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Detection of carbapenemase-producing Enterobacteriaceae in various scenarios and health settings

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ABSTRACT

Detection of carbapenemase-producing Enterobacteriaceae (CPE) is an important task at microbiology laboratories in hospitals. As the prevalence of CPE is increasing worldwide, the implementation of phenotypically based screening as well as confirmatory procedures to detect CPE are important for microbiologists. In addition to detection of carbapenem hydrolysis, the inhibition of activity against a carbapenem in the presence of several inhibitor compounds specific to class A, B, or class C beta-lactamases is a useful method to confirm the presence of carbapenemases in bacterial isolates. There is also a proteomic approach that compares the MALDI-TOF spectrum generated by the intact carbapenem (non-hydrolyzed) with that obtained after hydrolysis of the beta-lactam ring by beta-lactamase to reveal the presence of carbapenemases in bacterial isolates. Proteomic methods will probably be more frequently implemented in laboratories in the near future. Finally, molecular methods to directly or indirectly detect the presence of a carbapenemase genes are increasingly being used in microbiology laboratories. One of the main advantages of these methods is their speed, and also that they can be used directly with the clinical sample without the need for an isolated bacterial colony. Multiplex PCR, real-time PCR, DNA microarrays and pyrosequencing are some examples of molecular-based tests. Their main disadvantage is their cost, although prices are going down as the range of services increases. Surveillance of carriers is also an important task for infection control purposes. In this case, commercially available chromogenic medium supplemented with low carbapenem concentrations has shown an excellent ability to detect CPE. Moreover, molecular methods to detect specific carbapenemase genes directly from rectal swabs, stools, or other colonization sources have had excellent results.

Detección de enterobacterias productoras de carbapenemasas en diferentes escenarios y niveles de salud

Palabras clave:
OXA-48
KPC
IMP
VIM
NDM
Ácido borónico
Cloxacilina
Ácido dipicolínico
EDTA
Temocilina
MALDI-TOF
PCR a tiempo real

RESUMEN

La detección de enterobacterias productoras de carbapenemasas (CPE) es una tarea muy importante de los laboratorios de microbiología en los hospitales. Debido a que la prevalencia de CPE se está incrementando en todo el mundo, la implementación de métodos fenotípicos de cribado, así como de confirmación posterior, orientados a la detección de CPE es una tarea muy relevante para los microbiólogos. Además de la detección de la hidrólisis del antibiótico carbapenémico, la inhibición de la actividad de la enzima sobre estos antibióticos en presencia de compuestos inhibidores específicos para las betalactamasas de clase A, B o C es un buen método validado en la actualidad para la confirmación de carbapenemasas en los aislados bacterianos. Además existen métodos proteómicos que comparan el espectro MALDI-TOF generado por un antibiótico carbapenémico (no hidrolizado), con el que se obtiene tras la hidrólisis del anillo betalactámico por la betalactamasa, en este caso, una carbapenemasa, lo que revela de manera directa la presencia de estas enzimas en los aislados bacterianos. Los métodos basados en proteómica se irán implementando, de manera paulatina, en los laboratorios en un futuro cercano. Últimamente, métodos moleculares capaces de detectar de manera directa o indirecta la presencia de un gen que codifique una carbapenemasa se están in-

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troduciendo de modo creciente en los laboratorios de microbiología. Una de sus principales ventajas es su rapidez, además de que pueden usarse directamente en la muestra clínica sin requerir una colonia bacteriana aislada para su realización. Entre ellos destacar la multiplex-PCR, la PCR en tiempo real, los *microarrays* de ADN o la pirosecuenciación como ejemplos de tests basados en métodos moleculares. Su principal desventaja es el coste, aunque los precios se están abaratando a la par que se incrementa la cartera de servicios. El control y vigilancia de portadores es una tarea importante para el propósito de control de la infección. En este caso, medios cromogénicos disponibles comercialmente y suplementados con bajas concentraciones de carbapenémicos han mostrado un excelente rendimiento para detectar CPE. De igual manera, métodos basados en biología molecular capaces de detectar genes que codifican carbapanemasas directamente de muestras como frotis rectales, heces u otras fuentes de colonización han revelado excelentes resultados.

Introduction

Carbapenems are often the last line of effective therapy available for the treatment of infections caused by multiresistant Enterobacteriaceae.[1] The clinical microbiology laboratory acts as an early warning system, alerting the medical community to new resistance mechanisms present in clinically important bacteria. The presence of carbapenemases among Enterobacteriaceae is an infection control emergency, and the detection of these bacteria in clinical microbiology laboratories is a critical step for appropriate management of patients as well as for infection prevention and control efforts.[2] Clinical microbiology laboratories should be able to rapidly detect these enzymes among Enterobacteriaceae, especially when these enzymes are first introduced into the local bacterial population. In many areas, carbapenemase detection and characterization are currently recommended or mandatory for infection control purposes.[2] The most important and extensively distributed enzymes include the class A carbapenemases (KPC, GES types), the class B or metallo- beta-lactamases (MBLs) (VIM, IMP, and NDM types), and the class D oxacillinases (OXA-48-like enzymes).[3]

Accurate detection is required for both clinical specimens and carriers, which may be related to hospital outbreaks or surveillance programs.

The production of a given carbapenemase might confer a particular β-lactam resistance phenotype and the level of conferred resistance can vary depending on the bacterial species, the expression level, the enzyme type or variant, and the presence of additional resistance mechanisms such as permeability reduction, efflux, and/or the activity of other beta-lactamases.[4] For these reasons, molecular methods based on carbapenemase gene detection may offer advantages with respect to either phenotypic or proteomic methods.

