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Chapter 6

# Phenotypic and Molecular Methods for the Detection of Antibiotic Resistance Mechanisms in Gram Negative Nosocomial Pathogens

Meletis Georgios, Tzampaz Egki and
Sianou Effrosyni

Additional information is available at the end of the chapter

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## 1. Introduction

Antibiotic resistance among clinical isolates of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Enterobacter* spp and *Proteus* spp is causing worldwide concern [1-5] especially when mediated by transferable genetic elements.

The role of the Clinical Microbiology Department in this regard is crucial for the isolation of non-susceptible bacteria and the detection of the underlying mechanisms leading to their resistant phenotype. Rapid and reliable results are of the utmost importance in order to apply the appropriate treatment and to contain the spread of resistance determinants within hospital settings.

In the present chapter, laboratory procedures for the detection of antibiotic resistance mechanisms will be discussed focusing mainly on those more frequently used for Gram negative clinical isolates around the world.

## 2. Antibiotic resistance mechanisms among Gram negative nosocomial pathogens

Antibiotic resistance may be intrinsic (the microorganism is by definition resistant against a certain antibiotic) or acquired. Acquired refers to resistance that is a consequence of mutational events or gene acquisition via horizontal gene transfer.

Four general mechanisms leading to acquired antibiotic resistance have been described: (1) decreased entrance of the antibiotic into the bacterial cell; (2) increased extrusion of the antibiotic by bacterial efflux systems; (3) mutational modification of the antibiotic's target and; (4) production of antibiotic-inactivating enzymes. Characteristic examples for each mechanism are presented in Table 1.

| Mechanism | Examples |
|---|---|
| **Decreased permeability** | Diminished expression or loss of the OprD porin in *Pseudomonas aeruginosa* and OmpK35, OmpK36 porins in *Klebsiella pneumoniae* [6-9] |
| **Efflux** | Overexpression of MexAB-OprM and MexXY-OprM in *Pseudomonas aeruginosa* and OqxAB in *Klebsiella pneumoniae* [10-13] |
| **Target modification** | Mutations of gyrases and topoisomerases leading to fluoroquinolone resistance [14-16] |
| **Inactivating enzymes** | Production of beta-lactamases and aminoglycoside modifying enzymes [17-19] |

**Table 1.** Examples of antibiotic resistance mechanisms.

Among the aforementioned mechanisms, the production of beta-lactamases is considered of major importance because these enzymes are commonly transferable and inactivate multiple beta-lactam antibiotics. Within this large enzymatic family, carbapenemases (class B metallo-beta-lactamases (MBLs) [20] that contain zinc in their active center and class A KPC [21]) hydrolyze in vitro all or almost all beta-lactams, including carbapenems [22]. Class A extended spectrum beta-lactamases (ESBLs) hydrolyze penicillins, monobactams and cephalosporins whereas are inhibited by the beta-lactamase inhibitors [23,24]. Class C cephalosporinases (AmpC) present various spectrums of cephalosporin hydrolysis but are not inhibited by the beta-lactamase inhibitors [25]. Additionaly, AmpC enzymes may be inducible in *Serratia* spp, *Pseudomonas* spp, Indole-positive *Proteus*, *Citrobacter* spp and *Enterobacter* spp (SPICE group of bacteria) complicating the treatment of infections caused by these pathogens. Finally, molecular class D beta-lactamases (OXA) comprise numerous enzymes with variable spectrums of beta-lactam hydrolysis [26].

## 3. Phenotypic tests

Phenotypic tests may be used in the everyday laboratory practice in order to identify the presence of acquired resistance mechanisms among frequently isolated nosocomial pathogens. In the present chapter, the procedure and the interpretation of seven useful phenotypic tests are described. Special attention has been given to the phenotypic detection of beta-lactamases and especially those hydrolyzing carbapenems together with other beta-lactams (carbapenemases).

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### 3.1. Double Disc Synergy Test (DDST)

The DDST is used for the detection of beta-lactamases that are inhibited by beta-lactamase inhibitors such as clavulanic acid (Ambler class A beta-lactamases and especially ESBLs). For SPICE organisms, cloxacillin should be incorporated in Mueller-Hinton agar during its preparation in order to prevent any AmpC interference [27].

#### *3.1.1. Procedure*

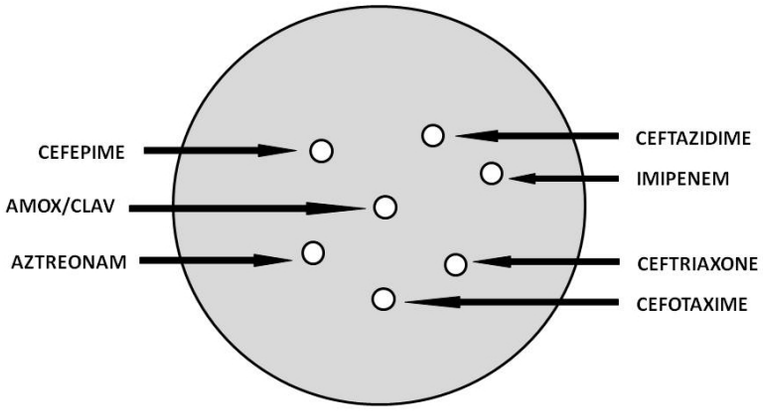


**Figure 1.** Double Disc Synergy Test preparation.

Step 1: Prepare agar plates containing 200μg/ml cloxacillin (by adding 1ml solution containing 80 mg cloxacillin in 399 ml Mueller-Hinton agar at the liquid phase). Omit this step when testing non-SPICE bacteria.

