated AmpC production because these organisms lack a chromosomal AmpC β-lactamases. [9,10]

Screening

i. Requirement
   - Test organism
   - Normal saline
   - Blood agar plates
   - Mueller-Hinton agar (MHA) plates
   - 30-μg cefoxitin disk

ii. Procedure
   - Make 0.5 McFarland bacterial suspension in normal saline prepared from an overnight blood agar plate
   - Inoculate surface of MHA plate with this suspension by swabbing
   - Place 30-μg cefoxitin disk on inoculated MHA
   - Invert the plate and incubate overnight at 35°C

iii. Plate reading and interpretation
   - After overnight incubation measure zone diameter around 30-μg cefoxitin disk
   - Select the isolates with zone diameters less than 18 mm for confirmation of AmpC production.

Phenotypic confirmatory tests

Three-dimensional extract test

i. Requirement
   - Test organism
     - Escherichia coli ATCC 25922 or E. coli ATCC 11775
     - Normal saline
     - Blood agar plates
     - MHA plates
     - 12 ml brain heart infusion (BHI) broth
     - 30-μg cefoxitin disk
     - Sterile blade

ii. Procedure
   - Prepare 0.5 McFarland bacterial suspension from an overnight blood agar plate
   - Inoculate 12 ml BHI broth with 50 μl of 0.5 McFarland bacterial suspension and incubate for 4 h at 37°C
   - Concentrate cells by centrifugation and freeze-thaw 5 times to prepare crude enzyme
   - Prepare 0.5 McFarland bacterial suspension using one of two E. coli ATCC 25922 or ATCC 11775 and inoculate surface of MHA plate by using this suspension
   - Place 30-μg cefoxitin disk on the inoculated agar plate
   - With a sterile scalpel blade, cut a slit beginning 5 mm from the edge of the disk in an outward radial direction
   - By using a pipette, dispense 25-30 μl of enzyme preparation into the slit, beginning near the disk and moving outward, avoiding slit overfill
   - Incubate inoculated media overnight at 37°C

iii. Plate reading and interpretation
   - After overnight incubation check the enhanced growth of the surface organism at the point where the slit intersected
   - If there is a zone of inhibition of surface organism, the test is positive three-dimensional test [Figure 1].
**Boronic acid disk test method**

i. Requirements
   - Test organism
   - Normal saline
   - 10 µg imipenem disk
   - 30 µg cefoxitin disk
   - 20/10 µg amoxicillin-clavulanate disk
   - 30 µg ceftazidime disk
   - MHA Plates

ii. Procedure
   - Prepare 0.5 McFarland bacterial suspension from an overnight blood agar plate
   - Inoculate surface of MHA plate using this suspension as per standard disk diffusion method
   - Place a 30 µg cefoxitin disk on the inoculated surface of the MHA
   - Place inoculated AmpC disk almost touching the antibiotic disk with the inoculated disk face in contact with the agar surface
   - Invert the plate and incubate overnight at 35°C

iii. Plate reading and interpretation
   - After overnight incubation, compare the zone diameter around the antibiotic disk with added boronic acid and the antibiotic-containing disk alone
   - An organism that demonstrates a defined increase (≥5-mm) in zone diameter around the antibiotic disk with added boronic acid consider to be an AmpC producer [Figure 3].

**Disk approximation test**

i. Requirements
   - Test organism
   - Normal saline
   - Imipenem disk
   - Cefoxitin disk
   - Amoxicillin-clavulanate disk
   - Ceftazidime disk
   - MHA Plates

ii. Procedure
   - Prepare 0.5 McFarland bacterial suspension from an overnight blood agar plate
   - Inoculate surface of MHA plate using this suspension as per standard disk diffusion method
   - Invert the plate and incubate overnight at 35°C
Place a 30 µg ceftazidime disk at the center on the plate
Place 10 µg imipenem, 30 µg cefoxitin, and 20/10 µg amoxicillin-clavulanate disks at a distance of 20 mm from ceftazidime disk
Invert the plate and incubate overnight at 35°C

After overnight incubation, examine the plate for any obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the inducing substrates (imipenem, cefoxitin and amoxicillin-clavulanate disk)
If there is any blunting or flattening of the zone, consider as a positive result for AmpC production [Figure 4].