Detection of carbapenemases by phenotypic methods

Screening by susceptibility tests

Phenotypic methods for detection are generally easy to perform, interpret, and introduce into the work flow of a clinical laboratory. The use of the most affected carbapenem as an indicator of the presence of a resistance mechanism, or better yet several carbapenems simultaneously, can lead the microbiologist to suspect their presence. Broth microdilution and disk diffusion methods are considered to be reliable for the detection of all types of carbapenemase-mediated resistance. Gradient diffusion methods (e.g., E-tests) are not considered to be appropriate for screening due to their heterogeneous growth, which makes interpretation difficult.[5]

The carbapenem MICs (>0.12 μg/mL or <25 mm for ertapenem and/or meropenem and >1 μg/mL or <23 mm for imipenem) for Enterobacteriaceae, which is in accordance with the guidelines issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), will detect the majority of carbapenemases. Carbapenem MICs for carbapenemase producers are highly variable. Ertapenem has excellent sensitivity but poor specificity, especially in species such as *Enterobacter* sp., due to its relative instability with extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases in combination with porin loss. Imipenem should not be used for *Proteus* spp., *Providencia* spp., and *Morganella* spp.[6] Meropenem offers the best compromise between sensitivity and specificity in terms of detecting carbapenemase-producers.[6] Imipenem is therefore not recommended for use as a stand-alone screening test compound. Nevertheless, due to factors such as low carbapenem MICs and inoculum effects, no single carbapenem-screening criterion can be used to identify all isolates.[7] A concomitant examination of additional beta-lactam antibiotics could assist in detection of carbapenemase producers. Reduced susceptibility to either carbapenems and resistance to at least one cephalosporin of subclass III (cefotaxime, ceftriaxone and/or ceftazidime) are indicators of possible class A (KPC-like or GES-like) or class B (VIM, IMP) carbapenemase production.[8] Nevertheless, the activity of expanded-spectrum cephalosporins against class D carbapenemase producers is not necessarily affected[9] and activity against organisms with other, rarer class A carbapenemases (e.g., SME, IMI and NMC-A) is usually not affected at all.[8]

Phenotypic confirmation tests

Several phenotypic confirmation tests have been described for the detection of carbapenemase-producing Enterobacteriaceae. These include: *a)* spectrophotometric assays; *b)* bioassays that detect the ability of these enzymes to hydrolyze the carbapenems (e.g., the modified Hodge test [MHT]); *c)* inhibitor-based methods, and *d)* the colorimetric method.

a) Spectrophotometric measurement of carbapenem hydrolysis is considered to be the reference standard method for detection of carbapenemase production in a suspected carbapenemase producer organism. Hydrolysis of carbapenems in the presence or absence of inhibitors (e.g., EDTA or dipicolinic acid for MBLs, tazobactam or clavulanic acid for KPCs, NaCl *in vitro* for most oxacillinases), performed with crude cell extracts or partially purified enzymes, could provide additional information concerning the enzyme type. These assays should be performed in reference laboratories because they are not available in most clinical laboratories.[10]

b) The cloverleaf method, also known as the modified Hodge Test (MHT), has been extensively used as a general phenotypic method for the detection of carbapenemase activity, and so far, it has been the only method of detection recommended by the CLSI.[11] The test is based on the inactivation of a carbapenem by either whole cells or cell extracts of the organisms, which enables a carbapenem-susceptible indicator strain to extend its growth towards a carbapenem disk along the streak of inoculum of the test strain or extract thereof. The test does not provide information on the type of carbapenemase involved. In the case of MBL producers, the addition

of zinc sulfate to the medium has been suggested to improve both the interpretation of the test and its sensitivity (e.g., weak detection of NDM producers).[12] Cloverleaf lacks specificity, and there have been reported false-positive results with CTX-M-producing strains with reduced outer membrane permeability.[13] Other false positive results have been reported for high-level AmpC producers (e.g., *Enterobacter* spp.). Nevertheless, the test works well for detection of KPC and OXA-48-like enzyme producers.[14] The use of the MHT is not recommended by EUCAST because the results are difficult to interpret, the specificity is poor, and in some cases the sensitivity is also suboptimal. Some novel modifications of the technique have been described, such as performing the MHT test on cloxacillin-containing plates or with the carbapenem and cloxacillin in the same disk to eliminate the false positives due to high-level AmpC producers. However, the modifications are cumbersome in routine clinical laboratories and do not solve all the problems of sensitivity and specificity.

c) Concomitantly with the MHT, inhibition studies should be performed. The most clinically significant Ambler class A carbapenemases hydrolyze all beta-lactams and their activity is inhibited by boronic acid and at least partially by clavulanic acid and tazobactam. Phenotypic assays for the identification of class A carbapenemases are based on the inhibitory effect of boronic acid, usually 3-aminophenylboronic acid (APB) but also phenylboronic acid (PB). Various formats (disk diffusion and agar dilution) have been used. The double-disk synergy test and the combined disk test with the use of meropenem disks (10 µg) with or without among 400-600 µg of APB provide high sensitivity but low specificity caused by organisms with reduced susceptibility to carbapenems due to high-level expression of AmpC-type beta-lactamases and porin alterations. A comparison between the zone diameters of meropenem and meropenem plus cloxacillin (750 µg) disks can suggest the presence of a strain that overproduces the chromosomal AmpC or produces a plasmid-encoded AmpC.[15] MICs of carbapenems can also be evaluated in agar dilution in the presence and absence of APB (0.3 g/L) with a three-fold or greater reduction of carbapenem MIC in the presence of the inhibitor.[16] Inhibition of cephalosporinase activity by growing those strains on cloxacillin-containing plates or by using cloxacillin-containing tablets may help differentiate them. The APB-based assays fail to detect the co-production of KPC- and VIM-enzymes.[17] The combination disk test has the advantage that it has been well validated in various studies, and moreover it is commercially available (MAST®, UK; Rosco®, Denmark).[15] The main disadvantage of these methods is the time required for completion (overnight incubation), which has led to a search for quicker methods.