Step 2: Make a 0.5 McFarland bacterial suspension.

Step 3: Inoculate with a sterile cotton swab and place an amoxicillin/clavulanic acid disc at the center of the plate (20 μg amoxicillin+10 μg clavulanic acid).

Step 4: Place ceftazidime, imipenem, ceftriaxone, cefotaxime, aztreonam and cefepime discs around the central amoxicillin/clavulanic acid disc (Figure 1).

Step 5: Incubate at 37$^{o}$C for 18-24h.

#### *3.1.2. Interpretation*

The DDST is considered positive when the inhibition zone of any of the antibiotics is larger towards the clavulanic acid disc (Figure 2-lower left plate) or a ghost inhibition zone appears between the central disc and any of the other antibiotics (Figure 2-lower right plate). This is happening because of the ESBL's inhibition by the clavulanic acid. In proximity to the central disc the enzyme's activity is blocked. Thus, the growth inhibition zone appears only towards the clavulanic acid disc. If resistance to cephalosporins is not due to ESBL production, the test results negative (Figure 2-upper plates).

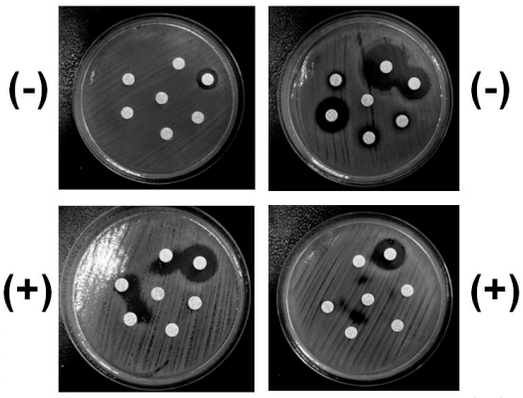


**Figure 2.** Interpretation of the DDST. (-): Negative; (+): Positive.

### 3.2. Imipenem-EDTA synergy test

EDTA (ethylene-diamine-tetraacetic acid) is a polyamino carboxylic acid that binds metal ions like zinc and can inactivate the metallo-beta-lactamases. Therefore, it is used for the phenotypic detection of MBL production in clinical isolates [28].

*3.2.1. Procedure*

Step 1: Soak paper discs within a 0.1 M EDTA solution.

Step 2: Make a 0.5 McFarland bacterial suspension.

Step 3: Inoculate with a sterile cotton swab and place an imipenem and a ceftazidime disc at the center of the plate.

Step 4: Place the EDTA discs at both sides in respect to the antibiotics as shown in Figure 3.

Step 5: Incubate at 37$^{o}$C for 18-24h.

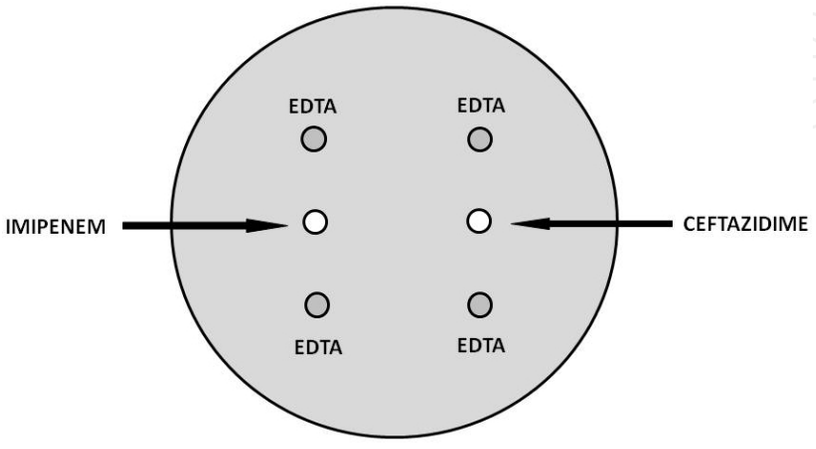


**Figure 3.** Preparation of the imipenem-EDTA synergy test.

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*3.2.2. Interpretation*

The imipenem-EDTA synergy test is positive when the inhibition zone takes a characteristic keyhole shape because of the MBL inactivation by the EDTA (Figure 4). In proximity to the EDTA discs, the hydrolytic activity of MBLs is blocked. Consequently, imipenem and ceftazidime inhibition zones may appear larger towards the EDTA discs.

### 3.3. Boronic acid test

Phenylboronic acid acts as an inhibitor for KPC carbapenemases and class A and C beta-lactamases. The boronic acid test has been proposed for the phenotypic detection of KPC-producers because it is easier to perform than the DDST and also presents less false positive results because of the presence of ESBLs or AmpC beta-lactamases [29-31].

*3.3.1. Procedure*

Step 1: Make a 0.5 McFarland bacterial suspension.

Step 2: Inoculate with a sterile cotton swab and place two meropenem discs (Figure 5).

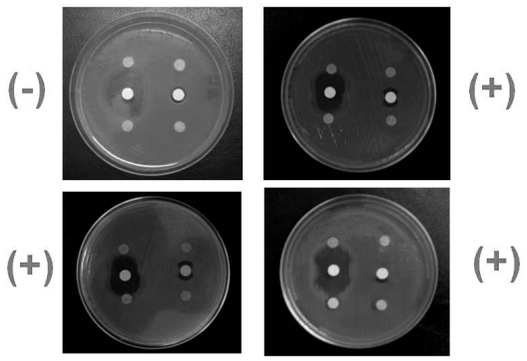


**Figure 4.** Interpretation of the imipenem-EDTA synergy test. (-): Negative; (+): Positive. Note that the upper left isolate is negative for MBL production but shows positive D-test between imipenem and ceftazidime indicating for the presence of inducible AmpC beta-lactamases (The D-test is described in paragraph 3.6).

