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REVIEW

PHENOTYPIC METHODS FOR DETECTION OF BETA-LACTAMASE-MEDIATED RESISTANCE IN E. COLI AND KLEBSIELLA PNEUMONIAE

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Abstract. Enterobacteriaceae are major pathogens in both hospital and community. Recently the number of MDR phenotypes (multi drug resistant) increased, with the emergence of XDR phenotypes (extremely drug resistant – susceptible only to colistin and tigecycline). It is extremely important to detect and correct the reporting mechanisms for resistance. Many clinical laboratories have problems detecting extended-spectrum beta-lactamases, class C beta-lactamases and carbapenemases. Confusion exists regarding the importance of these resistance mechanisms, optimal testing methods and appropriate reporting conventions. Failure to correctly detect these enzymes contributed to their spread and sometimes, to inappropriate use of antibiotics. Clinical laboratories need to have expertise to provide a rapid and clinically relevant antibiogram result in hospitals where these resistance mechanisms are encountered. This review aims to summarize the most important methods for detection of extended spectrum β-lactamases, class C beta-lactamases and carbapenemases. We studied recent and relevant literature retrieved from PubMed and the internet.
Keywords: ESBL, AmpC, carbapenemases, clavulanic acid, resistance

Introduction

Constant growth variety of β-lactamases reported in strains of Enterobacteriaceae, especially *E. coli* and *K. pneumoniae*, raise important issues regarding the diagnosis. Many methods have been described for detection of ESBL (extended spectrum β-lactamases), AmpC β-lactamases (class C) and carbapenemases but some of these methods are technically complicated, raise certain questions of interpretation, others require special reagents difficult to obtain. Failure to detect resistance to antibiotics can have severe consequences for the patient. Microbiology laboratories must constantly improve testing methods and provide accurate results to clinicians.

Extended spectrum β-lactamases (ESBL)

The increasing prevalence of ESBL-producing *E. coli* and *Klebsiella pneumoniae* worldwide has created a great need for simple and accurate techniques of laboratory testing for identification [1].

In ESBL producing bacteria, all the members of penicillins, cephalosporins and monobactam families are ineffective. Therefore only beta-lactamase inhibitors and carbapenems are drugs of choice.

In the microbiology laboratory, detection of ESBL can be done with phenotypic or genotypic testing. The phenotypic methods are based on resistance that gives ESBL to oxymino beta-lactam and beta-lactamase inhibitors ability (usually clavulanate) to block this resistance [2].

Phenotypic methods can be divided into screening tests and confirmatory tests [3].

The following tests have been applied in clinical microbiology. The initial screening step consists of testing for resistance with cephalosporin disks: ceftriaxone, cefotaxime, ceftazidime, cefpodoxime or aztreonam. The sensitivity of the method can vary. If it is not possible to use more than one antibiotic, it is advisable to use ceftazidime or cefpodoxime. Latest studies establish the important

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role of cefepime in testing methods [4].

The confirmatory step is based on the synergy between cephalosporins and clavulanic acid. These tests distinguish between AmpC enzymes which are not inhibited by inhibitors of β-lactamases and ESBL. Several methods can be used: double disc synergy (DD), the combination disc method, ESBL E-tests.

The DD method proposed by CA-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie) is performed by inoculating the test strain (inoculum of 0.5 McFarland) on a Mueller-Hinton agar plate and then placing a disc containing amoxicillin-clavulanate (20 + 10 mg) in the center of the plate and, at a distance of 25-30 mm from the center, standard 30 μg discs of ceftazidime, ceftriaxone, cefotaxime, aztreonam, or 10 μg cefpodoxime disc. Enlargement or distortion of the inhibition zone between the discs of cephalosporins and the clavulanic acid disc indicates ESBL [5,6].