The class B beta-lactamases possess the highest carbapenemase activity, exhibiting a broad spectrum of hydrolytic activity against beta-lactams including all penicillins, cephalosporins, and carbapenems, and to a lesser extent aztreonam. Their activity is not inhibited by classical inhibitors (clavulanic acid, tazobactam, or sulbactam). In the case of MBLs, resistance to ceftazidime and susceptibility to aztreonam might indicate the presence of these enzymes. Nevertheless, this phenotype should be used with caution, as MBL producers sometimes have other resistance mechanisms that affect monobactams such as ESBLs, plasmid-mediated AmpCs or overproduction of chromosomal beta-lactamases. The mechanism of hydrolysis of class B enzymes is dependent on the interaction of the beta-lactam with Zn^{2+} ion(s) in their active site, which explains the inhibition of their activity by chelating agents of divalent cations as EDTA. Several inhibitor-based tests have been developed for the specific detection of MBL producers. These tests are based on the synergy between MBL inhibitors such as EDTA,[18] EDTA plus 1,10-phenanthroline,[19] thiol compounds (2-mercaptopropionic acid or sodium mercaptoacetic acid)[12] or dipicolinic acid[20] and a carbapenem (imipenem and/or meropenem) and/or an oxyimino-cephalosporin (ceftazidime) with and without clavulanic acid as an indicator of beta-lactam compounds. The inclusion of ceftazidime with clavulanic acid in the synergy test is to rule out a possible concomitant ESBL enzyme. This is due to the fact that ceftazidime is a better substrate for the carbapenemase, which should be inhibited if a class A enzyme is present. These tests take advantage of metalloenzyme dependence on zinc ions, and use the chelating agents to inhibit beta-lactam hydrolysis. Various formats (disk diffusion or broth dilution) of EDTA-based synergy tests have been the most commonly used and evaluated. The double-disk synergy test using various distances and the combined disk test using various amounts of EDTA exhibit high sensitivity, but the possible intrinsic activity of EDTA should be tested. The E-test MBL has been shown to be inappropriate for the detection of MBL-producing Enterobacteriaceae with low imipenem MICs, whereas new E-test® strips, with low meropenem concentrations, have been developed.[21]

The Ambler class D enzymes with carbapenemase activity in Enterobacteriaceae are mostly OXA-48 and OXA-181. They have a hydrolysis profile that spares ceftazidime, thus cefotaxime hydrolisis is very low, and there is no inhibition by clavulanic acid/tazobactam. This enzyme class often has other co-resistant mechanisms, such as ESBL, which gives rise to a broader composite resistance profile. There is no inhibition test suitable for detecting OXA-48/OXA-181 producers because the activity of these enzymes is not inhibited by clavulanic acid, tazobactam, sulbactam or any zinc chelator. High-level resistance to both temocillin (MICs >64 mg/L) and piperacillin-tazobactam in strains showing reduced susceptibility or resistance to at least one carbapenem may be used as a first step towards identifying possible OXA-48 producers.[22] However, temocillin as a marker is not specific to OXA-48-type carbapenemases because other resistance mechanisms might confer this phenotype. The presence of OXA-48-like enzymes therefore must be confirmed with a genotypic method. Avibactam, a novel, covalent, non-beta-lactam beta-lactamase inhibitor acts as a potent inhibitor of the class D OXA-48 carbapenemase.[23] However, avibactam can inhibit other beta-lactamases. A phenotypic method has been proposed, which is based on the potentiation of ertapenem by avibactam on cloxacillin-containing plates to detect class A and D carbapenemases. The results are placed into a flow chart that identifies the different types of carbapenemases.[24]

Figure 1 summarizes the diagnostic algorithm for phenotypic confirmation of carbapenemases.

d) A rapid colorimetric method, the Carba NP test (CNP), based on the colorimetric detection of hydrolysis of the imipenem beta-lactam ring, has been described.[6] The CNP, with slight modifications from the originally described protocol,[25] is performed on isolates recovered from either zinc-supplemented Müller Hinton agar or chromogenic medium. The test is not validated to identify carbapenemase producers grown on Drigalski agar and McConkey agar plates.[25] A color change from red to yellow/orange indicates carbapenemase production (i.e., as a result of the pH change induced by imipenem hydrolysis) in 30 min to 1 h.

Surveillance of carriers

Colonization with carbapenemase producers has been associated with several healthcare-related factors, and patients with asymptomatic colonization are at risk of invasive infection.[26] The appropriate biological materials for surveillance are rectal swabs or stools for isolation of Enterobacteriaceae.[27] Agar or broth media supplemented with a low-concentration of a carbapenem may be used. MacConkey agar containing imipenem at a concentration of 1 mg/L, or a 5-mL aliquot of tryptic soy broth containing a 10 µg disk of imipenem (resulting in a final concentration of imipenem of 2 mg/L) are suitable. In addition, the use of MacConkey agar onto which a 10 µg disk of imipenem or ertapenem is placed has also been reported.[28] In addition, chromogenic media supplemented with