Step 3: Add 20 μl of phenylboronic acid 20 g/L on one of the two meropenem discs.

Step 4: Incubate at 37°C for 18-24h.

*3.3.2. Interpretation*

In case of KPC production, the phenylboronic acid that has been added to the second meropenem disc will block the hydrolytic activity of the enzyme. As a consequence, the second disc will have a larger inhibition halo. The test is considered positive when the inhibition zone of the meropenem+phenylboronic acid is ≥ 5mm larger than the inhibition zone of meropenem alone (Figure 6).

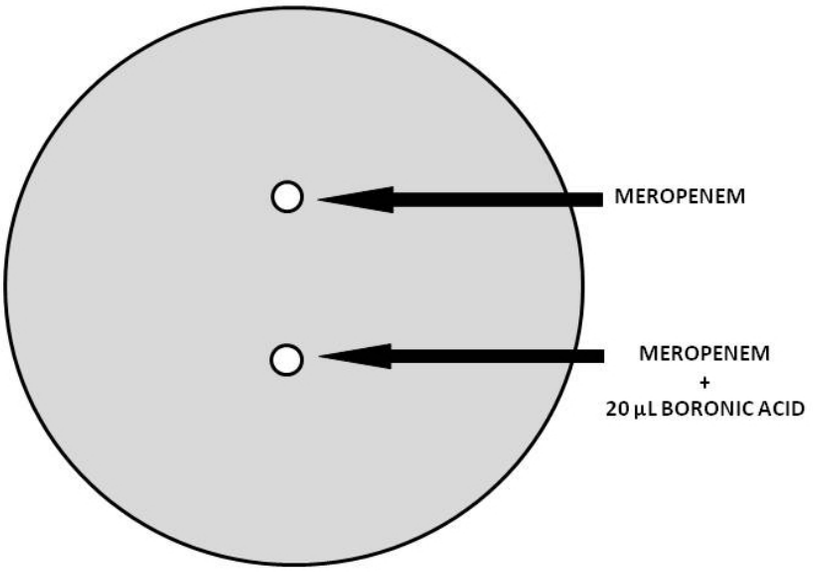


**Figure 5.** Preparation of the boronic acid test.

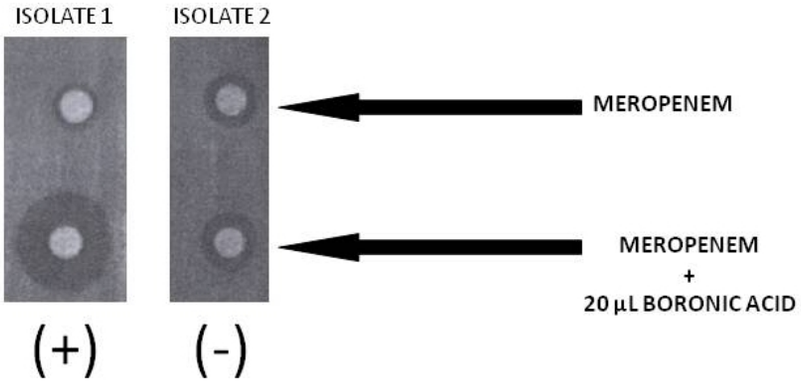


**Figure 6.** Interpretation of the boronic acid test. (+): Positive; (-): Negative.

### 3.4. Hodge test

The Hodge test is used to reveal carbapenemase production [32]. This is achieved by inoculating the study isolate together with a carbapenem-susceptible indicator strain and evaluating the distortion of the indicator strain's inhibition zone because of carbapenemase production by the study isolate. Despite its usefulness, this test presents a disadvantage: it detects the presence of carbapenemases only, without being able to discriminate between different carbapenemase types (KPC or MBLs).

#### *3.4.1. Procedure*

Step 1: Make a 0.5 McFarland suspension of the indicator strain (for example *E. coli* ATCC 25922).

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Step 2: Inoculate with a sterile cotton swab and place a carbapenem disc at the center of the plate.

Step 3: Streak 3-5 colonies of the test isolate from the center to the periphery of the plate (Figure 7).

Step 4: Incubate at 37°C for 18-24h.