The combined disc method (fig. 1) consists in comparing the inhibition zone diameter on a disc of cephalosporin with and without clavulanic acid. 10 μg of clavulanic acid are added to the cefotaxime disc (30 μg) and the ceftazidime disc (30 μg). If the strain is ESBL producer the inhibition zone for the disc with clavulanic acid increases with ≥ 5 mm compared to that of the disc without inhibitor. Another variant of the test uses cefpodoxime (10 μg) and cefpodoxime/ clavulanate (10 + 1 μg). One can calculate the ratio between the zone diameter with and without inhibitor. A report > 1.5 signifies the strain is ESBL [5,6].

Figure 1. The combination disc method: ceftazidime, cefotaxime and cefepime alone and with clavulanic acid. ESBL production is confirmed by a ≥ 5mm increase in zone diameter of cephalosporin discs combined with 10 μg of clavulanic acid (personal archive of the author)

The sensitivity and specificity of the method were originally stated as 96% respectively 100% by both the Clinical and Laboratory Standards Institute (CLSI) and the British Society for Antimicrobial Chemotherapy (BSAC). The cefpodoxime and clavulanic acid disc distinguishes ESBL producers from over expression of AmpC and *K. oxytoca* K1 strains [7, 8].

Another method is the E-test method (fig. 2). The method uses double plastic strips that have ceftazidime on one half and ceftazidime plus clavulanic acid on the other half, both in a stable concentration gradient. Cefotaxime and cefotaxime clavulanic acid can also be used. Interpretation of E-test results is delicate and requires expertise. Studies show that in approximately 30% of cases, laboratories fail to correctly interpret the results. Failure may appear when the obtained MIC is situated outside the strip scale [5, 6, 7].

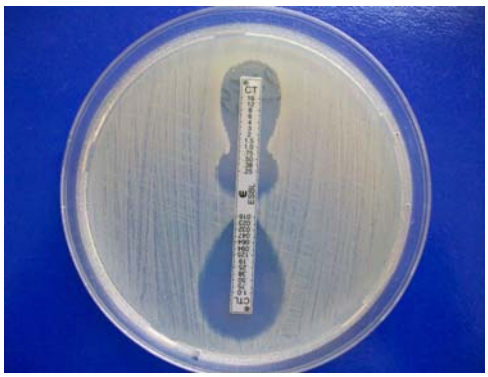

Figure 2. E-test strip with cefotaxime and cefotaxime/clavulanic acid. A ≥ 3 twofold reduction in the MIC on the side containing clavulanic acid is indicative of ESBL production (personal archive of the author)

Compared with the disc method, the E-test method proved to be equal or more sensitive. Yet, it still remains expensive and problematic for the interpretation of certain underrepresented enzymes and it is unable to distinguish between ESBL and hyper-production of *K.oxytoca* K1 strains. E-tests with cefepime/ clavulanic acid have been shown to be capable of detecting all kinds of ESBL but have problems for ESBL TEM-12 [8].

Comparing the E-test method with the disc method we have 94% sensitivity versus 93% and a specificity of 85% versus 81%. The double-disc method (ceftazidime, cefotaxime, cefpodoxime and cefpirome) has the highest specificity and the highest positive predictive rate (97% and 98%) [9].

Quality control recommendations when performing screening and phenotypic confirmatory tests, require simultaneous testing with a non ESBL strain (*E.coli* ATCC 25922) and an ESBL strain (*Klebsiella pneumoniae* ATCC 700603) [10].

Several commercial methods incorporating the same principle have been developed: Vitek2, Phoenix and Microscan Walkaway. With these systems, growth in wells with cephalosporins alone and in

combination with clavulanic acid is detected and analyzed with algorithms.

Starting with 2010, there was a revolution in terms of testing and reporting the sensitivity to cephalosporins in Enterobacteriaceae. Breakpoints to cephalosporins were modified and reporting of results is made according to the results obtained with the new breakpoints without the transformation of S in R. According to EUCAST and CLSI 2010, screening for ESBL is no longer necessary. According to CLSI, laboratories can continue testing for ESBL in epidemiology or infection control purposes and the recommendation of EUCAST is to continue testing. In this situation, some ESBL strains will be reported susceptible to ceftazidime and cefepime [10, 11].

