Review

Current methods for the identification of carbapenemases

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Detection of carbapenemases in clinical microbiology labs is a challenging issue. Comparison of the results of susceptibility testing with the breakpoint values of carbapenems is the first step in the screening of carbapenemase producers. To date, screening of carbapenemase-producing (CP) bacteria has been mostly performed by a selective medium. Although these media are practical for the detection of most CP isolates, the inoculated plates have to be incubated overnight. Subsequently, we need the confirmation of the carbapenemase producers present in the culture medium by additional testing [e.g. inhibition studies with liquid or solid media, modified Hodge test (MHT), or gradient strips], which can take up to another 48hours. Despite the lack of discrimination between the three different classes of carbapenemases (KPC, MBL and OXA) and difficulties in the interpretation of the results, the MHT is usually deemed as the phenotypic reference method for the confirmation of carbapenemase production. Molecular techniques, such as real-time polymerase chain reaction (PCR) assays, in contrast to phenotypic methods that are very time consuming, are faster and allow for the quick identification of carbapenemase genes. These techniques can detect and characterize carbapenemases, including NDM- and KPC-mediated resistance, which is critical for epidemiological investigations. The aim of this review is to gather a summary of the available methods for carbapenemase detection and describe the strengths and weaknesses of each method.

Keywords: Carbapenemases, Phenotypic methods, Molecular methods, Gram-negative, Identification method

Introduction

With the advent of antimicrobial resistance to several bacterial agents, a group of antibiotics, namely carbapenems, has been regarded as the last line of treatment against infections caused by resistant pathogens.[1] Carbapenems [e.g. meropenem (MEM), imipenem (IPM)] with a broad spectrum of antibacterial activity are safe and potent beta-lactam antibiotics that are generally an effective therapeutic choice for the treatment of serious Gram-negative bacterial (GNB) infections when resistance to other classes of antimicrobials is present.[2]

The last decade has witnessed the worldwide dissemination and dramatic emergence of carbapenem-resistant isolates among GNB, especially *Pseudomonas* spp., *Acinetobacter baumannii* and *Enterobacteriaceae*. Resistance to carbapenems among the multi-drug-resistant Gram-negative bacteria (MDR-GNB) is mostly related to the production of carbapenemases. These enzymes are able to hydrolyze not only the carbapenems themselves but also all the other beta-lactam agents.[3] Because of their association with resistance to beta-lactam antibiotics and other classes of antibiotics, such as fluoroquinolones, cotrimoxazole and aminoglycosides, they have now received an increasing concern in the healthcare centres worldwide.[4] The carbapenemase genes are mainly located on highly mobile genetic elements, including plasmids, which facilitate their transfer to other organisms.[5] Thus, because of this ability, they turn many pathogens-producing beta-lactamases into MDR ones.[6]

A variety of carbapenemases has been reported, some of which are as follows. Ambler class A, KPC-type (mostly identified in *P. aeruginosa* and *Enterobacteriaceae*) and GES-type (mostly in *A. baumannii*).[7] This class is mostly encoded chromosomally and includes carbapenemases sensitive to inhibition by clavulanic acid. The Ambler class B metallo-beta-lactamases (MBL) is another one, which mostly consists of GIM-, IMP-, VIM- and NDM-types. Finally, the Ambler class D carbapenemases include OXA-23, OXA-24/-40, OXA-58 and

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OXA-143 types in *Acinetobacter* spp. and OXA-48 type in *Enterobacteriaceae*. The presence of porin alternations in the class D carbapenemases most frequently confers resistance.[6,7]

Carbapenemase-producing GNB can cause a wide spectrum of infections, including bacteraemia, endocarditis, wound infections, urinary tract infections and nosocomial pneumonia. These infections are mostly associated with high mortality rates, treatment failures and long hospital stay; for example, it has been reported that attributable mortality for carbapenem-resistant *P. aeruginosa* infections is between 51.2 and 95%.[8] Therefore, for choosing antibiotic therapy schemes, especially in intensive care units (ICU), and implementing infection control measures, accurate and rapid identification of patients colonized by CP bacteria in the clinical microbiology laboratory is a matter of major importance.[9,10] In addition, studies have confirmed that, the order to have a less number of laboratory tests, early availability of antimicrobial susceptibility data can result in the decreased number of invasive procedures; as a result, hospital stay and healthcare costs are reduced.[11] However, their detection poses a number of problems, since the relevant methodology based on specific tests has not yet been well standardized and it cannot be based simply on the resistance pattern.[12] Generally, detection of CP isolates may rely on phenotypic and advanced molecular-based methods.[12,13] The ideal assay for identifying CP MDR GNBs should have a short turnaround time to ensure the timely implementation of control measures and able to detect carbapenem hydrolysis mediated by various enzyme classes. This issue could be challenged by difficulties in terms of detecting CP isolates, since minimum inhibitory concentrations (MICs) to carbapenems could be elevated, but within the susceptible range or even low, as described in *A. baumannii* and *Enterobacteriaceae*.[8] Therefore, the detection of these enzymes is thought to be a critical activity in many medical centres and could define robust standardized screening methods for the effective detection of CP bacteria in order to control their spread.

To screen the horizontally acquired mechanisms of reduced susceptibility in the clinical microbiology laboratory, practical and accurate phenotypic approaches are urgently required. Before the application of more expensive molecular techniques, non-molecular assays may provide crucial information. For the identification of carbapenemases, several non-molecular methods have been studied, mainly based on the use of specific inhibitors.[14] Although standard methods for its identification are based on molecular techniques, these methods are restricted in their ability to detect new carbapenemase variants. Moreover, the competencies and equipment required for molecular diagnostics are mainly available in the reference laboratories.[15] The overall detection scheme includes a screening step that is followed by a phenotypic and genotypic confirmation step presented in Fig. 1.

In the present review, we aim to gather a summary of the current methods for the identification of carbapenemase and describe the strengths and weaknesses of each method. To access this goal, almost all of the articles published in PubMed, Scopus and Google scholar are evaluated.

Carbapenemase Detection by Antibiotic Susceptibility Methods

Although novel phenotypic and molecular-based methods for carbapenemase production in Gram-negative pathogens are introduced, we still need simple and reliable tests. The first step in the detection of carbapenemase is based on the methods for characterizing antibiotic susceptibility testing in clinical isolates. These methods include disc diffusion, broth microdilution, antimicrobial gradient methods (e.g. Etest strips) and multiple commercially available automated systems [e.g. Vitek systems from bioMérieux (Marcy L'Etoile, France), Phoenix Automated Microbiology System from BD Diagnostics and MicroScan WalkAway System from Siemens (Dade Behring, West Sacramento, CA)].[11]

A clinical subject should qualify a clinical isolate for further testing of carbapenemase production by specific methods such as a significantly decreased size of inhibition zone using disk diffusion method or elevated MIC of a carbapenem. However, achieving to accurate susceptibility testing results for carbapenem drugs is often challenging in clinical microbiology laboratories. To indicate the appropriate antimicrobial treatment, phenotypic breakpoints established by European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) are useful (Table 1). However, the correct assessment of the resistance gene in the case of hospital outbreaks is fundamental to ascertaining the route of dissemination and employing the most appropriate containment measures.[16] According to CLSI breakpoints, to permit better detection of carbapenem-resistant isolates, the MICs of CP strains are significantly reduced.[17] According to the guidelines, the breakpoints for IPM and MEM are susceptible (S) ≤ 1 and resistant (R) ≥ 4mg/l, and for ertapenem (ETP), they are S ≤ 0.5 and R ≥ 2mg/l. Nevertheless, in the surveys performed to delineate the epidemiology of these pathogens and control their spread, the application of reliable detection methods is essential.[18] Detection of CP isolates based only on MIC values of ETP has no specificity. However, for detecting most of them, this agent seems to be a good candidate, since MICs of ETP are generally higher than those of other carbapenems.[16,19]

The disk diffusion and broth microdilution methods are considered more reliable for the detection of all types of carbapenemase-mediated resistance. Agar and broth microdilution methods aim to determine the MIC of assayed antimicrobial agent, which under the defined test conditions, inhibits the visible growth of the bacterium being investigated.[20] In the broth microdilution method