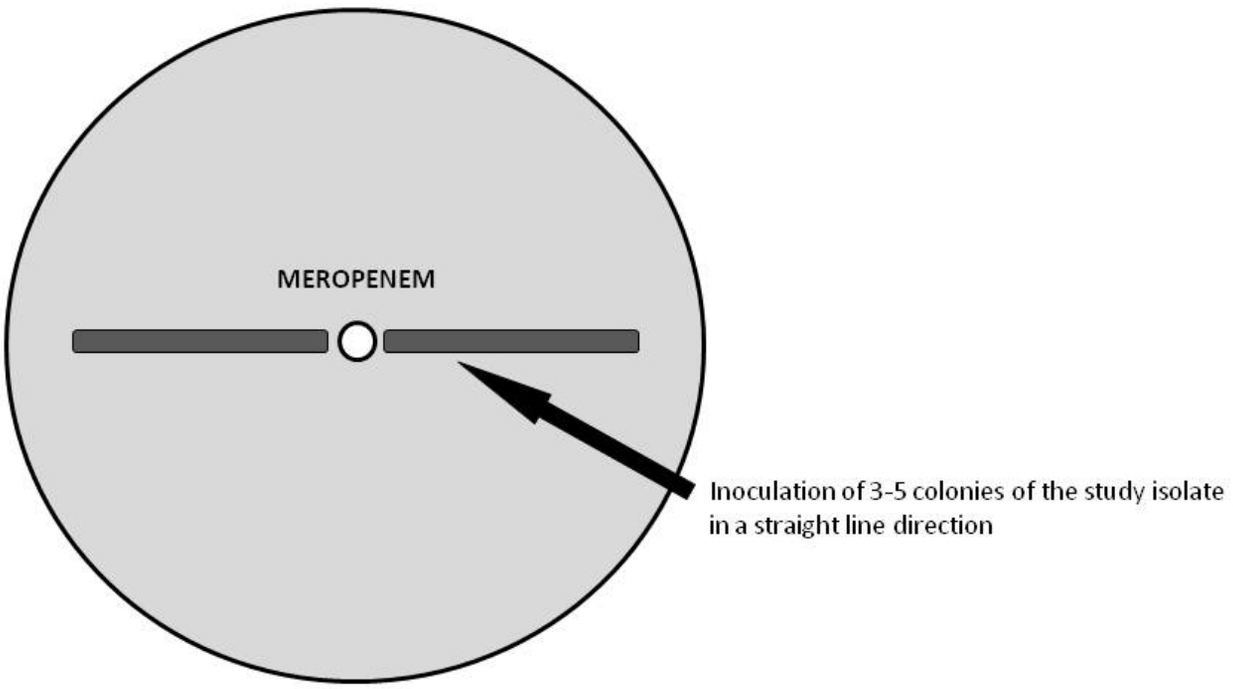


**Figure 7.** Preparation of the Hodge test.

*3.4.2. Interpretation*

The presence of a distorted inhibition zone due to growth of the indicator strain toward the carbapenem disc is interpreted as a positive result (Figure 8). This occurs due to carbapenemase production by the study isolate. Uncertain results need to be confirmed by other tests or molecular methods.

### 3.5. Combination meropenem disc test

This test is a combination of the EDTA and the boronic acid test in a single plate and has been introduced in Greece after the emergence of Gram negative isolates co-producing KPC and MBL carbapenemases [33-37]. The advantage of the test is that it discriminates between carbapenem-susceptible, KPC-producing, MBL-producing and double-carbapenemase-producing bacteria.

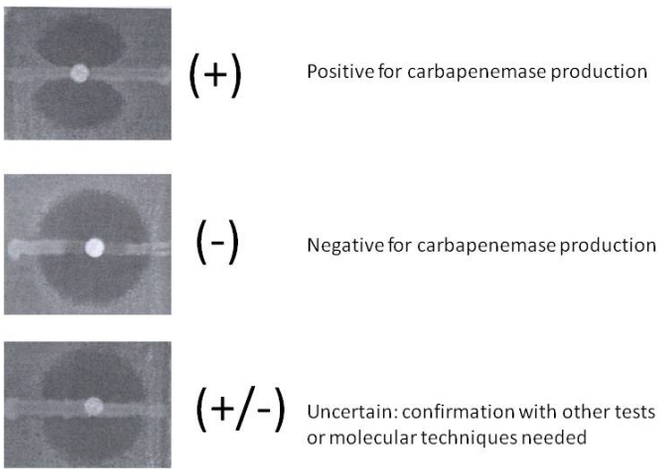


**Figure 8.** Interpretation of the Hodge test.

*3.5.1. Procedure*

Step 1: Make a 0.5 McFarland bacterial suspension.

Step 2: Inoculate with a sterile cotton swab and place four meropenem discs (Figure 9).

Step 3: Add 10 μl EDTA 0.1 M on the second disc, 20 μl of phenylboronic acid 20 g/L on the third disc and 20 μl of phenylboronic acid 20 g/L+10 μl EDTA 0.1 M on the fourth disc.

Step 4: Incubate at 37ºC for 18-24h.

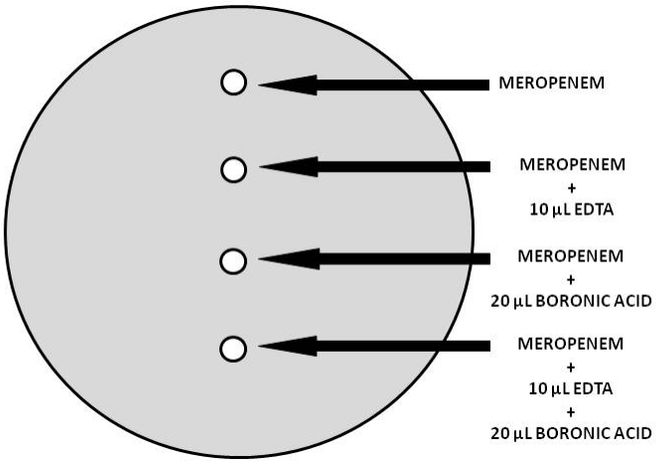


**Figure 9.** Preparation of the combination meropenem disc test.

*3.5.2. Interpretation*

The interpretation of the combination meropenem disc test is based on the comparison between the inhibition zones of the four meropenem discs as presented in Figure 10. If no carbapenemase is present, the zone diameters of the discs where inhibitors have been added

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will not present significant differences (≥5mm) from the meropenem disc alone. In case of KPC production, an increase of ≥5mm in the discs that are supplemented with boronic acid will be observed. MBL production will become evident by an increase of ≥5mm in the discs that are supplemented with EDTA. In case of a KPC+MBL-producer, the fourth disc will present the larger zone diameter of all. The EDTA-supplemented and boronic acid-supplemented discs may or may not have a ≥5mm larger zone diameter than that of the meropenem disc alone.