AmpC

AmpC β-lactamases (also termed class C or group 1) have gained importance in the late 1970s, being cephalosporinases able to hydrolyze all β-lactams, with some exceptions.

There are several types of AmpC β-lactamases: constitutive and inducible, chromosomal or plasmid mediated [12]. Chromosomal AmpC β-lactamases are found in *Enterobacter spp.*, *C. freundii*, *M. morganii*, *Providencia spp, Serratia spp.* and can be inducible or derepressed [13].

Inducible cephalosporinases or AmpC β-lactamases are inhibited by aztreonam and not by clavulanic acid, tazobactam, sulbactam. For detection, cefoxitin or imipenem can be used as inducers and piperacillin, cefotaxime or ceftazidime as indicators, placed at a distance of 20-25 mm centre to centre. After overnight incubation at 35°C, antagonism, indicated by a visible reduction in the inhibition zone around the indicator disc, is regarded as positive result. The best method is association between imipenem and piperacillin/tazobactam. The results are reported R to penicillins (except temocillin), penicillins/inhibitors, cephalosporins (except for cefpirome and cefepime) cephamycins and monobactam, irrespective of their inhibition zone diameter [14].

Plasmid AmpC β-lactamases are encoded on highly mobile plasmids; they hydrolyze penicillins, extended and broad-spectrum cephalosporins, cephamycins, monobactam and they do not affect carbapenems and cefepime and are not inducible and are resistant to beta-lactamase inhibitors. Resistance to extended-spectrum cephalosporins may be due to expression of a class C β-lactamases chromosomally encoded in *Enterobacter spp*, *Citrobacter spp* and *Serratia spp.* In *E. coli*, a chromosomal gene is present although not expressed. The transfer of the AmpC gene on plasmid allowed the dissemination to *Klebsiella spp.*, *E. coli*, *Proteus mirabilis* and *Salmonella* [14].

It is very important that microbiology laboratories be able to detect plasmid AmpC beta-lactamase for a better interpretation of antibiograms results. There are currently no standardized phenotypic methods for screening. Reduced sensitivity to cefoxitin may be an indicator for AmpC activity but resistance to cefoxitin can occur by altering the outer membrane permeability; it can be a species-specific resistance and some AmpC producing strains remained susceptible to cefoxitin [15].

Extraction methods for the enzyme have been described but are complicated and do not address a routine laboratory. There are several enzyme inhibitors for AmpC, such as boronic acid and cloxacillin. These inhibitors may be incorporated onto disks of antibiotics such as cefoxitin (30 μg) or cefpodoxime (10 μg), dispensed with 20 microliters of stock solution of cloxacillin (200 μg) or *phenylboronic* acid (400 μg) [16, 17]. Enhancement of the inhibition zone around the antibiotic disc with inhibitor, compared to that of the antibiotic disc without inhibitor, is considered positive. Results vary depending on the antibiotic used. Best results were obtained with the cefoxitin disc [16].

Another method used is the approximation using imipenem discs of 10 μg, cefoxitin 30 μg, amoxicillin/clavulanate 20/10 μg as an inducer, ceftazidime 30 μg as substrate [18]. The discs are applied at 20 mm distance and track the flattening of the inhibition zone between the ceftazidime disk and the inductors. The method was found to be lacking in sensitivity [19].

Phenotypic methods cannot differentiate between chromosomal and plasmid AmpC. Molecular tests are the only ones able to correctly detect the AmpC β-lactamase

The studies of Coudron, Jacoby et al. and Yagi et al. have validated the use of boronic acid derivatives for the detection of Gram-negative bacilli with AmpC beta-lactamase. Coudron's study included the recommendation that all strains positive for the boronic acid test must be reported as resistant to all cephalosporins but it is unclear if they refer to cefepime and cefpirome as well, or just to extended spectrum cephalosporins (ESC). Resistance to ESC may be due to ESBL, or to a mutation that activates chromosomal AmpC β-lactamase production; it also may result from the acquisition of a plasmid AmpC [16, 20, 21].