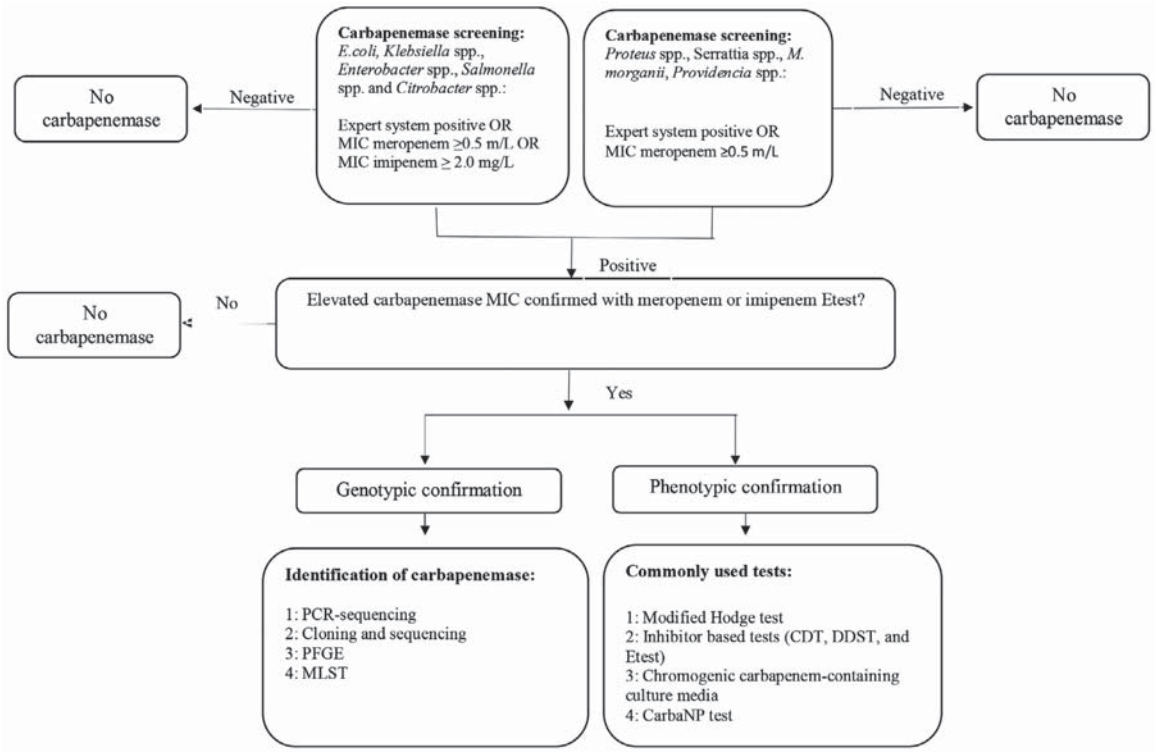

Figure 1 Detection scheme for carbapenemases in Enterobacteriaceae. The zone diameter screening breakpoint for meropenem has been set at ≤23mm with a disk content of 10 µg. For *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp., the zone diameter screening breakpoint for imipenem has been set at ≤21mm with a disk content of 10 µg (adapted from Cohen Stuart *et al.*[9]).

recommended by CLSI, the incubation time is set between 16 and 20hours for determining the antimicrobial susceptibility of pathogenic bacteria. However, the incubation time may differ for some microorganisms, which possess resistance difficult to detect.[21] Data suggest that the MICs determined by the microdilution method increase with increasing incubation time for these drug-resistant bacteria. Thus, the extension of the incubation time might be necessary to obtain the consistent MIC for drug-resistant bacteria.[22] According to the CLSI guideline for the phenotypic screening of CP *Enterobacteriaceae*, MICs of ETP, IPM and MEM related to 2, 2–4 and 2–4 µg/ml, respectively [or a zone of inhibition by ETP or MEM of ≤21mm in diameter in the disk diffusion (DD) assay] may indicate the production of carbapenemase isolates and this phenotype should be confirmed by specific methods.[23]

Automated susceptibility testing methods, because of their convenience and efficiency, are widely used in clinical settings. However, some of them have difficulty with the detection of carbapenemase production.[24] Indeed, probable errors reported by test system can have a serious effect on the clinical outcome for patients. A limited instrument quality control procedure has been a problem with automated susceptibility test instruments, which use both conventional and short incubation periods. This issue has occurred largely, because the standard control strains often result in many off-scale values, i.e. MICs greater than the highest concentration or less than or equal to the lowest

Table 1 Current BSAC, EUCAST and CLSI clinical breakpoints for carbapenems

		Zone breakpoints (mm)							
		BSAC		EUCAST		CLSI		MIC (µg/ml)	
Antibiotic (10 µg disc content)	Bacteria	R≤	S≥	R<	S≥	R≤	S≥	R>	S≤
Doripenem	*Enterobacteriaceae*	18	24	18	24	19	23	4	1
	Acinetobacter spp.	14	22	15	21	14	18		
	Pseudomonas aeruginosa	24	32	19	25	15	19		
Ertapenem	*Enterobacteriaceae*	15	28	22	25	18	22	1	0.5
Imipenem	*Enterobacteriaceae*	16	21	16	22	19	23	8	2
	Acinetobacter spp.	13	25	17	23	18	22		
	Pseudomonas aeruginosa	16	23	17	20	15	19		
Meropenem	*Enterobacteriaceae*	19	27	16	22	19	23	8	2
	Acinetobacter spp.	12	20	15	21	14	18		
	Pseudomonas aeruginosa	15	20	18	24	19	15		

BSAC: British Society for Antimicrobial Chemotherapy; EUCAST: European Committee on Antimicrobial Susceptibility Testing; CLSI: Clinical and Laboratory Standards Institute; MIC: minimum inhibitory concentration; S: sensitive; R: resistant.

concentration tested by the instrument.[25] According to the CLSI recommendations, the phenotypic detection of KPC-producing organisms is based on decreased susceptibility to MEM or ETP and includes a statement that the IPM disc test performs weakly as a screening test for carbapenemases.[26] Automated susceptibility testing methods also do not reliably detect KPC-mediated resistance[27,28]; however, many automated systems truly report KPC isolates as susceptible to MEM, while the MICs for most KPCs are above the susceptibility breakpoint of Food and Drug Administration (FDA) (4 µg/ml).[29]

Currently, several automated identification systems are available; most of these systems use colourimetry, turbidity or fluorescent assay principles. In microbiology laboratories, to decrease the laboratory turnaround time, automated antimicrobial susceptibility testing (AST) systems, such as Vitek, are commonly used,[30] that for approval when used for MEM- and IPM-satisfied FDA criteria.[31] The Vitek AutoMicrobic System (AMS; bioMerieux Vitek) was first introduced in the 1980s. A newer and more automated instrument is Vitek II (introduced into the market in 1999), which automates the initial sample processing steps to a greater degree than Vitek I. This system, which has been evaluated in several studies, automatically performs rapid detection and antimicrobial susceptibility of both Gram-positive cocci and Gram-negative rods after an inoculum has been prepared manually.[32,33] By providing definitive identification results for Gram-negative rods (including both members of the family of non-enteric bacilli and *Enterobacteriaceae*) within 3hours, Vitek II system is fundamentally different from the previous Vitek system.[34] Vitek II could be applied with assurance for the detection of resistance to several clinically important antimicrobial agents.[35] It has been shown that only 31.6% of MBL producers are flagged as potentially carbapenemase producers, while the sensitivities of carbapenemase detection with Vitek II by the aid of the advanced expert system has been reported to be 74–76%.[36] It would be interesting to determine whether Vitek II system versus agar dilution and Etest is more effective for the detection of carbapenemase producers with low-level carbapenem resistance.[37]

Phoenix™ Automated Microbiology System (BD Diagnostics, Sparks, MD, USA) is another system released in 2003, which was planned for the rapid bacterial detection at the species level and accurate determination of AST of clinically important human bacterial pathogens.[38] The very major error (VME) rate of 1.9% was noted with this system for both IPM and MEM, which was above the acceptable rate of ≤1.5%.[31] Screening based on the carbapenem MIC of ≥2 µg/ml (MEM for Vitek 2; ETP in combination with either IPM or MEM for BD Phoenix, Becton Dickinson Diagnostic Systems, Sparks, MD) was more sensitive and less prone to misinterpretation than the expert system screening.[39]

It is suggested that clinical microbiology laboratories routinely using MicroScan automated system for validating IPM susceptibility should consider using an independent AST method.[40] Conventionally, MicroScan system has the highest VME rate for IPM susceptibility testing (2.8%).[31] With the evaluation of results upon the acceptable performance criteria for susceptibility tests, the error rates of BD Phoenix and Vitek II are found to be in acceptable limits, while MicroScan has unacceptable results with very minor and major error rates in detecting IPM susceptibility.[40]

Phenotypic Screening Tests

For the identification of carbapenemase activity, several phenotypic, in-house and commercially available laboratory tests have been described. Some tests have good sensitivity and specificity; but, none of them approach 100%. In recent years, many phenotypic tests for the detection of carbapenemases have been presented, which include modified Hodge test (MHT), inhibitor-based methods and use of specific culture media.