**Multiplex PCR**

**Requirements**
- 0.5-ml thin-walled PCR tubes
- Molecular biology grade water
- Taq DNA polymerase (5U/µl)
- 10X Taq buffer with KCl
- Tris-HCl (pH 8.4)
- 25 mM MgCl₂
- DNTPs, 10Mm

**Procedure**
- Make a master mix containing 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 0.2 mM each dNTPs; 1.5 mM MgCl₂; 0.6 µM primers MOXM, MOXMR, CITMF, CITMR, DHAMF, and DHAMR; 0.5 µM primers ACCMF, ACCMR, EBCMF, and EBCMR; 0.4 µM primers FOXMF and FOXMR; and 1.25 U of Taq DNA polymerase. Add 2 µl DNA template. The list of all the primers are given in Table 3.
- Set the PCR program on an initial denaturation step at 94°C for 3 min, followed by 25 cycles of DNA denaturation at 94°C for 30s, primer annealing at 64°C for 30s, and primer extension at 72°C for 1 min. After the last cycle, a final
extension step at 72°C for 7 min
• Set the tube in the PCR machine and run the program.

Electrophoresis
i. Requirements
• Agarose
• Ethidium bromide
• Loading die
• 100-bp DNA ladder

ii. Procedure
• Prepare 2% agarose gel in 1X TE buffer
• Analyze 5 µl PCR product mixed with 1 µl 6X loading die
• Use 100-bp DNA ladder as a marker
• Stain gel with ethidium bromide (10 µg/ml) and analyze the presence of bands in ultraviolet transilluminator
• Use the PCR mixtures with the addition of water in place of template DNA as negative control.

Plate disposal
• Keep all culture plates sealed inside blue plastic bags and seal in an autoclave bag
  • Autoclave at 121°C for 30 min
  • Discard the sealed sterilized bags in the site designed for this purpose.

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REFERENCES
1. Kaurthe J. Increasing antimicrobial resistance and narrowing therapeutics in typhoidal salmonellae. J Clin Diagn Res 2013;7:576‑9.
2. Laxminarayan R, Klugman KP. Communicating trends in resistance using a drug resistance index. BMJ Open 2011;1:e000135.
3. Drawz SM, Bonomo RA. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev 2010;23:160‑201.
4. Hanson ND. AmpC beta-lactamases: What do we need to know for the future? J Antimicrob Chemother 2003;52:3‑4.
5. Pérez‑Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 2002;40:2153‑62.
6. Papanicolaou GA, Medeiros AA, Jacoby GA. Novel plasmid-mediated beta-lactamase (MIR‑1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of Klebsiella pneumoniae. Antimicrob Agents Chemother 1990;34:2200‑9.
7. Mirelis B, Rivera A, Miró E, Mesa RJ, Navarro F, Coll P. A simple phenotypic method for differentiation between acquired and chromosomal AmpC beta-lactamases in Escherichia coli. Enferm Infecc Microbiol Clin 2006;24:2153‑62.
8. Papanicolaou GA, Medeiros AA, Jacoby GA. Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of Klebsiella pneumoniae. Antimicrob Agents Chemother 1990;34:2200‑9.
9. Mirelis B, Rivera A, Miró E, Mesa RJ, Navarro F, Coll P. A simple phenotypic method for differentiation between acquired and chromosomal AmpC beta-lactamases in Escherichia coli. Enferm Infecc Microbiol Clin 2006;24:2153‑62.
10. Thomson KS. Extended-spectrum-beta-lactamase, AmpC, and Carbapenemase isolates. J Clin Microbiol 2010;48:1019‑25

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