Pai and colleagues have demonstrated that strains producing AmpC β-lactamase, similar to those producing ESBL, may appear susceptible to ESC and can lead to erroneous treatment recommendations. Strains producing AmpC, in contrast to those producing ESBL, remain sensitive to cefepime or cefpirome [22].

Thus it becomes important for laboratories to detect the correct type of beta-lactamase to unduly reduce the use of carbapenems.

Carbapenemases

Identification of carbapenemase-producing bacteria remains a challenge. Availability of guidance and sufficient capacity of laboratories to routinely detect and confirm carbapenemases-producing isolates are of great importance. According to Grundmann et al. carbapenemase producing strains are underreported in at least one third of European countries [23].

Struelens et al. showed that 11 European countries have developed guidelines for infection control and that laboratories are capable to routinely determine and confirm the carbapenemase producing strains [24].

Carbapenems resistance occurs when an Enterobacteriaceae acquires a carbapenemase or produces an excess of ESBL or AmpC β-lactamase or in combination with porin loss. Carbapenemases fall into three classes according to their amino acid sequence.

Class A carbapenemases are:

- Chromosomal: SME (Serratia marcescens enzyme 1-3), NMC (non-metalloenzyme carbapenemase) , IMI (imipenem hydrolyzing β-lactamase 1-2)
- Plasmid: KPC and GES (Guyana extended spectrum 1-12). KPC 1-10 are more frequent in *K.pneumoniae* but we can find them also in *Enterobacter spp*. and *Salmonella spp.* They have as substrate penicillins, extend-spectrum cephalosporins, carbapenems, aztreonam and they are not inhibited by EDTA.

Class B carbapenemases are:

- Metallo-carbapenemases IMP, NDM (New Delhi metallo-β-lactamase)

Class D carbapenemases are:

- OXA carbapenemases

Within these classes, further divisions are made and new variants are frequently encountered [25]. They all have in common the ability to rapidly hydrolyze most beta-lactam antibiotics including carbapenems, thus conferring resistance to these antibiotics. In most cases, they are encoded by genes located on transferable elements that allow the transfer of genes between species of Enterobacteriaceae [26]. EUCAST (The European Committee on Antimicrobial Susceptibility Testing) and EARSS (European Antimicrobial Resistance Surveillance System) experts have recommended a series of methods to detect class A and B enzymes in Enterobacteriaceae. The detection includes a screening step followed by a phenotypic and genotypic confirmation step. Screening is based on detection of reduced susceptibility to carbapenems.

According to CLSI, potential carbapenemase producing strains are considered to be the strains that meet the following conditions: ertapenem zone diameter ≤ 21 mm, MIC ≥ 2 μg/ml, meropenem zone diameter ≤ 21 mm, MIC ≥ 2 μg/ml, imipenem zone diameter ≤ 21 mm, MIC ≥ 2 μg/ml. Ertapenem has a lower specificity compared to imipenem and meropenem because isolates with ESBL or AmpC and decreased permeability have higher MICs for ertapenem than for imipenem or meropenem [27].

Use of a correct inoculum is important since a moderate decrease in the inoculum may lead to inaccurate susceptibility results. Strains with an MIC above the carbapenemase screening breakpoint but below the clinical breakpoint might have a carbapenemase gene. E-tests can be used to determine the MIC of carbapenemase producing strains, but the interpretation can be complicated because mutant colonies with higher MICs may be found in the inhibition ellipse [25].

Phenotypic confirmation of carbapenemase producing strains is based on detection of a diffusible carbapenemase and in vitro inhibition of carbapenemase activity upon addition of an inhibitor [25].