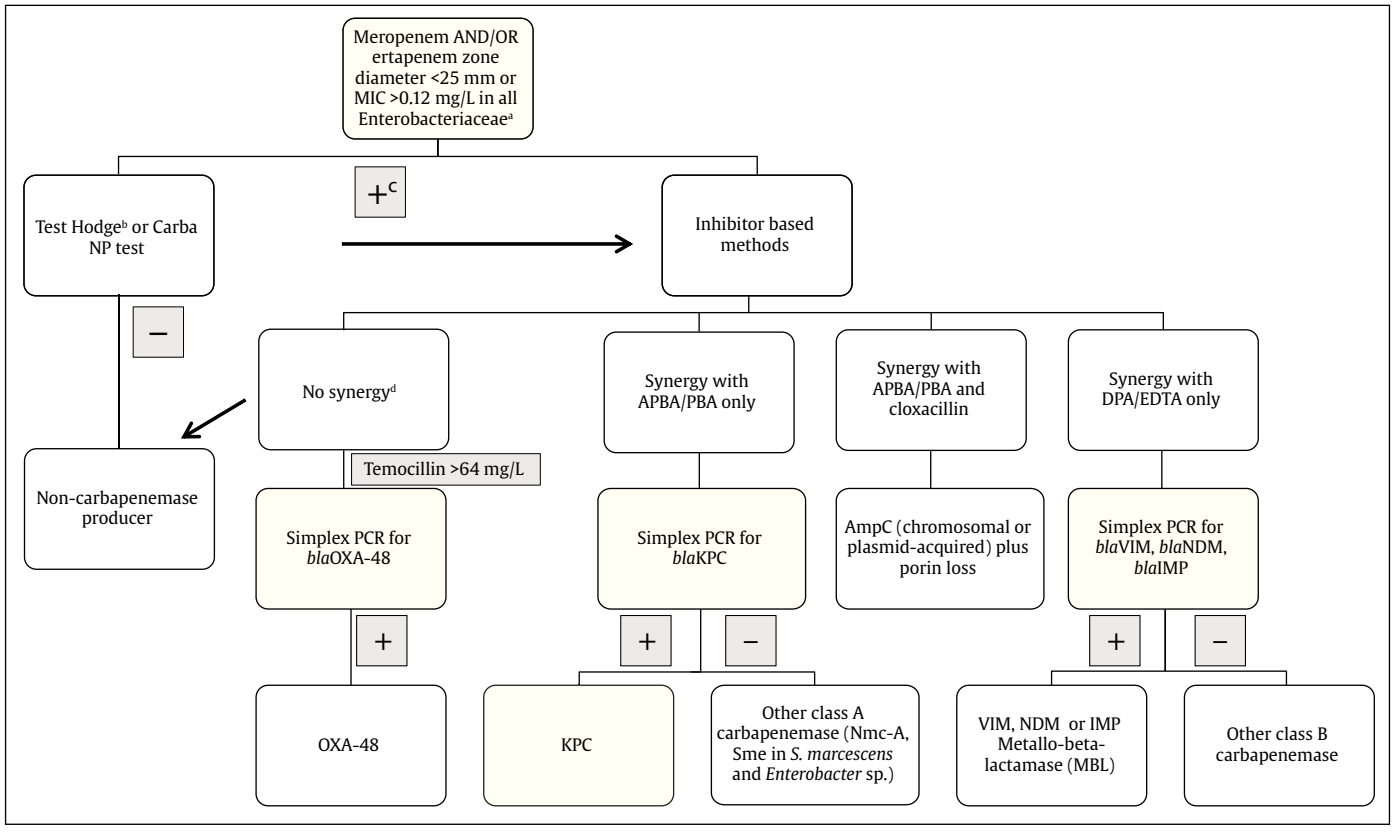

Figure 1. Diagnostic algorithm for phneotypic confirmation of carbapenemases. APBA: aminophenyl boronic acid; DPA: dipicolinic acid; EDTA: ethylenediaminetetraacetic acid (all of them beta-lactamase inhibitors added to disks or tablets cantaining meropenem in combination disk testing assays); PBA: phenyl boronic acid. [a]Exception: for enterobacterial species for which natural distribution of MIC of imipenem is around 1 mg/L (*Proteus/Providencia/Morganella* sp.), only meropenem should be considered. For *Enterobacter* sp., due to its relative instability to extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases in combination with porin loss, only imipenem and meropenem should be considered. [b]False-positive results with high AmpC producers (test plating on cloxacillin-containing plates). False negative results could be due to weak detection of NDM producers (test EDTA or DPA inhibition). [c]Positive or hard to interpret. [d]KPC and MBL combination may not show synergy but isolates are normally highly resistant to carbapenems. They are easiest to detect with molecular methods.

agents that inhibit the growth of carbapenem-susceptible organisms have been developed. The chromogenic, chromID CARBA medium (bioMérieux, France), a carbapenem-containing medium, has an excellent ability to detect KPC and MBL producers.[29] ChromID OXA-48 medium (bioMérieux, France) is specific for detection of OXA-48 producers, and chromID CARBA SMART (bioMérieux, France) is a media bi-plate that combines of ChromID CARBA on one side and chromID OXA-48 media on the other. The SUPERCARBA medium, which contains ertapenem, cloxacillin and zinc sulfate and Brilliance CRE Agar (Thermo Fisher Scientific, UK), has been developed to detect any type of carbapenemase. The SUPERCARBA or Brilliance CRE Agar, the combination of ChromID CARBA/chromID OXA-48 media, and the chromID CARBA SMART offer the highest sensitivity for detecting any type of carbapenemase.[30]

Detection of carbapenemases by proteomic methods

Several recent studies aimed to detect carbapenemase activity using a mass spectrometry platform based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). This strategy compares the MALDI-TOF spectrum generated by the intact carbapenem (non-hydrolyzed) with that obtained after hydrolysis of the beta-lactam ring by beta-lactamase, in this case a carbapenemase. Due to the biochemical nature of the procedure, this article will focus on methodological aspects of theses procedures.