Modified Hodge test

Cloverleaf test (or MHT), a modified version of Hodge test, which was recommended by the CLSI in 2009, is a phenotypic screening test,[41] for the isolates susceptible to a carbapenem. However, it demonstrates reduced susceptibility either by MIC testing or disk diffusion while performing a phenotypic test for carbapenemase activity. Modified Hodge test provides a high level of sensitivity and specificity (>90%) in terms of detecting Ambler class A (KPC type) and class D (OXA-48) carbapenemase producer among Enterobacteriaceae.[42] However, it suffers from poor sensitivity for MBL detection, lack of specificity for serine carbapenemases and a long turnaround time (Table 2).[43,44] But, to resolve these problems, after adding zinc in the culture medium, MHT method would be highly sensitive for detecting class A, B and D carbapenemases.[45] Although this method is relatively easy to perform and feasible in clinical laboratories, interpreting the results requires some experience. It is based on the inhibition of carbapenem activity along the streak of testing strains inoculum by CP strains that enable a carbapenem-susceptible indicator strain to extend growth towards a carbapenem-containing disk.[45]

Modified Hodge test and similar methods, such as Masuda assay (MAS), directly analyze the carbapenemase activity in unbroken cells and enzyme crude extracts, respectively. For the detection of CP bacteria, these tests are performed better than routine phenotypic methods, especially when combined mechanisms are present.[47] Studies suggest that MHT advocated by Centres for Disease Control and Prevention (CDC) and CLSI has specificity inferior to that of the indirect carbapenemase test (ICT) for the detection of carbapenemase production by non-*Klebsiella Enterobacteriaceae* KPC-producing isolates. Indirect carbapenemase test can assist clinical laboratories in accurately identifying *C. freundii*, *E. coli*,

Table 2 Main characteristics of methods for the detection of carbapenemase producing bacteria (adapted from Dortet et al.[46])

Test parameters	Modified Hodge test (MHT)	Culture method	Carba NP test	UV spectrophotometry	MALDI-ToF	Polymerase chain reaction (PCR)+ sequencing	Microarray assay
Sensitivity	100	96.5–43	100	100	100	100	98.9
Specificity	<90	57–68	100	100	100	100	100
PPV	85	60.4	100	100	100	100	100
NPV	100	100	100	100	100	100	99.7
Rapidity (hours)	24–48	24–48	2>	12–24	<4	24–48	0038–24
Cost[a]	$	$	$$	$	$$	$$$	$$$$
Expertise needs[b]	+	+	+	+++	++	++	++
Complete gene identification[c]	–	–	–	–	–	+	±

PPV: positive predictive value; NPV: negative predictive value. [a]The number of $'s correlates with the effective (relative) price of the test. [b]The number of +'s correlates with the expertise and training needed to perform and interpret the test. [c]+ means that the technique is able to give a complete gene identification, – means that the technique is not able to give a complete gene identification, and ± means that the technique is able to give a partial gene identification

Enterobacter and sp. isolates that do not harbour bla_{KPC}, yet have an elevated ETP MIC.[44]

Modified Hodge test in the detection of carbapenemase activity of candidate isolates can be used in the first step. In addition, it is useful for the evaluation of carbapenemase activity as a part of the infection control process for the outbreaks caused by carbapenemase producers.[16] It is also suggested to be used as a confirmatory test for carbapenemase production when the initial screening tests are indicative (carbapenem MICs 1mg/l).[48] However, the MHT-negative isolates with reduced zone diameters in Kirby Bauer disc diffusion method must be cautiously dealth with.[49]

False detection of carbapenemase production by MHT may occur in the species of *Enterobacteriaceae*, including *K. pneumoniae* and *E. coli* as well as intrinsic chromosomal AmpC beta-lactamase of *Serratia marcescens* and *Enterobacter cloacae*. These false-positive results probably result from low-level carbapenem activity by ESBLs and AmpC enzymes, or loss of porins.[42] This hypothesis is strengthened considering the fact that the frequency of false positive results is directly related to the tested inoculum.[50] Positive results could also be due to other carbapenemases.[51] Additionally, MHT may detect the expression of functional carbapenemases and used by some laboratories. However, it adds significant time (24–48hours) for detection and is recommended only for the detection of KPC by CDC.[52] Therefore, strains with positive results from MHT need to be further investigated in terms of the presence of class A carbapenemase genes.[42]

For any indeterminate or positive isolates in MHT, performing the combination disk test is requested by laboratories, essentially routine disk diffusion susceptibility testing with each carbapenem, together with the same carbapenem disk incorporating the inhibitor of class A carbapenemases and that of class B carbapenemases into boronic acid and ethylenediaminetetraacetic acid (EDTA), respectively.[53] Modified Hodge test has been found to be useful for the phenotypic detection of KPC enzymes in hospitals where these beta-lactamases are endemic. However, the test cannot discriminate between KPCs and other carbapenemases. Obviously, its positive predictive value (PPV) for KPC detection is low in the regions where other carbapenem-hydrolyzing enzymes, like MBLs, are also prevailing.[54] The proposed positive MHT category has 98% agreement with the molecular bla_{KPC} results, highlighting the good PPV of KPC detection among *Enterobacteriaceae* when a standardized method for interpretation is in place.[51] However, EUCAST and British Society for Antimicrobial Chemotherapy (BSAC) do not recommend carbapenemase detection in *Enterobacteriaceae* as the routine method (Table 1).[50]

Pasteran *et al.*[55] used a new indicator strain, *K. pneumoniae* ATCC 700603 instead of *E. coli* ATCC 25922 to optimize MHT for a more reliable and accurate method in terms of detecting carbapenemase production in *P. aeruginosa*; so, they called the test *P. aeruginosa* MHT (PAE-MHT). This test will enable routine laboratories to identify CP *P. aeruginosa* isolates, including some of the most important epidemiological challenges of recent times, such as carbapenem susceptible MBLs and KPCs.

Inhibitor-based methods

Series inhibitor-based methods have been developed for the detection of carbapenemase producers. Although, for OXA-48 (like) carbapenemases, no specific phenotypic identification method is available, the sensitive and specific detection of class A and B carbapenemases may be performed using carbapenemase inhibition disc diffusion tests (synergy tests).[56] However, in the case of MBL detection, there are no standard guidelines available; thus, standardization of a phenotypic method for screening MBL-producing isolates is of crucial importance. The selection of the appropriate MBL test should be based upon studies providing sensitivity and specificity results for that specific pathogen.[57] With regard to the laboratory detection of MBLs in *Enterobacteriaceae*, the MBL-inhibitory action of chelating agents has shown their usefulness.[15] Several screening methods have been developed for the detection of MBL-producing organisms based on the fact

that the MBL activity is blocked by chelating agents, such as EDTA, dipicolinic acid (DPA) and 2-mercaptopropionic acid (2-MPA).[58] Unfortunately, inhibitor-based disc tests with MBL inhibitors combined with carbapenems can be associated with low PPV and specificity for *A. baumannii* and *P. aeruginosa* due to the unspecific impact of inhibitors on the bacterial cell. EDTA, like many other metal chelators, in general increases the permeability of the outer cell membrane, which potentially results in false-positive MBL test results.[15] It is worth mentioning that EDTA is a polyamino carboxylic acid, which is primarily used to lyse bacterial cells and release beta-lactamases but also binds metal ions like zinc and can inactivate MBLs.[59]

In biochemical studies, DPA has been described as an effective chelating agent against some MBL chelators.[60] It neither hydrolyzes the antibiotics nor inhibits the growth of bacteria.[61] Data have suggested that DPA–IPM disk and DPA disk synergy tests (D-DST) for the detection of MBL-producing isolates could be a useful screening method due to their high efficacy and relatively easy procedures.[62] In addition, among the phenotypic detection methods introduced so far, DPA–IPM disk test is considered to be the best phenotypic screening method for detecting MBL (at least VIM-2 and IMP-1 types)-producing isolates of *K. pneumonia*, *Acinetobacter* spp. and *Pseudomonas* spp.[62,63]

On the other hand, the moxalactam, an extended-spectrum cephamycin, in the moxalactam-EDTA (MOX-EDTA) DST can be utilized as a confirmation method to differentiate MBL production and outer membrane impermeability. This assay is suitable for specific MBL screening in the countries with limited resources, due to requiring inexpensive reagents.[64]

In the comparative evaluation of MEM-inhibitor discs and chromogenic agar medium for carbapenemase detection in *Enterobacteriaceae*, the MEM-plus-inhibitor approach, despite the limitation of being unable to detect class D carbapenemases, was more sensitive and specific than the other methods.[65] Rosco Diagnostica Neo-Sensitabs (RDS) and Mastdiscs ID inhibitor combination disks (MID) containing MEM with different inhibitors plan for the detection of *Enterobacteriaceae* that produces different types of carbapenemases. Doyle *et al.* showed that the sensitivity and specificity of RDS are 80 and 93%, respectively. He also demonstrated the sensitivity and specificity of Mastdiscs ID inhibitor combination disks as 78 and 93%, respectively.[66]

