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How do I do it

Detection of *Amp*C β Lactamases in Gram-negative Bacteria

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ABSTRACT

*Amp*C 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 *Amp*C 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]

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*Amp*C β-lactamases can be chromosomally or plasmid mediated. The plasmid mediated *Amp*C β-lactamases hydrolyze all β-lactam antibiotics except cefepime and carbapenems. The plasmid-mediated *Amp*C 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 *Amp*C β-lactamases, which also involve all β-lactam antibiotics except for carbapenems and cefepime.[7]

Detection of *Amp*C 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 *Amp*C mediated resistance in Gram-negative clinical isolates and hence, it usually poses a problem due to misleading results, especially so in phenotypic tests.[8]

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 *Amp*C 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 *Amp*C-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).

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

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 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	*Amp*C disk (i.e., filter paper disks containing Tris-EDTA)	*E. coli* ATCC 11775	Sterile culture plates
Autoclave	MacConkey agar	Phenylboronic acid	Imipenem (10 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	Ceftazidime (30 μg)		Sterile 1.5 ml Appendorf
Micropipettes (1000 μl, 100 μl, 20 μl and 10 μl)		PCR reagents			Sterile storage vials
Centrifuge		Taq DNA polymerase (5 U/μl)			Forceps
PCR thermocycler		10X Taq buffer with KCL			Parafilm
Gel electrophoresis		Tris-HCl (pH 8.4)			
Gel Doc		25 mM $MgCl_2$			
		DNTPS 10 mM (fermentas)			
		Agarose			
		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

- 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 H_2O)
- To determine the amount of H_2O 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 H_2O 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 H_2O.

TEST PROCEDURE

What is unnecessary?

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

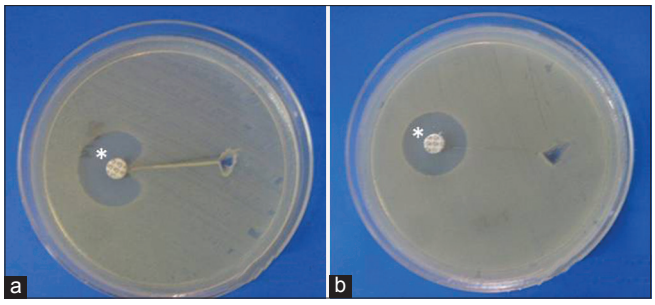

Figure 1: Representation of three dimensional extract test. (a) Zone of inhibition showing positive test, (b) no zone of inhibition showing negative test. *30 µg cefoxitin disk

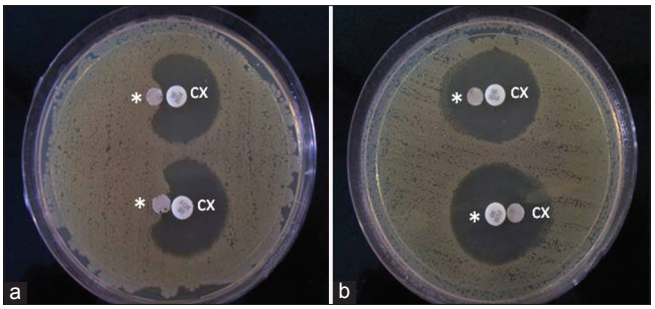

Figure 2: Representation of *AmpC* disk test. (a) Zone of inhibition showing positive test results, (b) no zone of inhibition showing negative test results, *AmpC disks (filter paper containing tris-EDTA), CX: 30µg cefoxifin disks

AmpC disk test

i. Requirements
- *Amp*C disks (filter paper disks containing TE)
- 30 µg cefoxitin disk
- Blood agar plates
- MHA plates
- Test organisms
- *E. coli* ATCC 25922
- Normal saline

ii. Procedure
- Prepare 0.5 McFarland bacterial suspension of *E. coli* ATCC 25922
- Inoculate surface of MHA plate using this suspension as per standard disk diffusion method
- Immediately prior to use, rehydrate *Amp*C disk with 20 µl of saline and several colonies of each test organism apply to a disk
- Place a 30 µg cefoxitin disk on the inoculated surface of the MHA
- Place inoculated *Amp*C 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 in ambient air

iii. Plate reading and interpretation
- After overnight incubation, examine the plate for either an indentation or a flattening of the zone of inhibition
- If there is any zone of inhibition, it indicates enzymatic inactivation of cefoxitin (positive result)
- If no zone inhibition, indicates no significant inactivation of cefoxitin (negative result) [Figure 2].