Hydrolysis of the beta-lactam ring leads to a change in the molecular mass of +18 Da that can be easily identified by MALDI-TOF. However, depending on the antibiotic, other derivatives can be generated, for instance decarboxylation (–44 Da). Figure 2 shows the meropenem spectrum after hydrolysis with the indicated carbapenemase (h: hydrolyzed; d: decarboxylated). In general, it is preferable to not only monitor the degradation peak of the antibiotic but also the appearance of peaks of hydrolysis and other derivatives. To carry out this assay, a suspension of the bacteria is prepared in a

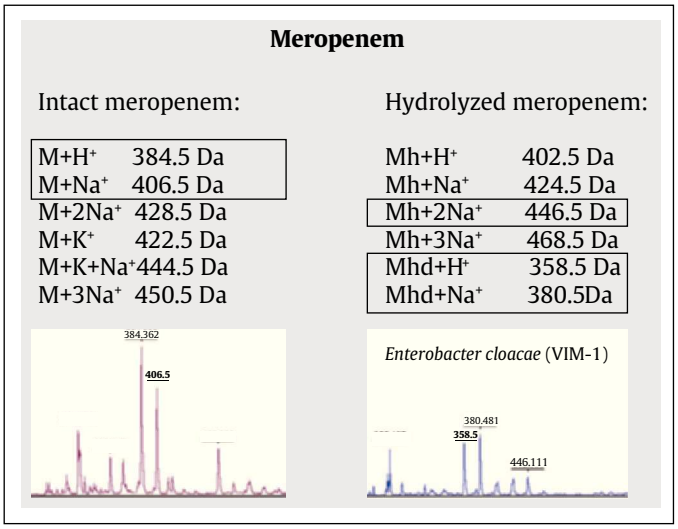

Figure 2. Meropenem spectrum after hydrolysis with VIM-1 carbapenemase. h: hydrolyzed; d: decarboxylated.

solution of antibiotic, which is incubated for a period of time ranging from 1 to 4 h. Next, the suspension is centrifuged and the supernatant is analyzed by MALDI-TOF to observe the degradation of carbapenem. The assay determines if there is production of carbapenemases but not the type of carbapenemase. However, if a bacterial inoculum with the antibiotic in the presence of a carbapenemase inhibitor (e.g., EDTA for class B carbapenemase or boronic acid for class A carbapenemase) is used in parallel, the type of carbapenemase can be inferred.

Several studies have evaluated this methodology. Burckhardt and Zimmermann[31] chose ertapenem as the substrate to detect carbapenemase activity. It was observed that the time necessary to hydrolyze the antibiotic depended on the type of carbapenemase and not to the MIC of the antibiotic. Thus, strains with NDM-1 and IMP -1 needed only 1 h to complete the degradation of ertapenem, while others such as VIM-2 took about 2.5 h. In another study published by Hrabaket al[32] in which meropenem was used as the substrate, various buffer solutions (MOPS, HEPES, TRIS) to dissolve the antibiotic were tested. They showed that Tris-HCl with a pH of 6.8 generated a minor background of peaks. Moreover, several matrix solutions were also tested. They found that the use of 2,5-dihidrobenzoic acid (DHB) diluted in 50% ethanol at a concentration of 10 mg/mL as a matrix produced fewer background peaks in the mass spectral range analyzed (360-600 m/z). In this study, two false positive and one false negative result were obtained among the 25 strains of *Pseudomonas aeruginosa* studied. Both strains with a false-positive result had a high MIC of meropenem and imipenem but were classified as non-carbapenemase producers by a spectrophotometric assay of the hydrolysis of imipenem. The authors attributed this false-positive result to other mechanisms, such as an interaction of meropenem with components of the cell wall or ESBL or AmpC hyperproduction, while the false-negative result was explained by the low expression of the carbapenemase. Later, the same authors published a slight modification of the assay,[33] which consisted of the addition of 0.01% SDS to the Tris-HCl buffer used to dissolve the antibiotic.

In a broad, comprehensive study, Sparbier et al[34] developed an assay based on MALDI-TOF MS for the rapid detection of resistance to a range of beta -lactam antibiotics such as ampicillin, piperacillin, ceftazidime, cefotaxime, imipinem, ertapenem and meropenem. All of these antibiotics except ceftazidime were dissolved in ammonium citrate. To carry out the assay, the amount of bacteria that fit in a loop of 1 µL was resuspended in 10 µL of solution of antibiotic and incubated for 3 h at 37 °C. Following a quick spin at high speed, 1 µL of supernatant was used for analysis with MALDI-TOF MS using (10 mg/mL of acid α-cyano-4-hydroxy-cinnamic acid [α-CHCA] dissolved in 50% acetonitrile-2,5% trifluoroacetic acid as a matrix. On comparing the hydrolysis of ertapenem, meropenem and imipenem it was shown that ertapenem presented a strong decarboxylation with the subsequent formation of the sodium and potassium adducts. However, imipenem did not form adducts. Thus, only one peak was observed on incubation with a non-producing carbapenemase strain. Incubation with a carbapenemase-producing strain led to the disappearance of any peak of imipenem and its hydrolyzed derivative. This outcome was probably due to various reasons, such as the extreme instability of the hydrolytic products, difficulties in ionization that lead to a lack of detection by MALDI-TOF, or an interaction with the cellular components of the bacteria that prevented its presence in the supernatant of the sample. In the same publication, the assay was applied to bacteria obtained directly from positive blood cultures with good results.