Etest MBL strip

Etest is a quantitative technique for determining the MIC of antimicrobial agents against microorganisms and for detecting resistance mechanisms. Etest MBL strip [IPM–IPM+EDTA (Ip–IpI)] is one of the methods proposed for the detection of IMP and VIM-type MBLs (both chromosomal and plasmid mediated) based on the inhibition of MBL activity by EDTA.[67,68] It has been evaluated in different works and found to be a sensitive method for the detection of MBL production in *Pseudomonas* and *Acinetobacter* species.[69] Etest MBL is efficient in detecting MBL producers exhibiting high-level resistance, but may fail to detect MBL producers exhibiting low-level resistance to IPM.[16]

It contains increasing concentrations of IPM on one end and IPM overlaid with EDTA on the other. Although this test is simple to perform, it is costly and highly insensitive in terms of detecting carbapenem-susceptible MBL-carrying organisms (MIC $\leq$ 4 µg/ml).[70] Considering these defects, many clinical microbiology laboratories use alternative screening methods, such as combined disk (CD) assay and double-disk synergy test (DDST) (see below).[57]

The EDTA chelates the zinc ions required by MBLs to catalyze the hydrolysis of IPM and MEM, thereby inhibiting MBL activity.[71] Therefore, in the presence of EDTA, susceptibility to IPM will be restored, confirming the significance of MBL as a source of carbapenem resistance among MBL producers. However, several MBL-negative strains producing OXA-23 or OXA-40 provide false-positive results.[67] It has been noted that Etest MBL is highly sensitive and specific for detecting $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-2}}$ allele-positive isolates of *Acinetobacter* spp. and *Pseudomonas* species.[69] The choice of the Mueller–Hinton agar brand may affect the performance of Etest MBL, but may have also been influenced by the level of IPM resistance of the MBL-producing strains and possibly other testing conditions.[69] The main advantage of the KPC and MBL Etests over MHT and Carba NP tests is the rapid discrimination between class A and class B carbapenemase producers.[72] However, Carba NP test II, which includes inhibition by EDTA and tazobactam, may be also used for the detection of carbapenemase types (see below).[72]

Boronic acid-based method

To limit the spread of the bacteria, laboratory identification of class A carbapenemases-harbouring clinical isolates, such as KPC, is crucial. However, the detection of KPC-harbouring stains in the clinical laboratory has remained a difficult task. The phenotypic methods based on the inhibitory activity of boronic acid compounds are very easy to perform and interpret and may be applied from the first day of the isolation of the suspected resistant *Enterobacteriaceae.*[73]

Boronic acid compounds are serine-type beta-lactamase inhibitors, which were reported in the early 1980s and are known to be rapid and reversible inhibitors of AmpC enzymes belonging to class C beta-lactamases; their inhibition is not based on the beta-lactam structure.[74] Class A carbapenemases, like KPC, can be screened by synergy with boronic acid; but, false-positive synergy test results occur if AmpC beta-lactamases are coproduced.[75] However, they also have an inhibitory activity against multiple class A beta-lactamases, such as the chromosomal penicillinase of *Bacillus cereus* and some of the CTX-M-type ESBLs.[73,76] Recently, boronic acid compounds have

been proposed to be the inhibitors that can be used in disk potentiation tests and efficiently differentiate KPC producers from those producing MBLs or other broad-spectrum beta-lactamases.[54] These methods have been found to significantly increase the growth-inhibitory zones around the disks of cefotetan disks, allowing for the accurate differentiation of plasmid-mediated AmpC-producing isolates.[77] Recently, a boronic acid-based method, using 3-aminophenylboronic acid (APBA)-IPM CD, has been found promising in the detection of the presence of class A carbapenemases, with higher sensitivity and specificity than MHT.[42]

Combination disk test

Previously, combined disc tests (CDT) using different amounts of EDTA/disc have been evaluated for the detection of MBLs. Combined disc synergy tests, because of their low cost and convenience, have been extensively used. However, the performance of these assays may be compromised by changes in CP bacterial populations.[18] Combined disk tests are based on the inhibition of MBL enzymes by DPA or EDTA, leading to differences in the zone diameters of carbapenem disks with or without the inhibitor.[78] Recently, various CDT, particularly those using MEM, have been introduced for the differentiation of class A carbapenemases and MBLs.[14,36] This method of providing fast and reliable detection of carbapenemases among *P. aeruginosa* and *Enterobacteriaceae* isolates could be very practical in daily practice.[79] It has been shown that MEM CD test provides an accurate and uncomplicated phenotypic method for the specific differentiation of *Enterobacteriaceae* isolates possessing MBLs, KPCs or both carbapenemase types.[14] The diameter of inhibition zones produced by many beta-lactam/MBL inhibitor combinations may be different, depending on the technique by which the CDs are prepared.[80] A CDT using EDTA and IPM (EDTA–IPM CDT) due to growth inhibition by EDTA alone shows a high false-positive rate with *Acinetobacter* spp.[81] Conversely, the combined use of IPM and MEM MICs (cutoff values of $\geq 2\,\mu g/ml$ and $\geq 1\,\mu g/ml$, respectively) will allow routine labs to detect those isolates suspected of producing carbapenemases with high confidence levels.[30]

Double disc synergy test

Double disc synergy test is a method for the screening of beta-lactamases that are inactivated by inhibitors such as clavulanic acid (Ambler class A beta-lactamases, especially ESBLs).[82] Double-disk synergy test is easy to perform and can be incorporated into the daily task of clinical microbiology laboratories that routinely use disk diffusion as their preferential AST method.[83] Also, to screen for the emergence of MBL-producing strains for clinical and surveillance purposes, this method might be helpful.

Identification of MBLs is generally based on carbapenem-EDTA synergy tests in different formats.[84] By an IPM disk and an EDTA disk, or a ceftazidime (CAZ) disk and a thiol compound (2-mercaptopropionic acid) disk, DDST has been suggested as an effortless method for identifying MBL-producing isolates.[81] However, according to the inhibition zone diameters of the substrate and inhibitor disks, DDST requires the modification of the distance between the disks.[85] In the comparison of the double-disk with Etest methods and CD, 2-mercaptopropionic acid double-disk potentiation method using cefepime and CAZ with and without clavulanate has been found to be the most sensitive (100%) for detecting MBL-producing GNB.[58] Although DDST and CDT are effortless and cheaper than MBL Etest, depending on the employed methodology, MBL inhibitors (IMBL), beta-lactam substrates and tested bacterial genus, they have shown discordant results.[57] According to the previous studies, EDTA-DDST has better performance than MBL Etest and CDT.[86] When strains are highly resistant to MEM caused by the overexpression of efflux pumps, IPM disks perform better than MEM disks for DPA-DDSTs.[87] Hirsch *et al.* compared four phenotypic screening methods including the phenylboronic acid double disk synergy testing (PBA-DDST) using both meropenem (MePBA) and ertapenem (Ee PBA), MHT and one type of chromogenic agar.[88] In their study, MePBA-DDST showed the best operation with 100% sensitivity and specificity.

To detect MBL-producing strains, another technique using the similar principle based on the inhibition by EDTA was evaluated. Based on synergy with EDTA, this method is accessible for the detection of MBLs, but can exhibit false-positive results with some strains and cannot distinguish between MBL types.[75] Conversely, for the identification of class D carbapenemases such as OXA-48/OXA-181-producing strains, there is no specific inhibition test. The reason is that the effect of these enzymes is not inhibited by sulbactam, tazobactam, clavulanic acid or any zinc chelators;[16] but, there are reports on using temocillin disk (or in combination with avibactam) for this purpose.[8]

Carbapenemase detection with culture-based method

There are several culture methods for the detection of carbapenem-resistant GNB, including methods that use in-house-prepared selective media, such as tryptic soy broth or MacConkey agar containing a 10-μg carbapenem disk, or commercial chromogenic agar media, like CHROMagar KPC, ChromID ESBL, ChromID CARBA, Brilliance™ CRE Agar and SUPERCARBA.[89]

Currently, culturing perianal or rectal swab samples on selective and differential agar plates, such as MacConkey agar, is the standard technique for the screening of patients colonized with CP organisms. It is sometimes supplemented with a carbapenem disk and followed by AST of lactose-fermenting colonies.[90] Chromogenic media are widely used for the screening and rapid detection of carbapenem-resistant GNB. Indeed, detection of CP carriers

in stool is generally based on the application of screening culture media, such as CHROMagar and ChromID CARBA media (Table 2),[91] both of which contain chromogenic molecules related to the recognition of enterobacterial species.

