Chapter 6

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

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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

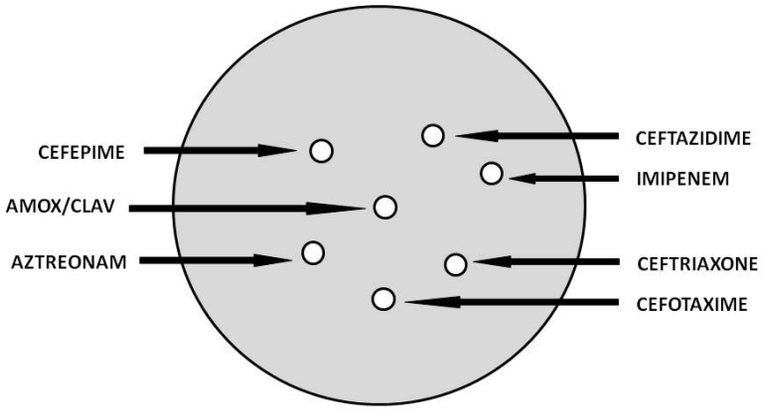

Figure 1. Double Disc Synergy Test preparation.

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).

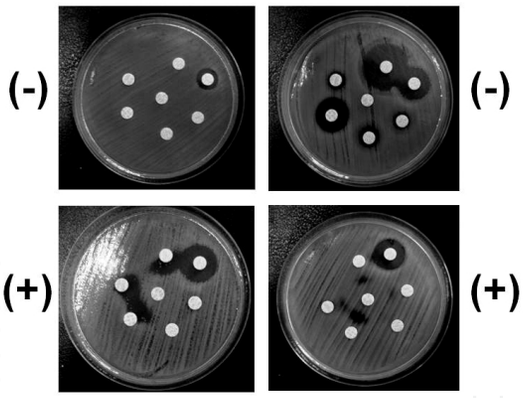

Figure 2. Interpretation of the DDST. (-): Negative; (+): Positive.

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.

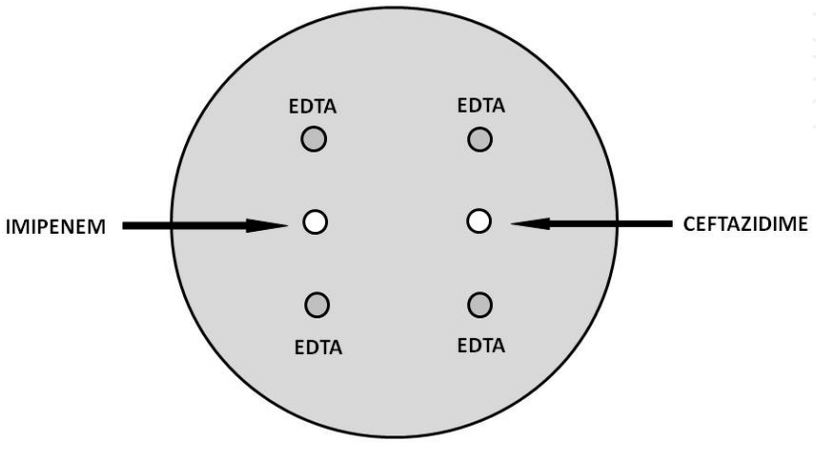

Figure 3. Preparation of the imipenem-EDTA synergy test.

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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).