However, it is of note that in the spectrum of the samples incubated with bacterial strains susceptible to imipenem, the presence of a low-intensity peak corresponding to the hydrolyzed form of imipenem (254 m/z) can be detected. This phenomenon of spontaneous hydrolysis has previously been described by several authors.[31,34]

In summary, MALDI -TOF can be a very useful tool for rapid and reliable detection of carbapenemases. However, several aspects must be considered when using the MALDI-TOF technology for carbapenemase detection, including the type and concentration of the substrate used, and the eventual spontaneous hydrolysis of the carbapenem. A marker for the presence of hydrolysis has been proposed to avoid misinterpretation and to standardize the analysis of the spectra obtained. This analysis includes the ratio of the area under the curve for peaks of imipenem and its hydrolytic derivative, which must be less than 0.5. Other aspects to consider are the parameters of the assay, such as the diluent of the antibiotic, the matrix solution and the calibrators used. Calibration plays an important role in preventing large deviations from the expected molecular masses of the antibiotic and hydrolytic derivatives. Finally, the incubation time depends on the type of carbapenemase, as previously mentioned. However this problem might be solved by taking readings during the incubation time. All these considerations highlight the need to standardize the various parameters of the assay to facilitate reproducibility.

Detection of carbapenemases by molecular methods

The rapid detection of Enterobacteriaceae-expressing carbapenemase-type enzymes can have a significant impact on the ability to make appropriate and efficient decisions. For example, it can impact those decisions that are made in specific clinical settings for infection control purposes, such as outbreaks, or decisions regarding patients with serious and life threatening infections. In these critical situations, in which the antimicrobial susceptibility test is still not available, it is imperative to provide a quick and accurate response. A rapid test is needed to identify the specific microorganism as well as any direct or indirect information on its antimicrobial susceptibility (resistance genes), in order to correctly manage these serious epidemiological or clinical situations.

For clarification on this topic, is important to consider what we mean by a "rapid" test. Those tests that require bacterial colonies or bacterial isolates in advance are not considered to be rapid tests but rather conventional tests. On the other hand, those tests that can be applied directly to a clinical sample, or those that use microorganisms after some previous preincubation step of the clinical sample, are considered rapid and quick tests. In other words, those tests that provide a result on the same day of taking the clinical sample will be defined as rapid tests. Once this criteria has been established, the spectrum of tests that fulfill this requirement is clearly reduced and focuses on two main laboratory tests: those based on molecular biology, or molecular (or genotypic) methods, and those based on proteomic methods from clinical samples or blood cultures. The latter have been discussed above.

Due to the fact that a standardized procedure has not yet been defined as a "gold-standard molecular-based method," in this section we will review several approaches described in the literature..

With respect to molecular methods, several rapid molecular approaches can be used to detect a specific gene associated with antimicrobial resistance (carbapenemases) in the Enterobacteriaceae family. The approach will depend on whether a single gene or multiplex genes are targeted (using single or multiplex PCR), whether real-time PCR will be used (Sybergreen®, TaqMan® or hybridization probes), or perhaps a DNA microarray, and finally if pyrosequencing will be performed.

– In the literature, there are few studies on the application of multiplex PCR for detection of carbapenemases in Enterobacteriaceae (Table 1). With respect to metallo-beta-lactamases, a commercial multiplex PCR called hyplex-MBL ID Multiplex PCR-ELISA® is available, and it has been proven reliable in detecting bla_{VIM} genes in blood, urine, pus, and sputum samples.[35]

Table 1
Several multiplex PCR assays for carbapenemase detection

Gene detection	Strains or samples	Year	Reference
Multiplex V (GES and OXA-48-like)	Enterobacteriaceae	2010	62
Multiplex VI (IMP, VIM and KPC)			
Multiplex real-time PCR (KPC, GES, NDM, IMP, VIM, OXA-48)	Enterobacteriaceae	2012	63
Multiplex I (IMP, VIM, SPM, SIM, GIM)	*Escherichia coli, Klebsiella pneumoniae* and *Citrobacter freundii*	2012	56
Multiplex II (KPC, NDM, OXA-48)			
SME, IMI/NMC-A, KPC, and GES	*K. pneumoniae*	2012	57
Triplex assay (KPC, NDM)	Enterobacteriaceae	2013	64
Multiplex PCR	Gram-negative isolates	2013	65
Multiplex TaqMan PCR assay (IMP, VIM, NDM, SPM, SIM, GIM)	Enterobacteriaceae	2013	66
Real-time multiplex PCR (OXA-48, VIM, IMP, NDM, KPC)	Gram-negative isolates and broth cultured rectal swabs	2014	67

Currently, these methods lack clinical validation from multicenter studies. Recently, Bogaertst al[36] validated and accredited a set of multiplex endpoint PCR assays targeting major carbapenemase genes according to the international ISO 15189. This validation scheme could be useful for microbiology laboratories attempting to accredit their own protocols.

– Real-time PCR for the detection of carbapenemases by Sybergreen® is less common than using TaqMan probes. Five types of non-metallo class A and D (GES, IMI/NMC, KPC, OXA-48 and SME) carbapenemases were rapidly detected by real-time TaqMan PCR in clinical isolates from this family.[37]

Recently, novel TaqMan real-time PCR® assays have been developed to detect carbapenemases in several members of the Enterobacteriaceae family, especially *Klebsiella* spp. producing KPC-type enzymes.[38] Moreover, a multiplex real-time PCR capable of identifying KPC-type genes with the ST258 clone of *K. pneumoniae* has been reported.[39] Commercial systems such as the NucliSENSEasyQ KPC® test for the rapid detection of *K. pneumoniae* harboring bla_{KPC} correctly identified all 111 isolates harboring bla_{KPC} with no false positives, and the results were available within 2 h.[40] Roth et al[41] developed a real-time PCR assay complemented with traditional high-resolution (HRM) analysis to detect bla_{KPC} and differentiate between KPC-2-like and KPC-3-like alleles. This real-time PCR assay was able to detect bla_{KPC} in all 66 clinical isolates (100% sensitivity and specificity) and the PCR/HRM assay can identify KPC-producing bacteria in 3 h after isolation of pure colonies, also distinguishing between $bla_{KPC\text{-}2\text{-}like}$ and $bla_{KPC\text{-}3\text{-}like}$ alleles.