Recently, CHROMagar KPC, a new chromogenic medium, has been developed. It is a strong medium for the screening of strains with high levels of resistance to carbapenems (>16 μg/ml); but, it is less sensitive for the screening of strains with low levels of resistance to carbapenems.[92] The phenotypic characteristics on CHROMagar KPC permit the easy differentiation of bacterial colonies. Moreover, this medium without the need for sub-culturing allows for the identification of isolates resistant to ertapenem and sensitive to other carbapenems.[93] In a clinical evaluation study, it was shown that MacConkey agar and CHROMagar-KPC screening plates supplemented with 1 μg/ml IPM had comparable sensitivities and negative predictive values (NPV) in the identification of highly resistant bla_{KPC}-producing CRE strain, which had an increase in healthcare centres and exhibited high-level resistance.[94] Compared to CHROMagar KPC and the ertapenem disk method, Spectra™ CRE has shown excellent overall sensitivity and specificity for detecting KPC-producing *Enterobacteriaceae* in perirectal swabs with superior performance.[95]

ChromID CARBA (bioMérieux) is another chromogenic agar medium planned for the detection of CPE, which supplemented with specific agents that restrain the growth of Gram-positive and non-carbapenemase producers.[89] This medium shows the best sensitivity and specificity and is at least as good as Brilliance CRE and CHROMagar KPC for the detection of *Enterobacteriaceae* strains that produce any of the conventional types of carbapenemase.[96] In a study designed for the recognition of carbapenemase-producing GNB, chromID CARBA demonstrated the highest sensitivity and specificity, followed by CHROMagar KPC and Brilliance CRE.[97] However, the chromID CARBA medium had some limitation in the detection of OXA-48-producing strains. To solve this problem, chromID OXA-48 (bioMérieux) has been introduced. Comparative assessment of this chromogenic culture medium with SUPERCARBA and chromID CARBA shows that chromID OXA-48 and SUPERCARBA media have the highest sensitivity for the identification of OXA-48 producing *Enterobacteriaceae* (91 and 93%) compared to chromID CARBA (21%).[98] ChromID OXA-48 has the highest specificity, with 100% compared to 68 and 53% for chromID CARBA and SUPERCARBA media in terms of detecting OXA- 48-producing strains, respectively.[98]

Brilliance™ CRE agar (Thermo Fisher Scientific, Waltham, MA, USA) is a novel commercially prepared chromogenic agar medium supplemented with a modified carbapenem that inhibits the growth of carbapenem-susceptible bacteria.[99] Although the detection of OXA-48 producers by this test is less optimal, it is an appropriate, simple and sensitive method for the detection of MBL- and KPC-producing *Enterobacteriacea*e.[100] Therefore, it can be a labour-intensive and less time-consuming alternative method recommended by healthcare preparedness activity (HPA) and CDC.[101] According to the available data, Brilliance CRE agar can be considered a reliable selective medium because of allowing the growth of the vast majority (92.6%) of the carbapenem-non-susceptible enterobacterial isolates while suppressing most (85.1%) of the susceptible ones.[102] However, relatively low specificity is a weakness of this medium, which needs the verification of carbapenemase production for the strains screened by CRE agar.[96] In a study by Stuart *et al.*, the sensitivity of CRE agar for the detection of CPE was 94% (89/95), but differed per carbapenemase gene (84% for OXA-48, 90% for VIM and 100% for KPC-, NDM- and GIM-producing strains); however, because of the growth of AmpC- and/or ESBL-producing strains, the specificity of CRE agar was 71%.[100] The medium could select carbapenem-nonsusceptible bacteria even if they exhibit low levels of resistance to carbapenems.[102] It permits the growth of carbapenem-resistant strains with low detection limits and could represent a practical screening medium for both non-fermentative and enterobacteria Gram-negative strains resistant to carbapenems.[99]

Nordmann *et al.* designed a Drigalski agar-based culture medium supplemented with a zinc sulphate, carbapenem and cloxacillin termed SUPERCARBA as a novel screening medium.[103] This is very first screening medium that may detect not only MBL- and KPC- but also OXA-48-producing strains. Ability to detect carbapenemase producers with low-level resistance and being as selective as possible by inhibiting the growth of carbapenem-resistant, but non-CP, isolates are the rationale for the design of this medium.[103] In the comparison of Brilliance CRE, CHROMagar KPC and SUPERCARBA screening media for the identification of *Enterobacteriaceae* with decreased susceptibility to carbapenems, as compared to CHROMagar KPC (43%) and Brilliance CRE (76.3%), the SUPERCARBA medium had the highest sensitivity (96.5%).[104]

Biochemical-Based Detection of Carbapenemases

Spectrophotometric methods

Based on the analysis of the IPM hydrolysis, a spectrophotometry method has been introduced that differentiates carbapenemase- from non-carbapenemase-producing strains.[105] This inexpensive method, adapted for the detection of CP strains, may be implemented globally in any reference laboratory. It can differentiate the CP strains from those that are carbapenem resistant due to carbapenemase-independent mechanisms, such as combined mechanisms of resistance (e.g. clavulanic acid-inhibited ESBL, outer membrane permeability failure, overexpression of cephalosporinases) and from the strains expressing

extended-spectrum beta-lactamases without carbapenemase activity (plasmid and chromosome-encoded cephalosporinases, ESBLs).[105]

In recent years, Raman spectroscopic analysis (RA) has been validated for the bacterial typing of different species. Raman spectroscopy is a label-free, optical technology based on the inelastic scattering of light by molecules.[106]

UV spectrophotometric assay

The biochemical method based on UV spectrophotometric assay for the identification of CP strains has also been introduced.[105] This method performs on a number of steps, including: (i) an 18hours culture (which can be reduced in some cases to 8hours); (ii) a step for protein extraction; and (iii) measuring IMP hydrolysis using a UV spectrophotometer.[107] Although this technique efficiently detects SIM, IMP and VIM producers, carbapenem-hydrolyzing class D beta-lactamase (CHDL) and NDM producers remain difficult to detect (Table 2).[67] UV spectrophotometric assay in a study carried out by Bernabeu *et al.* had 100% sensitivity and 98.5% specificity for detecting carbapenemase production.[105] These techniques are time-consuming, but require trained personnel and are usually applied for the research, in which specific activities of carbapenems for IPM at the wavelength value of 297nm are measured by UV spectrophotometry.[67] The use of mass spectrometry (MS) based on the analysis of the degradation of a carbapenem molecule for the detection of carbapenemase activity has been also proposed. Although this technique has to be further evaluated as a good method, matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF) apparatus is gradually more applicable in the diagnostic bacteriology laboratory (see below).[107]

Carbapenemase detection using mass spectrometry methods

The beta-lactamase mechanism is the hydrolysis of the beta-lactam ring via the addition of H_2O. Several authors have hypothesized that this mass alteration would be readily measurable by MS. Mass spectrometry method has been used to detect the production of beta-lactamase-catalyzed (e.g. hydrolyzed penicillin and carbapenem) from the microorganisms treated by drugs. Most of these assessments will detect the existence of hydrolyzed products qualitatively, but do not measure their concentrations and thus prevent the quantitative evaluation of beta-lactamase activity among different bacterial cells.[108] Resistance to beta-lactam antibiotics can be easily monitored by MS due to the vanishing of the original mass peak through the molecular mass shift of +18Da of the antibiotic agent after the hydrolysis of the beta-lactam ring by beta-lactamases.[109] In recent years, MS technology-based methods have been revealed to be able to detect CP bacteria. Some of these methods are ultra-performance liquid chromatography–tandem MS (UPLC-MS/MS) and matrix-assisted laser desorption ionization–time of flight MS (MALDI-TOF MS).[110]

An alternative technical solution could be capillary electrophoresis (CE) MS. Capillary electrophoresis is a highly efficient technique for peptide analysis because of its ability in separating and analyzing peptides with a wide range of physicochemical properties. Compared to LC–MS, it is particularly suitable for the analysis of smaller peptides.[111]

Recently, to detect beta-lactam ring hydrolysis, MALDI-ToF has been introduced in clinical microbiology for species identification. It is a technique in which material is ionized in a high-vacuum chamber and accelerated in an electric field. This assay allows for a very quick recognition and verification of class A and B carbapenemase activity and can be performed by standard matrix.[112] The MALDI-TOF MS is a cheap, highly accurate and rapid method, which is able to identify a great variety of isolated bacteria and fungi based on the composition of conserved ribosomal proteins (Table 2);[113] but, in the intricate diagnostic procedure, it may potentially be applied.[114]

During the last years, the concept regarding the recognition of beta-lactamases by means of this technology has been proved by a few studies.[112,115] However, this method is typically inadequate for the direct identification of resistant phenotypes and is not suitable for certain identification of the specific proteins responsible for this resistance.[111] Although MALDI-TOF MS has been incorporated with automated AST platforms, these systems usually do not accurately report the carbapenem MICs for CP *Enterobacteriaceae*.[116] Therefore, the accurate detection of carbapenemase production is still essential.