Boronic acid disk test method

i. Requirements
- Test organism
- 30 µg cefoxitin disk
- Phenylboronic acid
- 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
- Using sterile tips, dispense 20 µl of 15 µg/ml phenylboronic acid onto the disk
- Let the disk absorb it
- 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 *Amp*C producer [Figure 3].[5]

Disk approximation test

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

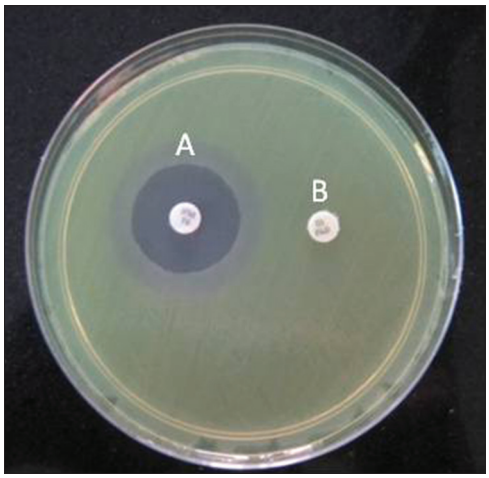

Figure 3: Representation of boronic acid disk test. A: 30 µg cefoxitin disk supplemented with 300µg of phenyl boronic acid. B: 30 µg cofoxitin disk alone

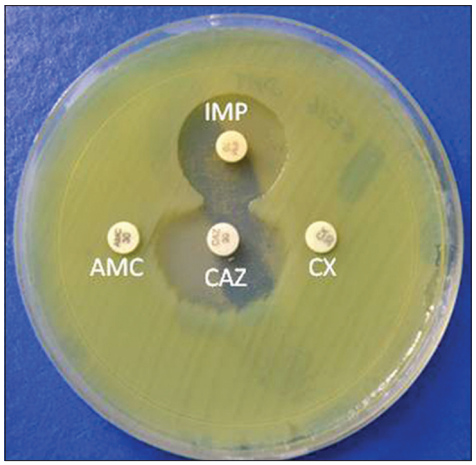

Figure 4: Representation of disk approximation test. Flattening of zone of ceftazidime toward imipenem disk (inducing substrate) showing positive result. IMP: Imipenem (10 µg), CAZ: Ceftazidime (10 µg), AMC: Amoxillin-clavulanate (20/10 µg)

- 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

iii. Plate reading and interpretation
- 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 *Amp*C production [Figure 4].

Multiplex polymerase chain reaction for plasmid mediated AmpC genes

Phenotypic tests do not differentiate between chromosomal and plasmid mediated *Amp*C β-lactamases. Plasmid-mediated *Amp*C β-lactamases are most accurately detected with the multiplex *Amp*C PCR test.

Preparation of template deoxyribonucleic acid (DNA)

i. Requirements
- Test organism
- Molecular biology grade water
- Microcentrifuge tubes
- Absolute ethanol
- DNA extraction kit

ii. Procedure
- Inoculate a single colony of each organism into 5 ml of Luria-Bertani broth and incubate for 20 h at 37°C with shaking
- Harvest cells from 1.5 ml of the overnight culture by centrifugation at 10,000 × *g* for 5 min
- Discard supernatant, re-suspend the pellet in 500 µl of distilled water
- Extract total DNA by using DNA extraction kit according to manufacturer's instructions
- Quantify total DNA prior to the multiplex PCR using spectrophotometer

Multiplex PCR

i. 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 $MgCl_2$
- $DNTP_s$ 10Mm

ii. 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 MOXMF, 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

Table 3: Primers for amplification of *AmpC* genes

Target (s)	Primer	Sequence (5′ to 3′)	Expected amplicon size (bp)
MOX-1, MOX-2, CMY-1, CMY-8 TO CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
	MOXMAR	CAC ATT GAC ATA GGT GTG GTG C	
LAT-1 TO LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
	CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
	DHAMR	CCG TAC GCA TAC TGG CTT TGC	
ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
	ACCMR	TTC GCC GCA ATC ATC CCT AGC	
MIR-1T ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
	EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOX-1 TO FOX-5B	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXMR	CAA AGC GCG TAA CCG GAT TGG	

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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