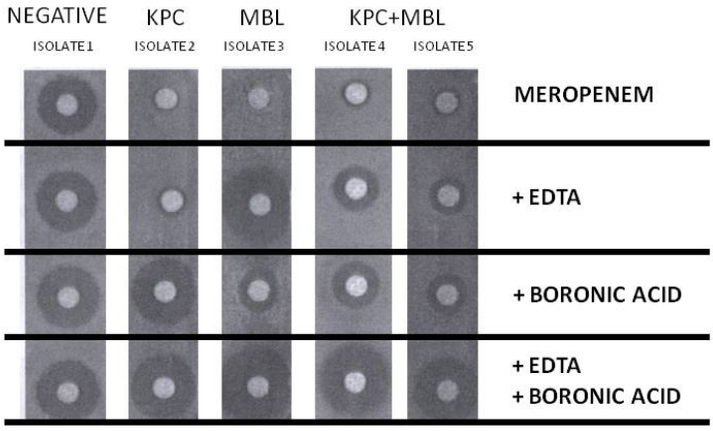


**Figure 10.** Interpretation of the combination meropenem disc test.

Recently, a novel variation of this test has been proposed [38] for surveillance cultures from rectal swabs. The same principle is generally followed, except that each swab is initially suspended in 1 ml sterile saline by rotating and agitating it to release the microorganisms. Afterwards, the suspension is cultured onto McConkey agar using a different swab. This method allows the identification and differentiation of carbapenemase-producing *Enterobacteriaceae* (Figure 11) directly at patient admission.

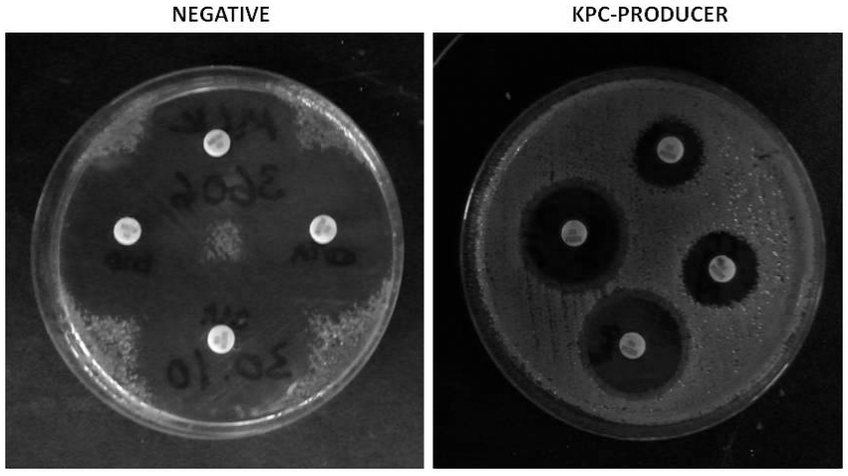


**Figure 11.** Application of the combination meropenem disc test for the direct differentiation of carbapenemase-producing *Enterobacteriaceae* in rectal swabs.

### 3.6. D-test

The D-test is used for the detection of inducible AmpC beta-lactamases [39]. An antibiotic is used as an inducer for AmpC production (imipenem or cefoxitin) whereas others are used as substrates (ceftazidime, cefotaxime, piperacillin/tazobactam).

#### *3.6.1. Procedure*

Step 1: Make a 0.5 McFarland bacterial suspension.

Step 2: Inoculate with a sterile cotton swab and place an imipenem disc.

Step 3: Place substrate discs (for example ceftazidime and piperacillin/tazobactam) near the imipenem disc as shown in Figure 12.

Step 4: Incubate at 37°C for 18-24h.

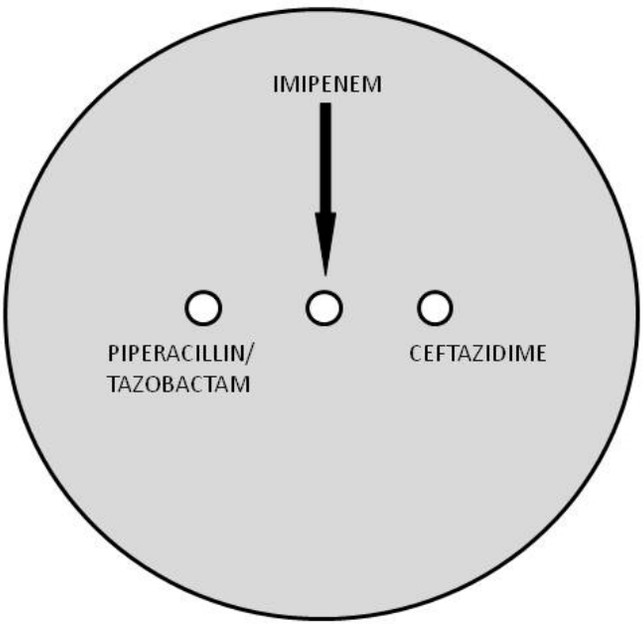


**Figure 12.** Preparation of a D-test.

#### *3.6.2. Interpretation*

The test is positive when a D-shaped inhibition zone is observed for one of the substrate discs (Figure 13) because of the imipenem-mediated induction of the AmpC production and the subsequent inactivation of the substrate antibiotic by the beta-lactamase. An important advantage of the test is that it can be easily incorporated within any routine antibiogram as shown in Figure 14.