As identification and typing with MALDI-TOF MS take only a few minutes and the ertapenem hydrolysis can be carried out within 2.5hours, MS-based antibiotic resistance detection in this case may help select better antibiotic therapy.[117] Using this technology, diagnostic spectral data can be acquired directly from fluids, such as blood and urine,[116,118] which indicate an advance in the treatment of septic patients. However, additional studies with more strains should be performed in order to evaluate sensitivity and specificity.[119] According to previous studies, the performance of liquid chromatography–tandem MS (LC–MS), MALDI-TOF MS and hydrolysis assays in detecting class A (GES-5 and KPC-2) and B (GIM-1, IMP-1, IMP-10, SPM-1, VIM-1 and NDM-1) carbapenemase activity is excellent for both 2- and 4-hours incubation times (100% sensitivity and 100% specificity).[120]

It is now well recognized that MALDI-TOF can be applied not only for bacterial identification but also for other important fields of diagnostic bacteriology, such as detection of antibiotic degradation mediated by the enzymatic reaction of diverse enzymes (carbapenemase, beta-lactamase).[121] However, for some beta-lactams, like MEM, due to binding the molecules to cell lysate

components, the revelation of degradation products by MALDI-TOF MS seems to be difficult.[122] For the peak detection, it is not easy to understand the causes behind the observed differences between the laboratories when different instruments, matrix, buffer, etc. are applied.[123]

The MALDI-TOF assay has higher sensitivity and specificity than other conventional carbapenemase detection methods, such as MHT and 3-D bioassay in VIM-2, IMP-6, KPC-1, NDM-1, SIM-1 and OXA-23/-51 CP *K. pneumonia*, *P. aeruginosa* and *Acinetobacter* spp.[124] Hrabak *et al.* described a modified method for detecting degradation products and reducing the turnaround time to ca. 2.5hours. The modified assay was confirmed with VIM-1-, KPC-2-, KPC-3-, NDM-1- and OXA-48/-162-producing members of *Enterobacteriaceae* and NDM-1-producing *A. baumannii* strains.[122] In comparison with molecular techniques, such as Check-MDR CT103 microarray and Check-MDR Carba PCR, MALDI-TOF-based method, utilizing a stable 10 µg disk of ETP, was highly efficient in detecting carbapenemase with higher sensitivity than microarray and PCR.[121]

The application of MALDI-TOF MS method for the recognition of carbapenem resistance has many advantages over other methods, such as PCR, because when the causative enzyme is unknown, it can distinguish low-level carbapenemase activity, even at low cost.[125] The main advantages of MALDI-TOF MS are the objective endpoint and the possibility of better detection of OXA-48 producers.[126] Thus, this method is appropriate both for finding novel carbapenemases and for quickly detecting resistance in clinical settings. However, the spectra should be manually interpreted by a specifically skilled technician, at least until an automated interpretation algorithm is developed and implemented in Bruker software.[121] The main disadvantage of MALDI-TOF MS is the extra time required for incubation (4 vs 2hours), processing of target plates and interpretation of the results (total hands-on time of several minutes).[126] Moreover, it cannot identify other mechanisms of carbapenem resistance. These issue would include efflux mechanisms and porin alterations for *P. aeruginosa* and *K. pneumonia* as well as efflux mechanisms, PBP alterations and porin alterations for *A. baumannii*.[125,127]

For antibiotic susceptibility testing (MAAST), Grundt *et al.* developed an MS-based test that allowed for providing significant health care data on the outcome of antibiotic susceptibility testing in <90minutes after primary microbial growth was detected in a blood culture that was turned out to be positive.[128] MAAST is based on the identification, detection and quantification of antibiotics and they are related to metabolization/inactivation products as generated by beta-lactamases.

Despite its wide use, MALDI-TOF MS is classically thought not to be well suited for small-molecule detection and LC–MS/MS remains the gold standard method for them.[129] LC–MS techniques can be considered as the methods of choice for the majority of drug detection and drug level determinations, even for the antibiotics like ETP.[130] This method, with its excellent analytical sensitivity, could reliably detect carbapenemase activity by monitoring the appearance of the hydrolyzed metabolite of a carbapenem antibiotic from a complex biological matrix.[52] It offers several advantages over other similar assays that use MALDI-TOF MS analysis. The system is easy to perform and robust for usual analysis in the concentration ranges in sub-microgram per millilitre, which is underlined by the investigated performance parameters. Finally, this LC–MS technique may be appropriate for other carbapenem agents.[130] Despite the fact that the UPLC–MS/MS platform is clearly more expensive than the real-time PCR, the detection of carbapenemase activity by the method could be simply implemented in the laboratories already using this technology for the determination of carbapenem antibiotic levels in patients.[110]

Carba NP test

Recently, Nordmann *et al.* introduced Carbapenemase Nordmann–Poirel (Carba NP) test as an inexpensive, rapid and easy to perform and interpret, with high specificity and sensitivity in terms of detecting not only carbapenemase activity but also carbapenemase types in *P. aeruginosa* and *Enterobacteriaceae* (Table 2).[131,132] It is based on the biochemical detection of the hydrolysis of the beta-lactam ring of a carbapenem molecule, followed by the colour change of phenol red pH indicator (red to yellow/orange) without the need for specialized equipment.[133,134] The principle is also used in a commercially available tablet format called Rapid CARB Screen Kit (Rosco Diagnostica A/S, Taastrup, Denmark).[135] For choosing a first-line therapy based on an aminoglycoside associated with a fluoroquinolone rather than beta-lactam-containing combinations in the case of positivity, use of this test would be helpful, in particular when treating pneumonia.[136]

Apart from the original publications, some studies have basically evaluated Carba NP test, and both the originally published protocol and modified versions have been used. For the labs concerned with the extensively distributed NDM- and KPC-producing isolates, the test eliminates the requirement for other more time-consuming and/or less accurate phenotypic methods to identify carbapenemase-producers, such as the CD tests or MHT.[134] Indeed, to quickly detect the potential carrier isolates, Carba NP test is a cost-effective and accurate technique, which could then be further confirmed by molecular techniques such as PCR.

With the exception of several GES-type producers, Carba NP test is able to differentiate the carbapenemase producers from those isolates that are carbapenem resistant because of carbapenemase-independent mechanisms, such as combined mechanisms of resistance (deficiency in outer membrane permeability associated or not with the overexpression of cephalosporinase and/or ESBLs)[133] or from the strains that are carbapenem sensitive, but produce

an ESBL without carbapenemase activity.[132] Also, it can be useful for screening CP strains in an attempt to control outbreaks and to quickly differentiate between carbapenemase producers (transferable resistance determinant) and non-carbapenemase producers (nontransferable resistance determinant).[136] It should be noted that, to recognize CP isolates grown on Drigalski agar and MacConkey agar plates, Carba NP test is not appropriate.[137]

Still, the combined sensitivity and specificity of Carba NP test are better than those of most other phenotypic methods, such as UV spectrophotometry and Rapid CARB Screen,[131,138] since Carba NP test is quicker (<2 vs 24 hours for UV spectrophotometry) and does not require any specific training to use.[46,139] In comparison with Rapid CARB Screen Kit, Carba NP test has better performance in terms of detecting carbapenemase production in *Enterobacteriaceae* and *P. aeruginosa.*[135] However, both tests have good performance, especially in terms of their high specificities, and are easy to perform and interpret together with reasonable costs. Also, compared with real-time PCR method, the application of this test has several advantages directly for blood culture. First, Carba NP test could identify any carbapenemase, whereas PCR-based techniques are planned to detect only one or a few carbapenemase genes. In addition, it could be implemented in low-income countries, which are known to be large reservoirs of CP strains, because of its inexpensive performance.[137] The test could also be used for the fast identification of carbapenem-resistant isolates from faecal specimens screened for multidrug-resistant bacteria.[132] For detecting carbapenemase activity in non-fermenters such as in *Pseudomonas* spp., Carba NP test has several benefits. It eliminates the need of *in vitro* identification of carbapenemase activity (Hodge test) and of beta-lactamase inhibitor-based phenotypic methods (EDTA for MBLs, boronic acid for KPC), both of which need at least 24–72hours to be performed.[133]

Among *Acinetobacter* spp., Carba NP test detects MBL producers, but fails to detect the production of other carbapenemase types. To solve this limitation, the newly designed CarbAcineto NP test (adapted from Carba NP test) has been introduced, which is rapid and reproducible. It is able to detect all types of carbapenemases with the exception of some GES-type producers at the specificity of 100% and sensitivity of 94.7%.[140] This method could help in the timely identification of these resistant clones and allow for earlier directed therapy and prompt infection control procedures.[141]

Pires *et al.* also proposed an adapted test (Blue-Carba) that was validated for the identification of carbapenemase-producers directly from bacterial cultures.[142] Blue-Carba is a cheaper and easier substitute for Carba NP test, which allows the detection of carbapenemase activity directly from the bacterial cultures of *Pseudomonas*, *Acinetobacter* and *Enterobacteriaceae* species.[142]

Carbapenemase Detection Using Molecular Methods

To screen the most clinically important mobile resistance genes, the accessibility of quick diagnostic techniques not only enables the surveillance of these multi-resistant infections but also allows for the earlier identification of outbreaks and, therefore, can facilitate the distribution of resistant bacteria.[143] Thereby, molecular techniques are additionally required for the fast, accurate and sensitive detection of carbapenemase genes. Due to the limitations of the phenotypic carbapenemase confirmation tests, gold standard methods for the detection of carbapenemases are based on molecular techniques, chiefly PCR, such as conventional PCR with sequencing, multiplex PCRs and DNA hybridization, and others are also applied to detect carbapenemase genes.[46] These techniques are comparatively complex and need particular costly equipment. Multiplex PCR analyses and DNA microarray assays are the most specific molecular techniques that have been developed.[144] PCR- and sequencing-based approaches are suitable for the detection of resistance genes even from primary material, while there are more expensive, but are the most frequently applied modes of testing (Table 2). Sequencing of the genes, mostly for epidemiological and research purposes, is interesting.