Commercial systems based on a multiplex Sybergreen® real-time PCR for BD Max instrument were able to detect 65/65 of the carbapenemase-positive and 87/87 of the carbapenemase-negative strains in other study.[42]

Probe ligation and real-time detection of KPC, OXA-48, VIM, IMP, and NDM carbapenemase genes by Check-MDR Carba test® were evaluated on 183 well-characterized Gram-negative bacilli, showing a sensitivity and specificity of 100%.[43] In addition, a novel PCR-based *in vitro* protein expression (PCR-P) method was used to detect $bla_{NDM\text{-}1}$ and its variants coding carbapenemases with varying activity. The method screened $bla_{NDM\text{-}1}$ within 3 h, with a detection limit of 5 copies, and identified functional variants within 1 day. This method provides the first integrated approach for quickly detecting the full length $bla_{NDM\text{-}1}$ and revealing variants with activity in clinical isolates.[44]

Moreover, there are important studies using real-time PCR with clinical samples for healthcare, or at least in surveillance samples related to outbreak controls for rapid identification of patients colonized with CPE. These approaches can prevent the introduction and the spread of CPE in hospitals.

The presence of bla_{KPC} carbapenemase by real-time PCR in rectal swabs preincubated in enrichment broth was compared to direct bacterial isolation on two selective screening agar plates (CHROMagar or VACC plates). Real-time PCR showed higher sensitivity (97%) than both cultures (77.3%). In addition, the turnaround time was significantly shorter for the PCR-based method than for the culture.[45]

Real-time PCR for detection of NDM-1 carbapenemase genes from spiked stool samples with 100% sensitivity and specificity has been demonstrated.[46] Although stool samples are considered the "gold standard" specimen for studying bacteria in general, and for detecting CPE specifically, Lerner et al[47] showed that rectal swabs are suitable for quantifying the carriage load of KPC-producing CRE. In addition, Nijhuis R et al[48] evaluated a new real-time PCR assay (Check-Direct CPE®) for the rapid detection of KPC, OXA-48, VIM, and NDM carbapenemases using spiked rectal swabs. By using 83 non-duplicated isolates, a 100% sensitivity and specificity was demonstrated. A new screening method based on a ligation-mediated real-time PCR was implemented to detect high-risk patients colonized with CPE within 24 h after hospital admission, using rectal and throat swabs incubated overnight. In 14 months, 454 patients were screened and six patients with CRE were detected (carriage rate 1.3%). In addition, in outbreak situations caused by OXA-48-like producing microorganisms, real-time PCR has been used to detect this carbapenemase in stools samples.[49] Another assay was a duplex real-time PCR assay for bla_{KPC} and bla_{NDM} performed directly on perianal and perirectal swabs and stool (D-PCR), which was compared to PCR after broth enrichment (BE-PCR) and two culture methods (HardyCHROM ESBL agar/ CDC screnning). Overall, D-PCR showed excellent sensitivity on perirectal and perianal surveillance swab specimens after a simple lysis procedure (when swabs are not visibly soiled with stool) and could be applied to stool and swabs soiled with stool after a nucleic acid extraction step.[50] Finally, a non-commercial multiplex PCR (with a new TaqMan probe) has been designed to detect all allelic variants of bla_{KPC} from easily accessible clinical specimens (perirectal and nasal swabs) in less than 2 h.[51]

With respect to multiplex TaqMan real-time quantitative PCR (qPCR) use in other clinical samples, Hindiyeh M et al[52] validated an assay for the detection of bla_{KPC} genes and the human RNase P gene in Bactec blood culture bottles. The sensitivity, specificity, positive and negative predictive value of this qPCR assay, compared to the results of culture, were all 100%.

– DNA microarrays for the detection of the CTX-M, TEM and SHV genes ESBLs in Enterobacteriaceae strains have been described, showing a sensitivity of 95% and specificity of 100% by using molecular characterization of ESBLs by PCR and sequencing as a reference.[53] Several commercial microarrays have been studied in clinical isolates. The Check-MDR CT101 microarray® (Wageningen, the Netherlands) has been used for the detection of the ESBLs in various studies.[54-55] Finally, one of the most promising approaches is the Check-MDR CT102® (Wageningen, the Netherlands) microarray that identifies bacteria that produce several beta-lactamases such as ESBL (SHV, TEM, and CTX-M) and carbapenemases (KPC, OXA-48, VIM, IMP, and NDM-1) with a sensitivity and specificity of 100% for most of the tested genes.[56,57] This technology has been used to detect ESBL and carbapenemase-producing Enterobacteriaceae directly from positive blood cultures with 100% agreement.[58] In 2013, Bush et al,[59] reported a need to include the gene of SME serine carbapenemase

in the detection system of the carbapenemases arrays, especially when carbapenem-resistant *S. marcescens* isolates are suspected.

Recently, Sullivan et al[60] evaluated the rapid Verigene Gram-Negative Blood Culture Test® (BC-GN), a microarray that detects 9 genus/species targets (*Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp., *E. coli/Shigella* spp., *K. oxytoca*, *K. pneumoniae*, *Proteus* spp., *P. aeruginosa*, and *S. marcescens*); and 6 antimicrobial resistance determinants (bla_{CTX-M}, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA}) directly from positive blood cultures. In total, 104 organisms were isolated from 97 clinical blood cultures. BC-GN detected 5/5 and 1/1 clinical blood cultures with bla_{CTX-M} and bla_{VIM}. All 6 results were corroborated by CT 102 microarray testing, which was used as comparator for detecting resistant determinants. Overall, The Verigene BC-GN test has the potential to expedite therapeutic decision making in pediatric patients with Gram-negative bacteremia. Its sensitivity was satisfactory but may be suboptimal for mixed Gram-negative blood cultures.