### 3.7. CCCP test

CCCP (Carbonyl cyanide m-chlorophenyl hydrazone) is an efflux pump inhibitor that can be added in Mueller-Hinton agar during its preparation. The test is used to detect efflux pump overexpression that contributes to or determines carbapenem resistance in the study isolate [40].

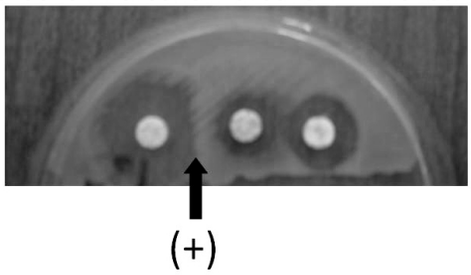


**Figure 13.** Interpretation of the D-test.

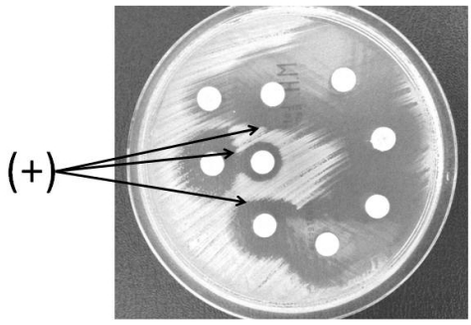


**Figure 14.** Incorporation of the D-test in a common antibiogram.

*3.7.1. Procedure*

Step 1: Prepare agar plates containing CCCP at a concentration of 12.5 μM.

Step 2: Make a 0.5 McFarland bacterial suspension.

Step 3: Inoculate with a sterile cotton swab on a CCCP-supplemented plate and in parallel on a CCCP-free plate. For economy reasons, two isolates may be inoculated on the same plate as shown in Figure 15.

Step 4: Place a meropenem disc on both plates for each inoculation.

Step 5: Incubate at 37°C for 18-24h.

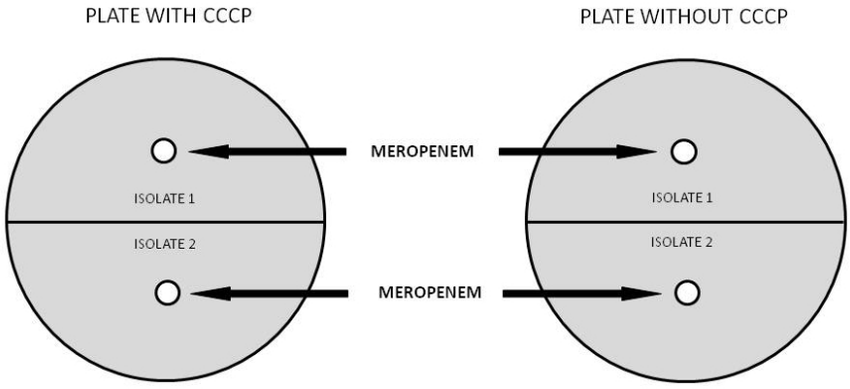


**Figure 15.** Preparation of the CCCP test.

*3.7.2. Interpretation*

The test is considered positive when synergy between meropenem and CCCP is observed on the CCCP-supplemented plate (Figure 16).

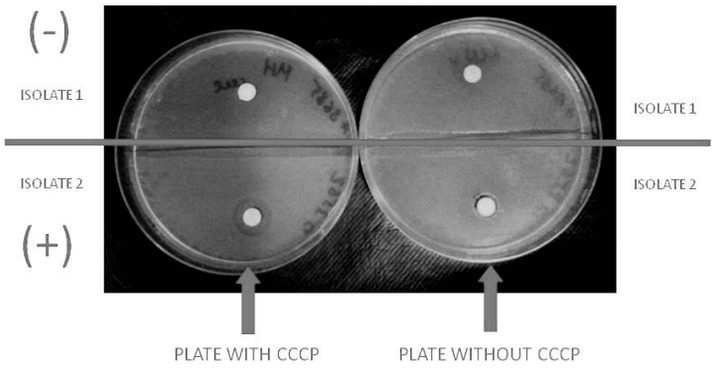


**Figure 16.** Interpretation of the CCCP test. Isolate 1 is inoculated on the upper side and isolate 2 is inoculated on the lower side of the plates. (+): Positive; (-): Negative.

## 4. Molecular methods

Genetic methods for the detection of resistance genes are based on nucleic acid hybridization and amplification. Therefore, the knowledge of specific primers (amplification nucleotides) and probes (labeled single-stranded ologonucleotides) is necessary in order to detect the genetic target of interest. The technique used depends on the type of resistance that is suspected. A simple polymerase chain reaction (PCR) may be applied searching for a gene that confers a certain level of resistance when it is expressed. This is the case for example, of genes encoding for antibiotic-inactivating enzymes. In Tables 2 and 3, primers for the detection of aminoglycoside or fluorquinolone resistance-conferring enzymes and beta-lactamases are shown, respectively.