For the detection of carbapenemase genes, molecular techniques have several advantages over culture-based methods, including rapid turnaround time and high sensitivity. In addition, they provide a significant supply for epidemiological studies. Despite their advantages, the molecular techniques have slow performance; first, because of the concerns about allelic diversity within target genes, the failure to detect new unidentified genes, primer cross-reactions, requirement for skilled personnel and high costs.[145] They also fail to correctly identify the simultaneous existence of more than a single beta-lactamase gene of a certain class.[146] However, the increasing number of new carbapenemases makes molecular tests unsuitable for the initial detection of carbapenemase production.

Real-time PCR assay and sequencing

Nowadays, due to the high variety of these versatile enzymes and because many clinical isolates harbour more than one beta-lactamase, PCR techniques are generally applied for their recognition in epidemiological studies.[147] This assay provides a quick, sensitive, specific and useful tool for the recognition and identification of these important resistance genes.[148] To determine the sequence of carbapenemase genes, DNA sequencing may also be used.

Multiple real-time PCR assays detecting KPC or NDM-1 have been described; but, each targets only one kind of carbapenemase gene.[149] In addition, for screening the presence of most common bla_{IMP}, bla_{VIM} and bla_{OXA} genes, several real-time PCR protocols have been introduced so that their wide distribution can be controlled.[150,151]

Compared with the present accessible techniques for the recognition of NDM – producing strains, i.e. screening for the carriers of NDM-1-producers with media, such as chromID ESBL culture medium, and final recognition as NDM-1-producers with standard PCR amplification and sequencing, the real-time PCR assay has multiple advantages, which includes sensitivity, specificity and possibility of identifying NDM-1-producing strains very rapidly (<2hours) either for quick screening of rectal swabs or for the identification of any carbapenem-resistant isolates.[152]

Real-time quantitative PCR (qPCR) is a standard PCR with the advantage of detecting the amount of DNA formed after each cycle with either fluorescent dyes or fluorescently tagged oligonucleotide probes.[153,154] Since its introduction, qPCR has modernized the field of molecular diagnostics and the technique is being used in a quickly expanding number of applications.[153] Molecular diagnostic techniques, in particular qPCR, have been shown to be accurate and quick methods for the detection of bla_{KPC} and bla_{NDM-1} genes.[91,155,156] This method is likely to decrease the time for carbapenemase detection, especially in the case of bla_{OXA-48} from 48 to 4hours. Also, it is an important tool for following up the disease outbreak in order to quickly isolate colonized patients and assign them to cohorts.[151] Recently, the development of qPCR for detecting carbapemenases directly from stool or rectal swab samples has been described.[91]

The PCR-P is another technique consisting of a new long-fragment quantitative PCR (LF-qPCR) to identify the full-length bla_{NDM-1} in clinical isolates. It is a functional assay of *in vitro*-synthesized protein to recognize whether LF-qPCR amplicons contain bla_{NDM-1} or its efficient variants or not; this process is performed via measuring the degradation rate of IPM.[157] This new technique for quickly detecting the full-length bla_{NDM-1} and making clear its functional variants in clinical isolates provides the first integrated approach.

Recently, to screen KPCs gene-containing isolates, an internally controlled real-time PCR assay based on SYBR Green has been developed that is confirmed for clinical strains or surveillance specimens.[158] SYBR Green-based method is the easiest and least expensive technique accessible for real-time PCR. Although the real-time PCR assay is unable to detect the bla_{KPC} gene subtypes in clinical isolates, this assay based on SYBR Green can be performed in <2hours, which would decrease the distribution possibility of the organism in the hospital.[158]

Sensitive and specific molecular techniques and detecting bla_{KPC} using real-time nucleic acid amplification or DNA microarrays provide the potential to prevail over these problems and may be able to provide same-day results, allowing prompt infection monitoring intervention.[159] Conversely, the identification of KPC with phenotypic tests can be labour-intensive and subjective; also, to obtain a perfect result, prolonged time of more than 72hours is also required. Another method for the qualitative detection of bla_{KPC} is EasyQ KPC assay that is a real-time nucleic acid sequence-based amplification (NASBA) assay.[13] EasyQ KPC assay is a sensitive and specific method for screening surveillance specimens for KPC-positive *Enterobacteriaceae*. It is planned for applying with the bioMérieux NucliSENS platform. Amplification, real-time detection and automated interpretation of the examination outcome are done on a NucliSens EasyQ analyzer.[160] The assay needs minimal interpretation and the results for the full run of 48 samples are obtained within 5hours.[159] On the whole, in comparison with the standard DNA sequence analysis, EasyQ KPC test should be of benefit, since it appears to allow for accurate, fast and cost-effective detection of KPC-producing isolates of *Enterobacteriaceae* with considerable savings in terms of work and time.[160]

For carbapenemase genes, a kind of multiplex PCR assays has been described.[75] However, it needs real-time PCR facilities or reliance on amplicon detection by gel electrophoresis; therefore, it might not be convenient for all laboratories. The multiplex real-time PCR method has several advantages. First, in contrast to the previously published real-time PCRs, it is able to detect the five most prevalent carbapenemases. Second, the good operation of the technique, when using pre-cultured broth, makes it suitable for the detection of carbapenemases in clinical swabs.[161] Recently, a multiplex real-time PCR assay has been introduced which, in a single reaction, is capable to detect the commonest types of serine carbapenemases and MBLs (KPC, GES, OXA-48, IMP, VIM and NDM-1) in *Enterobacteriaceae* isolates.[148] To detect and differentiate multiple class A carbapenamase genes in a single reaction, Hong *et al.* developed a multiplex PCR assay.[162] This kind of multiplex PCR offers a useful and simple approach for detecting and distinguishing class A carbapenamase genes in MBL-negative carbapenem-resistant strains. In another study, Dallenne *et al.* described the development of one simplex PCR and six multiplex PCRs that could detect common beta-lactamases.[163] This method is introduced as a fast, low-cost and reliable tool for the screening of frequently encountered beta-lactamases.

Check-Direct CPE (Check-Points Health B.V., Wageningen, the Netherlands) is a new multiplex real-time PCR assay that targets NDM, KPC, VIM and OXA-48 group. It has been developed to perform on rectal swabs or cultured isolates.[164] This sensitive tool is able to differentiate between the carbapenemase genes within 3hours, while the results of the method show 100% agreement with the previously defined genotypes and the results obtained by Check-MDR CT102 (see below).[165] A commercial multiplex PCR with amplicon detection by reverse hybridization was described by Kaase *et al.*, in which the sensitivity for the detection of VIM-, KPC-, NDM- and OXA-48-encoding genes was 100%, whereas two IMP variants were missed.[75]

Zheng *et al*. also described a new duplex real-time PCR assay for the prompt and simultaneous screening of bla_{NDM} and bla_{KPC} genes in a single reaction that had good sensitivity and specificity as well as excellent agreement with the conventional PCR and sequencing.[149] Its use would be particularly suitable for national epidemiological purposes in an outbreak situation, thanks to its comprehensive ability to detect all known bla_{NDM} and bla_{KPC} variants. LightCycler (Roche Molecular Diagnostics, Indianapolis, IN, USA) duplex real-time PCR assay is a method for detecting bla_{KPC} and bla_{NDM}, which employs fluorescence resonance energy transfer (FRET) hybridization probe based, when coupled with simple pre-PCR colony lysis, yields a 'colony-to-result' time of 90minutes, faster than any previously described assay and has 100% sensitivity and specificity compared with reference methods.[166] A triplex assay can also provide an accurate and rapid method for detecting bla_{KPC} and bla_{NDM}.[145] This assay provides considerable advantages over the methods that employ double-stranded DNA binding dyes and melting curve analysis, including the presence of an internal control to decrease false negatives, removal of the inherent variation in melting curves associated with allelic variation in target genes and ability to detect both bla_{KPC} and bla_{NDM} in the same target.[145]