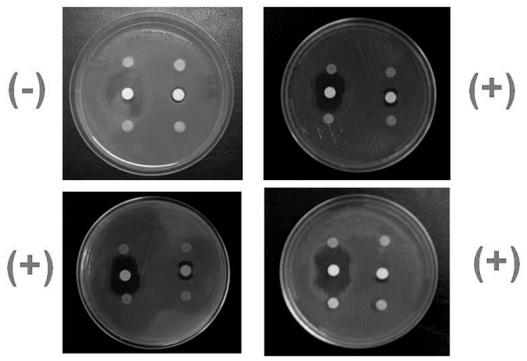

Figure 4. Interpretation of the imipenem-EDTA synergy test. (-): Negative; (+): Positive. Note that the upper left isolate is negative for MBL production but shows positive D-test between imipenem and ceftazidime indicating for the presence of inducible AmpC beta-lactamases (The D-test is described in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

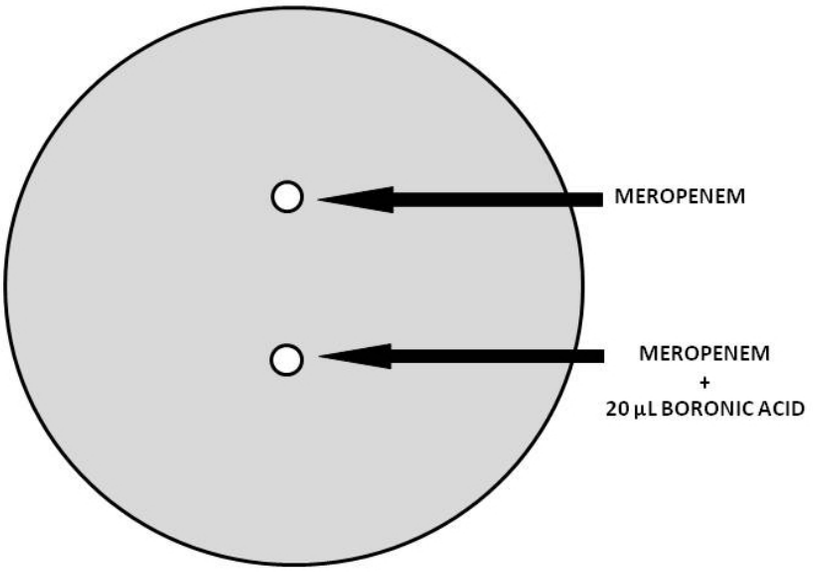

Figure 5. Preparation of the boronic acid test.

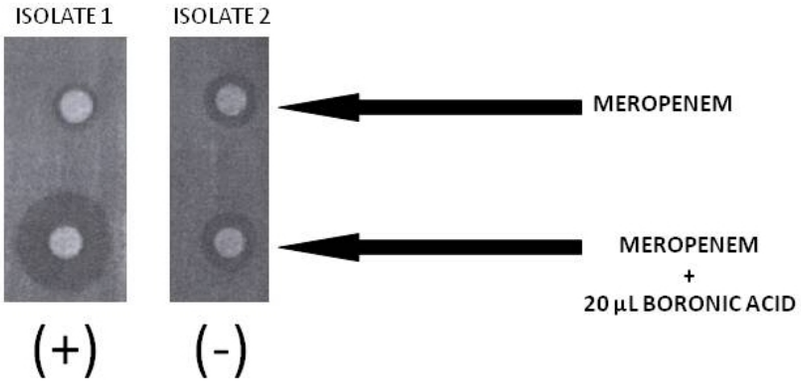

Figure 6. Interpretation of the boronic acid test. (+): Positive; (-): Negative.

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.