| Gene | Primers (5′- 3′) | Product size | Reference |
|---|---|---|---|
| **aac(6′)-Ia** | ATGAATTATCAAATTGTG | 558 bp | [41] |
| | TTACTCTTTGATTAAACT | | |
| **aac(6′)-Ic** | CTACGATTACGTCAACGGCTGC | 130 bp | [42] |
| | TTGCTTCGCCCACTCCTGCACC | | |
| **aac(3)-Ia** | ACCTACTCCCAACATCAGCC | 169 bp | [43] |
| | ATATAGATCTCACTACGCGC | | |
| **aac(3)-Ic** | GATGATCTCTACTCAAACC | 472 bp | [44] |
| | TTAGGCAGCAGGTTGAGG | | |
| **aac(3)-IV** | GTTACACCGGACCTTGGA | 675 bp | [45] |
| | AACGGCATTGAGCGTCAG | | |
| **aphA-3** | GGGACCACCTATGATGTGGAACG | 595 bp | [46] |
| | CAGGCTTGATCCCCAGTAAGTC | | |
| **aph(3′)-Via** | ATACAGAGACCACATACAGT | 235 bp | [47] |
| | GGACAATCAATAATAGCAAT | | |
| **aad(2′′)-Ia** | ATGTTACGCAGCAGGGCAGTCG | 188 bp | [48] |
| | CGTCAGATCAATATCATCGTGC | | |
| **aph(3′)-IIIa** | GGCTAAAATGAGAATATCACCGG | 523 bp | [49,50] |
| | CTTTAAAAAATCATACAGCTCGCG | | |
| **ant(4′)-Ia** | CAAACTGCTAAATCGGTAGAAGCC | 294 bp | [49,50] |
| | GGAAAGTTGACCAGACATTACGAACT | | |
| **strA-strB** | TATCTGCGATTGGACCCTCTG | 519 bp | [51] |
| | CATTGCTCATCATTTGATCGGCT | | |
| **armA** | AGGTTGTTTCCATTTCTGAG | 590 bp | [52] |
| | TCTCTTCCATTCCCTTCTCC | | |
| **rmtA** | CTAGCGTCCATCCTTTCCTC | 635 bp | [53] |
| | TTTGCTTCCATGCCCTTGCC | | |
| **rmtB** | ATGAACATCAACGATGCCCT | 769 bp | [54] |
| | CCTTCTGATTGGCTTATCCA | | |
| **gyrA** (***A. baumannii***) | AAATCTGCCCGTGTCGTTGGT | 343 bp | [55] |
| | GCCATACCTACGGCGATACC | | |
| **gyrA (*E. coli*)** | ACGTACTAGGCAATGACTGG | 190 bp | [56] |
| | AGAAGTCGCCGTCGATAGAAC | | |
| **qnrA** | TCAGCAAGAGGATTTCTCA | 627 bp | [57] |
| | GGCAGCACTATTACTCCCA | | |
| **Qnr** | CCGTATGGATATTATTGATAAAG | 661 bp | [58] |
| | CTAATCCGGCAGCACTATTA | | |

**Table 2.** Primers used for the detection of aminoglycoside and quinolone resistance determinants.

| Gene | Primers (5'- 3') | Product size | Reference |
|---|---|---|---|
| **$bla_{SHV}$** | GGTTATGCGTTATATTCGCC | 867 bp | [59] |
| | TTAGCGTTGCCAGTGCTC | | |
| **$bla_{TEM}$** | ATGAGTATTCAACATTTCCG | 867 bp | [59] |
| | CTGACAGTTACCAATGCTTA | | |
| **$bla_{CTX-M}$** | CGCTTTGCGATGTGCAG | 550 bp | [60] |
| | ACCGCGATATCGTTGGT | | |
| **$bla_{CTX-M-2}$** | ATGATGACTCAGAGCATTCG | 884 bp | [61] |
| | TTATTGCATCAGAAACCGTG | | |
| **$bla_{CTX-M-9}$** | GTGACAAAGAGAGTGCAACGG | 857 bp | [62] |
| | ATGATTCTCGCCGCTGAAGCC | | |
| **$bla_{CTX-M-10}$** | GCTGATGAGCGCTTTGCG | 684 bp | [63] |
| | TTACAAACCGTTGGTGACG | | |
| **$bla_{GES/IBC}$** | GTTTTGCAATGTGCTCAACG | 371 bp | [64] |
| | TGCCATAGCAATAGGCGTAG | | |
| **$bla_{PER-1}$** | ATGAATGTCATTATAAAAGC | 926 bp | [65] |
| | AATTTGGGCTTAGGGCAAGAAA | | |
| **$bla_{PER-2}$** | CGCTTCTGCTCTGCTGAT | 469 bp | [66] |
| | GGCAGCTTCTTTAACGCC | | |
| **$bla_{PSE}$** | ACCGTATTGAGCCTGATTTA | 321 bp | [67] |
| | ATTGAAGCCTGTGTTTGAGC | | |
| **$bla_{TLA-1}$** | TCTCAGCGCAAATCCGCG | 974 bp | [68] |
| | CTATTTCCCATCCTTAACTAG | | |
| **$bla_{VEB-1}$** | CGACTTCCATTTCCCGATGC | 643 bp | [69] |
| | GGACTCTGCAACAAATACGC | | |
| **$bla_{KPC}$** | TGTCACTGTATCGCCGTC | 331 bp | [70] |
| | TATTTTTCCGAGATGGGTGAC | | |
| **$bla_{SME-1}$** | AACGGCTTCATTTTTGTTTAG | 830 bp | [71] |
| | GCTTCCGCAATAGTTTTATCA | | |
| **$bla_{IMP}$** | CTACCGCAGCAGAGTCTTTG | 587 bp | [72] |
| | AACCAGTTTTGCCTTACCAT | | |
| **$bla_{IMP-1}$** | ATGAGCAAGTTATCTGTATTC | 741 bp | [73] |
| | TTAGTTGCTTGGTTTTGATGG | | |
| **$bla_{IMP-2}$** | ATGAAGAAATTATTTGTTTTATG | 741 bp | [73] |
| | TTAGTTACTTGGCTGTGATG | | |