– Pyrosequencing is so far a promising tool, which is based on real-time sequencing by a synthesis approach that has been applied to the single-nucleotide polymorphism (SNP) detection for ESBL identification in clinical strains.[61] Further work is still needed to implement it in clinical practice for carbapenemase detection.

Molecular methods for CPE detection are a challenge for microbiology laboratories, and they should be implemented in a multidisciplinary context and according to hospital resources, taking into account the specific situation of each diagnostic procedure. Although microbiological diagnostics based on molecular biology display high levels of sensitivity and specificity, they may not be the best option for a specific situation at an institution. Despite their increasing implementation in most tertiary specialized hospitals, these methods can never replace the microbiological culture that allows bacterial isolate recovery. Strain isolation is still the reference procedure in most protocols, and although its sensitivity is lower than with molecular methods, strain isolation remains in an acceptable range in certain conditions.

Lastly, Figure 3 summarizes the CPE diagnostic algorithm described in the present article. Proteomic and/or molecular methods can be applied either to direct patient samples (clinical or colonization samples) or to a bacterial isolate obtained after incubation of a primary clinical sample (to emphasize that MALDI-TOF is useful only with those clinical samples with high bacterial load/mass, such as positive blood cultures). However, the phenotypic test for screening and/or confirmation of a CPE can only be performed after a pure bacterial colony is isolated from a patient sample. The process will probably take 2 or 3 days (depending whether or not we consider positive blood cultures), and therefore will result in a delay in the diagnosis of a CPE. However, its main advantage is its low cost with respect to proteomic and molecular methods.

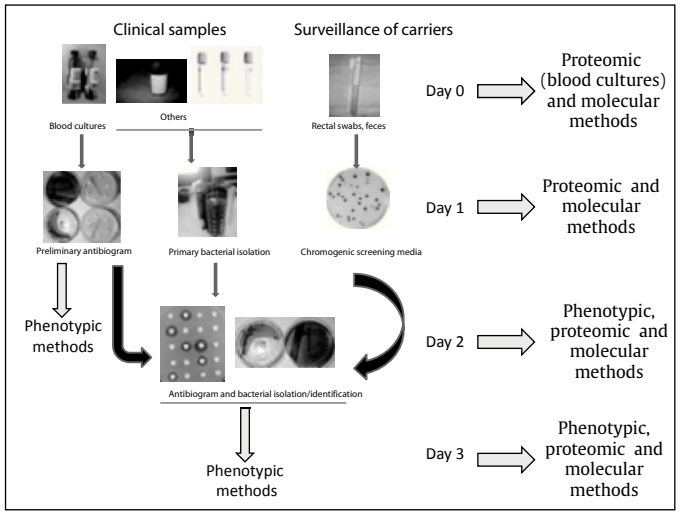

Figure 3. Carbapenemase-producing Enterobacteriaceae diagnostic algorithm.

Regarding which procedure should be used in the various scenarios, phenotypic tests for carbapenemase detection (either screening or confirmatory) are available in most microbiology laboratories regardless of the whether the hospital is a district, tertiary or reference hospital. These methods should be easily implemented in most microbiology laboratories. In addition, phenotypic tests are preferred because normalized protocols reported by CLSI or EUCAST are available (EUCAST can be freely downloaded from the Internet) and these methods are easy to perform and relatively inexpensive.

In terms of proteomic methods, at present their utility is mainly with positive blood cultures besides its usefulness in bacterial isolates. MALDI-TOF is currently an expensive tool, and it seems a reasonable tool/procedure for more complex or tertiary hospitals with greater resources. Moreover, improvements in the MALDI-TOF software related to the detection of beta-lactam resistance would be needed prior to implementing MALDI-TOF for detection of CPE.

In terms of molecular methods, their main disadvantages are the cost and the need for trained personnel in some situations, although they are expeditious and highly specific to detect carbapenemase genes. For the above-cited reasons, these methods seem like a reasonable option for tertiary and reference hospitals, although it may depend on the hospital's resources. A microbiology laboratory in a specific geographic area acting as a reference center for CPE identification must be capable of detecting the wide array of carbapenemase genes in a rapid manner. For these reasons, molecular methods based on either multiplex-PCR, RT-PCR, arrays or pyrosequencing are almost mandatory for such laboratories. In other cases it will depend on the availability of human and material resources.

Lastly, for surveillance of carriers several options are available. Among them, chromogenic or selective screening media are good options, in addition to the possibility of applying a specific molecular test directly from rectal swabs or stool samples. This decision will probably depend on the hospital situation (i.e., outbreak vs. endemia) and its resources. Despite the speed of the molecular test (a turnaround time within the same day) and increasing advantages associated with automation, its cost-effectiveness in situations with low prevalence of the searched carbapenemase-gene target is not yet clear. Molecular methods seem more appropriate in outbreak situations in which a rapid response is needed to implement patient isolation procedures. If the prevalence of CPE in a specific institution is low, it seems more reasonable and cost-effective to use chromogenic or selective screening media.

In summary, this article provided an overview of various phenotypic, proteomic, and molecular methods to detect CPE. The final choice of method will depend on the level of complexity of the hospital in addition to specific health situations, as well as human and material resources.

Conflicts of interest

The authors have no conflicts of interest to declare.

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