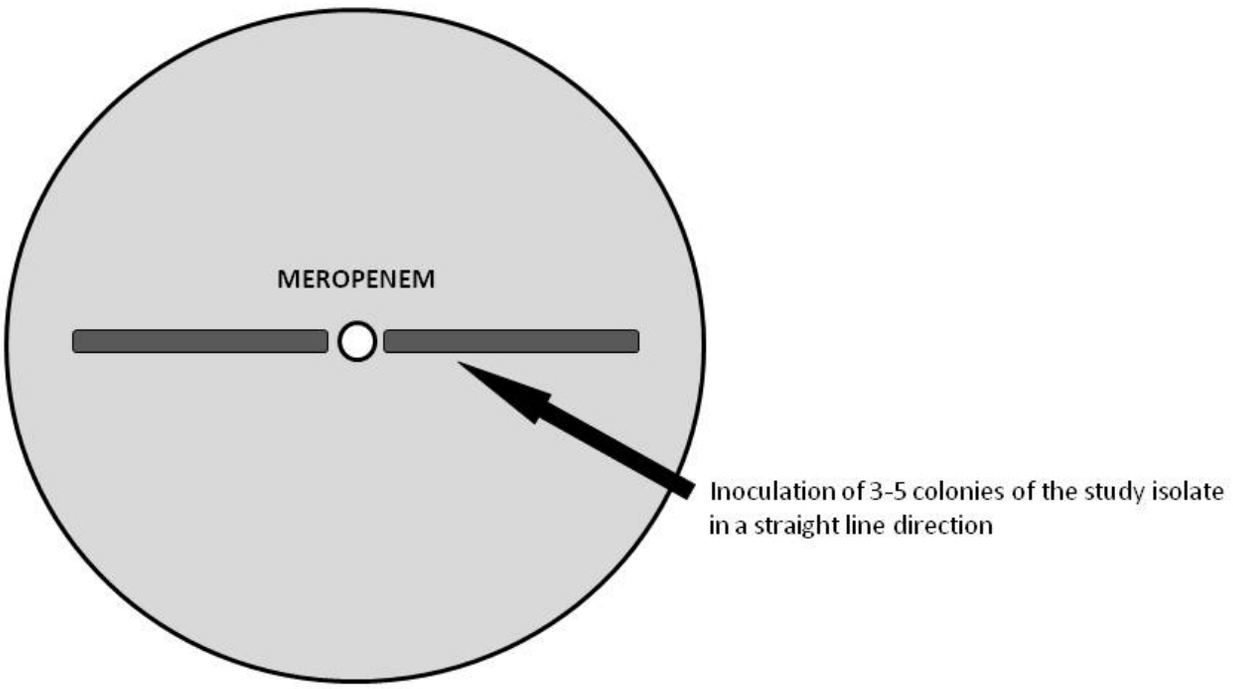

Figure 7. Preparation of the Hodge test.

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.

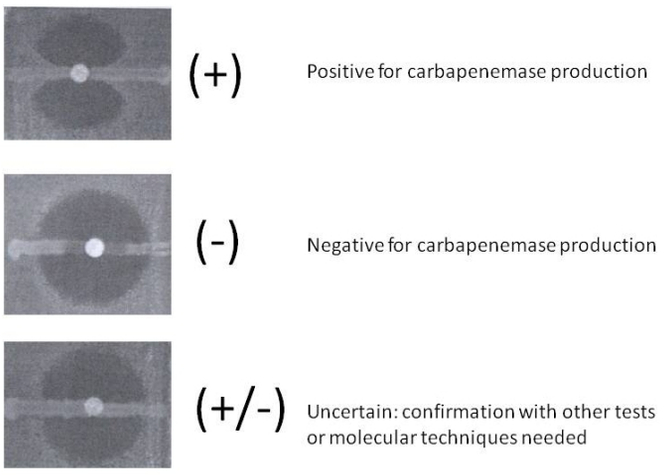

Figure 8. Interpretation of the Hodge test.

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.

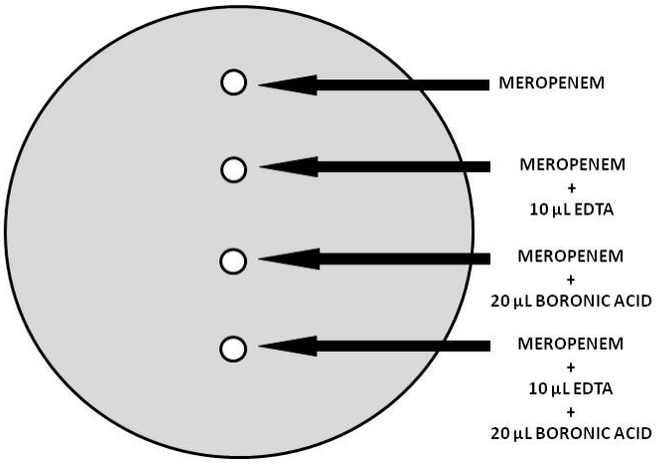

Figure 9. Preparation of the combination meropenem disc test.