| Gene | Primers (5'- 3') | Product size | Reference |
|---|---|---|---|
| **$bla_{VIM}$** | TCTACATGACCGCGTCTGTC | 748 bp | [74] |
| | TGTGCTTTGACAACGTTCGC | | |
| **$bla_{VIM-1}$** | GTTAAAAGTTATTAGTAGTTTATTG | 799 bp | [73] |
| | CTACTCGGCGACTGAGC | | |
| **$bla_{VIM-2}$** | ATGTTCAAACTTTTGAGTAAG | 801 bp | [73] |
| | CTACTCAACGACTGAGCG | | |
| **$bla_{SPM-1}$** | CCTACAATCTAACGGCGACC | 649 bp | [75] |
| | TCGCCGTGTCCAGGTATAAC | | |
| **$bla_{NDM-1}$** | GGTTTGGCGATCTGGTTTTC | 621 bp | [76] |
| | CGGAATGGCTCATCACGATC | | |
| **$bla_{OXA-1}$** | CCAAAGACGTGGATG | 540 bp | [77] |
| | GTTAAATTCGACCCCAAGTT | | |
| **$bla_{OXA-10}$** | CGTGCTTTGTAAAAGTAGCAG | 652 bp | [78] |
| | CATGATTTTGGTGGGAATGG | | |
| **$bla_{OXA-23}$** | CCTCAGGTGTGCTGGTTATTC | 513 bp | [79] |
| | CCCAACCAGTCTTTCCAAAA | | |
| **$bla_{OXA-24}$** | TTCCCCTAACATGAATTTGT | 1020 bp | [80] |
| | GTACTAATCAAAGTTGTGAA | | |

**Table 3.** Primers used for the detection of beta-lactamases frequently encountered among Gram negative pathogens.

In cases in which resistance depends upon the expression level (overexpression or downregulation) of the gene, real time Reverse Transcriptase-PCR (rt RT-PCR) is used to detect not only the presence, but also the mRNA expression of the gene. The results are consequently confronted with the expression level of the same gene in a control strain. This technique is useful for the study of the expression of specific porins and efflux pumps (primers and probes for such resistance determinants in *P. aeruginosa* are shown in Table 4).

| Gene | Primers (5'- 3') | Reference |
|---|---|---|
| **ampC** | CGCCGTACAACCGGTGAT | [81] |
| | CGGCCGTCCTCTTTCGA | |
| **probe** | [DFAM]TCAGCCTGAAAGGAGAACCGCATTACTTC[DTAM] | |
| **OprD** | CTACGGCTACGGCGAGGAT | [81] |
| | GACCGGACTGGACCACGTACT | |
| **probe** | [DFAM]CACCACGAAACCAACCTCGAAGCC[DTAM] | |
| **mexA** | AACCCGAACAACGAGCTG | [81] |

| Gene | Primers (5'- 3') | Reference |
|---|---|---|
| | ATGGCCTTCTGCTTGACG | |
| **probe** | [DFAM]CATGTTCGTTCACGCGCAGTTG[DTAM] | |
| **mexC** | GGAAGAGCGACAGGAGGC | [81] |
| | CTGCACCGTCAGGCCCTC | |
| **probe** | [DFAM]CCGAAATGGTGTTGCCGGTG[DTAM] | |
| **mexE** | TACTGGTCCTGAGCGCCT | [81] |
| | TCAGCGGTTGTTCGATGA | |
| **probe** | [DFAM]CGGAAACCACCCAAGGCATG[DTAM] | |
| **mexX** | GGCTTGGTGGAAGACGTG | [81] |
| | GGCTGATGATCCAGTCGC | |
| **probe** | [DFAM]CCGACACCCTGCAGGGCC[DTAM] | |

**Table 4.** Primers and probes used in real-time RT PCR for the determination of the expression levels for specific resistance mechanisms in *P. aeruginosa.*

Finally, sequencing [82-84] of the PCR product allows its confrontation with the already known gene sequences that are available in genetic databases. This can lead to the detection of mutations or to the characterization and classification of the gene within a genetic family.

## 5. Conclusion

There are several benefits and limitations using either phenotypic or molecular methods for the detection of resistance mechanisms in Gram negative pathogens. Phenotypic tests require bacteria in pure culture from a clinical sample thus needing 24-48h to obtain a final result. Molecular techniques on the other hand, can be performed directly with clinical speciments reducing significantly the procedure time.

The detection of low-level resistance is by definition problematic using phenotypic tests thus interpretation problems may appear. In such cases, molecular techniques are an option for clarifying the possible involvement of any known resistance mechanism.

Moreover, genetic detection gives a definite answer for the presence or not of specific resistance determinants within a study isolate (a specific beta-lactamase for example) whereas this is not possible with the phenotypic tests which provide only general information about the resistance mechanisms involved.

Genetic assays however, present also some major limitations: (i) It is possible to screen exclusively for known mechanisms and for one gene at the time (unless a multiplex PCR assay [85-88] can be applied) and; (ii) their cost is high and becomes higher when screening for multiple resistance determinants.

Consequently, the combined and rational use of the available methodologies seems to be the optimal solution for the cost-effective detection of resistance mechanisms in Gram negative pathogens by the Clinical Microbiology laboratory.

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## Author details

Meletis Georgios[1,2*], Tzampaz Egki[3] and Sianou Effrosyni[3]

*Address all correspondence to: meletisg@hotmail.com

1 Research Assistant, Aristotle University of Thessaloniki, School of Medicine, Thessaloniki, Greece

2 Clinical Microbiologist, Department of Clinical Microbiology, St. Paul's General Hospital, Thessaloniki, Greece

3 Clinical Microbiologist, Department of Clinical Microbiology, Hippokration General Hospital, Thessaloniki, Greece

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