Hyplex multiplex PCR-ELISA is a novel diagnostic method for the direct detection of MBL genes of IMP and VIM types in clinical specimens. Among the commercially available tests, Hyplex PCR-ELISA is capable to reliably identify different bacterial pathogens directly from blood culture or resistance mechanisms in *Enterobacteriaceae*.[167] Data indicated that Hyplex PCR-ELISA may be a helpful complementary test in the clinical laboratory for timely detection as well as high specificity (98.6%) and sensitivity (98.0%), both for KPC and MBL (bla_{VIM} and bla_{NDM}).[167] This method has been proved reliable in bla_{VIM} gene detection from urine, blood, sputum and pus samples and, as confirmed by the conventional methods, contains various VIM-producing species.[168]

The quick detection of CP *Acinetobacter* isolates is fundamental for adequate infection control and empirical treatment purposes. Loop-mediated isothermal amplification (LAMP) is a relatively simple and field-adaptable method. The technique for the clinical diagnosis and detection of parasites and bacteria involved in epidemics has been used widely.[139] The main advantages of the method reported here are the simplicity and ease of performance, potential for automation and shorter analysis time. In addition, water bath is the only specific equipment needed for the LAMP assay.[139] Finally, Eazyplex® system (Amplex Diagnostics GmbH, Munich, Germany) combines LAMP of the target and real-time photometric detection of amplified material for rapid and simple detection of carbapenemase-encoding genes.[169] This method allows for the amplification and detection of target genes in a single step at a constant temperature and provides highly reliable results in <15minutes.

Microarray-based assay

Despite the advantage of conventional PCR, these methods require time, manpower and resources. Therefore, common carbapenemases, only in the first series of tests, will be targetted. The procedure may hence cause a significant delay in the identification of carbapenemase genes, especially the rare ones. It could be also solved in a single test by combining all relevant targets using a microarray for the identification and detection of multiplex PCR products generated from suspect clonal culture material.[170] Nucleic acid microarray technologies, among the methods that are currently available, have proved to be important in the characterization of resistance genes and molecular epidemiology.[171] Microarray-technology hybridization is a promising method of identification that could detect a vast number of different genes simultaneously in a short period. This technique has considerable advantages over traditional methods, since it allows for the rapid identification of single nucleotide polymorphisms (SNPs), a multi-parametric analysis, and also uses a miniscule sample that reduces the time and cost needed to obtain the results (Table 2).[172,173]

For the detection of resistance in different genera, species or other groups of bacteria, several microarrays have been proposed.[174,175] To report accurate and valid resistance patterns, reduced time will be paramount as the number and density of drug-resistant pathogens increase in health cares.[171] By optimizing amplification, carbapenemases genes of molecular classes A, B and D can be amplified in a single multiplex PCR that takes about 40minutes. However, the entire hybridization analysis takes <4hours and can be used in clinical microbiology laboratories for expressing the diagnostics of antibiotic resistance caused by the production of carbapenemases.[176] The microarray covers the determinants for different mechanisms of resistance, like enzymatic modification, gene overexpression by promoter alterations, enzymatic inactivation of antibiotics, mutation of the target sites and mutations in regulatory genes.[174]

Check-MDR CT102 DNA microarray (Check-Points Health B.V.) is a novel molecular diagnostic test, which enables the detection of ESBL gene families (SHV, TEM and CTX-M) and most prevalent carbapenemases (IMP, KPC, VIM, NDM and OXA-48). Although the array for epidemiology of ESBL genes seems to be less suitable, this method is a robust and specific technique[177,178] and has 100% sensitivity and specificity for most isolates, suggesting that this assay allows for the accurate identification of carbapenemase producers and common ESBL from bacterial cultures.[175] It provides satisfactory outcomes on most of the DNA extracts of enterobacterial, *Pseudomonas* spp. and nonfermenter bacteria. Nevertheless, the present

format of Check-MDR CT102 microarray is more suitable for the member of *Enterobacteriaceae*, since multiple important genes are missing for an integrated approach to the detection of major transferable beta-lactamases found in non-fermenters, such as OXA-23/-24 and OXA-58 clusters of carbapenemases for *A. baumannii* or the several VEB, GES, PER, BEL-1 and OXA ESBLs for *A. baumannii* and *P. aeruginosa*.[175]

Epidemiological relatedness of carbapenemase genes by typing methods

The ability to accurately distinguish between multiple strains and resistant mechanism, within a bacterial species, is a fundamental requirement for epidemiological surveillance and micro-evolutionary studies. Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) are the methods for examining the molecular epidemiology of carbapenem-resistant isolate, which are used to better characterize the distribution of this resistance mechanism.[179]

Pulsed-field gel electrophoresis is basically the comparison of large genomic DNA fragments after digestion by a restriction enzyme. This digestion gives different linear molecules of DNA, because bacterial chromosome is typically a circular molecule.[180] Pulsed-field gel electrophoresis has also been successfully used for the isolates, which have been separated from different geographical regions; but, it is commonly considered over-discriminatory for this type of study, since the method identifies genetic diversity that is accumulated relatively rapidly, and even minor genetic variations (for example, loss of a restriction site or a point mutation resulting in creation) can result in a three-fragment difference in the PFGE gel banding pattern.[181] The variation in the electric field allows PFGE to resolve the very large fragments (>600kb) associated with this analysis.[182] For example, in a study in the United States, PFGE analysis of KPC-producing *K. pneumonia* from eight hospitals and five chronic care centres demonstrated a clonal relationship between many of these isolates, some of which appeared to be genetically related to the strains reported from outbreaks.[1]

The development of MLST indicates a major advance in this respect, since it relates organisms on the basis of the nucleotide sequences of the conserved housekeeping gene fragments.[181] Multi-locus sequence typing data are revealing evidence concerning the emergence and spread of antibiotic resistant clones,[183] define unambiguous strain types and provide results that can easily be exchanged between different laboratories.[184] This method is relatively expensive and therefore not an option for many clinical laboratories.

Careful attention should be given to time, cost and expertise needed to accomplish MLST compared with those required to perform PFGE when deciding on a typing system for a particular epidemiological need. For many strictly epidemiological applications, PFGE will remain the typing system of choice, with MLST reserved for long-term epidemiological and population genetic studies.[181] This balance may well change with the inevitable rapid improvements and advances in sequencing technology and its automation.

The repetitive extragenic palindromic sequence-based polymerase chain reaction (REP-PCR) is one of the most effective methods and is commercially available, known as DiversiLab microbial typing system (bioMerieux).[185] REP-PCR is a genomic fingerprinting method that generates specific strain patterns obtained by the amplification of repetitive DNA elements present along the bacterial genome.[186] This method has been proved as a reliable and rapid laboratory technique for the molecular confirmation of suspected nosocomial outbreaks.[187] Compared to automated REP-PCR with MALDI-TOF MS conducted by Treviño *et al.,*[188] both methods show a major genetic variation among CP strains, suggesting the acquisition of new resistance genes more than their clonal distribution. In addition, both techniques agree on the genetic variation found among the strains A type of REP-PCR is the amplification of genomic DNA located between enterobacterial repetitive intergenic consensus (ERIC) elements, which generates several distinct amplification products with the sizes ranging from approximately 50 to 3000bp.[189] This method is rapid and highly reproducible and has great potential as a reliable and simple method for fingerprinting diverse types of DNA samples.[189]

Conclusion

For the detection of carbapenemase in routine diagnostic laboratories, except MHT, there is no direct assay. However, the phenotypic techniques are not highly sensitive and specific. Detection of KPC- and MBL-producers may be based on the inhibitory properties of multiple molecules (boronic acid and EDTA, respectively) and needs a considerable degree of expertise. Molecular detection of carbapenemase genes is an interesting alternative, but also requires a high degree of expertise and remains costly, which makes it unavailable for non-specialized laboratories. The most specific molecular methods that have been expanded for carbapenemase detection are multiplex PCR analyses and DNA microarray assays. Both the phenotypic and molecular methods are time consuming and thus do not correspond to the real clinical demand. However, in order to prevent the development of nosocomial outbreaks, the identification of carbapenemase producers must really be followed by a quick adaptation of the antibiotic therapy and by the isolation of colonized patients. Finally, for infecting strains, Carba NP test with reduced susceptibility to carbapenems, which has higher specificity and sensitivity than others can be recommended. In positive cases, molecular method can be used as a confirmatory method that is mainly related to epidemiological reasons. But, in the case of screening the carriers, SUPERCARBA chromogenic medium, followed by Carba NP test, has high importance.

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Disclaimer Statements

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