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.

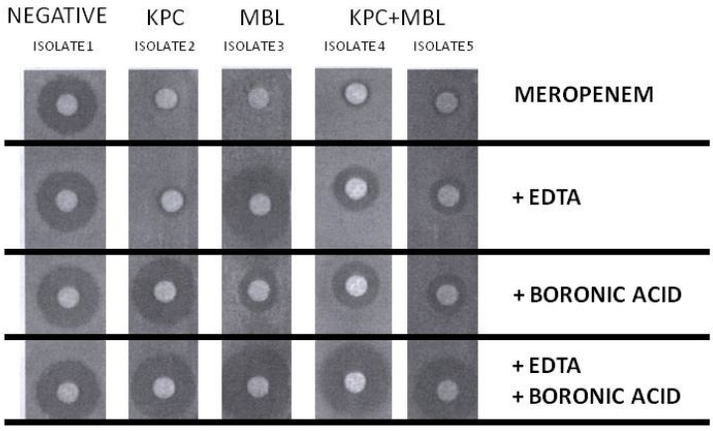

Figure 10. Interpretation of the combination meropenem disc test.

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.

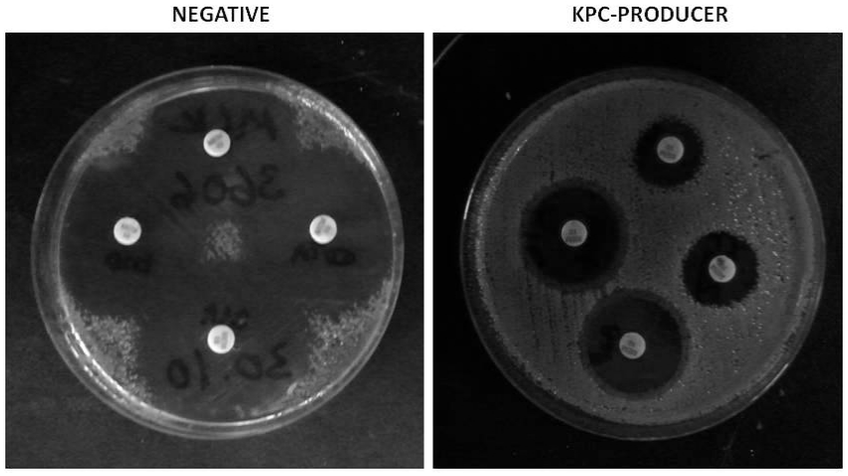

Figure 11. Application of the combination meropenem disc test for the direct differentiation of carbapenemase-producing *Enterobacteriaceae* in rectal swabs.

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.

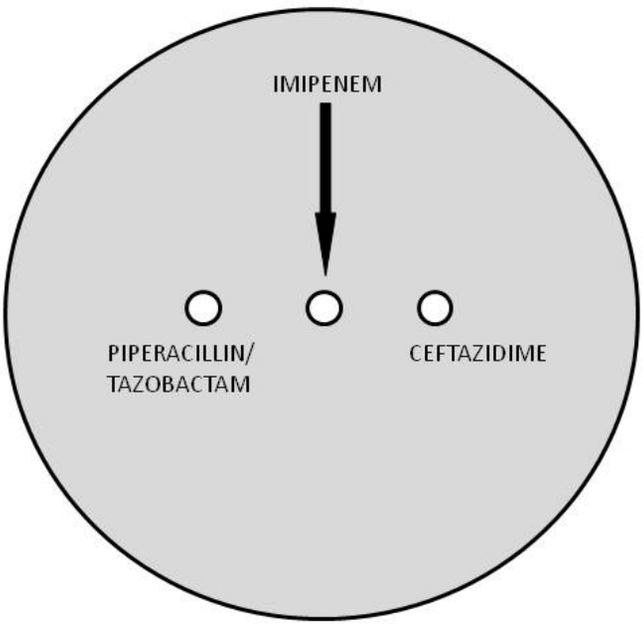

Figure 12. Preparation of a D-test.