The modified Hodge test (MHT) is a confirmatory test that detects the production of diffusible carbapenemase in strains of Enterobacteriaceae. The test should be performed according to the CLSI guidelines. The test detects carbapenemase production when the suspect strain produces an enzyme that will allow the growth of the control strain *E. coli* ATCC 25922, sensitive to carbapenems, towards the carbapenem disc, resulting in a characteristic clover leaf appearance [10]. Recommended quality control strains are *Klebsiella pneumoniae* ATCC BAA-1705 (KPC-positive) and *Klebsiella pneumoniae* ATCC BAA-1706 (KPC-negative) [10]. The method has limitations because it cannot determine the class of carbapenemases, its sensitivity is > 90%, its specificity is > 90% for the KPC, but unknown for MBL. False positive results have been described especially for CTX-M strains but false negative results may also occur for MBL strains [10]. For detection of class A carbapenemases, the specificity of MHT can be increased by modifying the test as described by Pasteran et al [28]. With the new breakpoints, since 2010 is has been no longer necessary to edit the results for carbapenems and no further testing for carbapenemases is required. the modified Hodge test is only required for epidemiological purposes [10, 11].

The inhibition tests are used to distinguish between the different classes of carbapenemases. Recently, several phenotypic methods have been described for the detection of KPC; these are based on the inhibitory effect of boronic acid (APB) [29, 30, 31]. The combined disc method proved to be

superior to the DDST method (double-disk synergy test) and as indicators both meropenem and imipenem are preferred by most authors [29]. Different cut-off values (≥ 4 to ≥ 7) have been described for the difference between diameters obtained with disks with carbapenem and disks with carbapenem and boronic acid [31].

For Class A we suspect KPC-type enzyme production when there is a noticeable difference_of more than ≥ 4 mm between the diameter of the inhibition zone around the meropenem disc (10 μg) and the diameter for the meropenem with boronic acid disk (600 μg). Boronic acid can inhibit class C enzymes, and therefore testing is recommended also with cloxacillin. In the presence of a diameter of more than ≥ 5 mm between meropenem and the meropenem disc combined with cloxacillin (750 mg) we can consider the strain to produce in excess a chromosomal or plasmid AmpC. Besides the disc method, a method for assessing the MIC for carbapenems has been described, both in the absence and in the presence of boronic acid (APB) [29].

Methods with boronic acid showed great sensitivity in detecting KPC positive strains but the specificity requires further evaluation [29]. It seems that false positive reactions occur mainly in strains with overproduction of AmpC cephalosporinases and porin modification [29]. False-negative strains were proven in strains that produce also VIM-type enzymes [32].

In group B we have metallo-beta-lactamases (MBL) which are not inhibited by inhibitors of β-lactamases; they require zinc ions in the active locus, and are inhibited by metal chelators. MBL phenotypic detection methods are based on the synergy between MBL inhibitors such as ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid and a carbapenem (imipenem or meropenem) or ceftazidime as the indicator [33]. Detection of class B enzymes rely on an increase of the diameter of more than ≥ 5 mm between the meropenem disc and the meropenem to which EDTA was added [33]. The addition of zinc in the culture media could increase method sensitivity. The MBL E-test is based on the synergy between imipenem and EDTA but it is considered inadequate for detection of MBL producing Enterobacteriaceae with MIC ≤ 4 mg/l [33].

For OXA carbapenemases found in group D, 37 in number, there is no phenotypic test for detection, PCR is the gold standard.

Conclusions

Failure to detect resistance to antibiotics can have adverse consequences for the patients. Microbiology laboratories must constantly improve testing methods and provide accurate results to clinicians.

Today many bacterial pathogens are more complex than a decade or two ago. Thus, previously reliable susceptibility tests may no longer be dependable. For example there are not only new resistance mechanisms, but also isolates that produce multiple beta-lactamases. Such changes necessitate new or modified tests to provide accurate and clinically relevant susceptibility reports.

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