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].

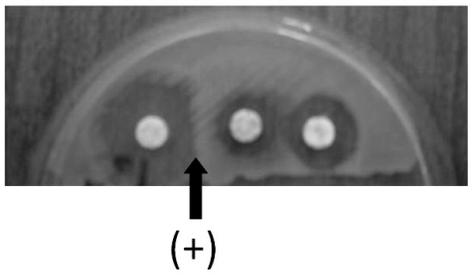

Figure 13. Interpretation of the D-test.

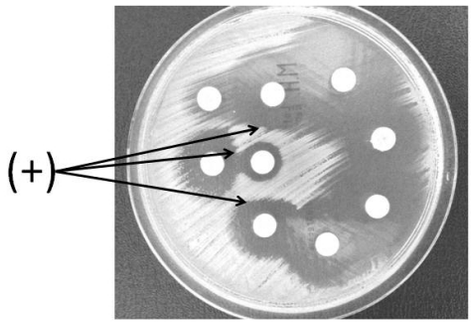

Figure 14. Incorporation of the D-test in a common antibiogram.

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.

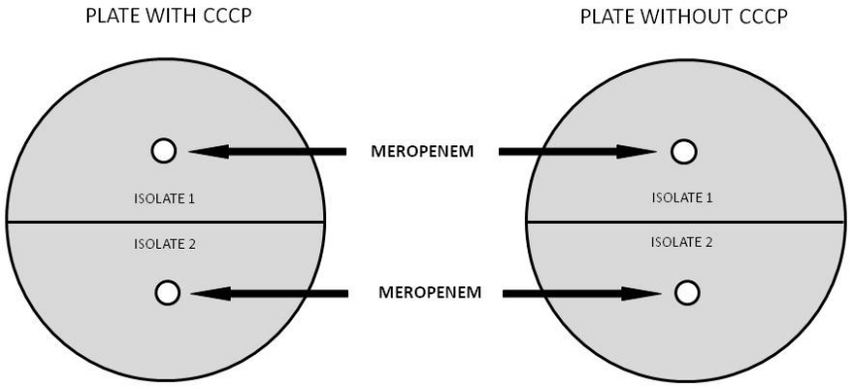

Figure 15. Preparation of the CCCP test.

3.7.2. Interpretation

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

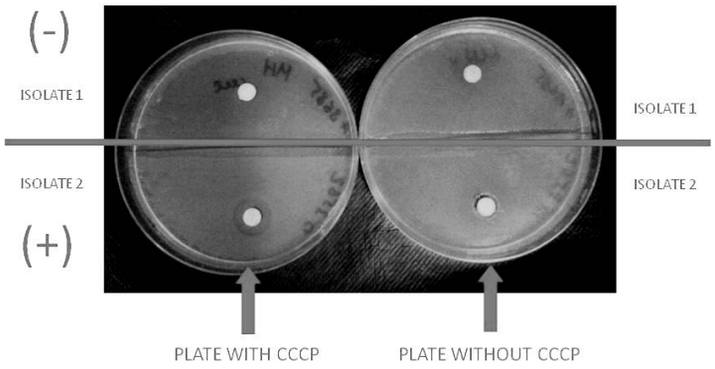

Figure 16. Interpretation of the CCCP test. Isolate 1 is inoculated on the upper side and isolate 2 is inoculated on the lower side of the plates. (+): Positive; (-): Negative.

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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