Recent advances in the laboratory detection of carbapenemase-producing Enterobacteriaceae

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Abstract

Carbapenemase-producing Enterobacteriaceae (CPE), mainly *Klebsiella pneumoniae* and *Escherichia coli*, have been increasing rapidly on a global scale and are considered to be significant health threats. The most common carbapenemases are KPCs, NDMs, OXA-48-like, IMPs and VIMs but their distribution and prevalence differs between countries. The accurate, simple, cost effective and rapid detection of carbapenemases in clinical laboratories is an important initial step to control the spread of CPE within institutions. The diversity of carbapenemases in general, has challenged a simple approach for the detection of most types of CPE. This article summarizes the current and describes newer techniques available for the detection of carbapenemases among Enterobacteriaceae. We also provided a simplified approach for the accurate and rapid detection of CPEs that can easily be implemented in a clinical diagnostic laboratory.

Keywords: Carbapenemase-producing Enterobacteriaceae, Laboratory detection.

Introduction

The Enterobacteriaceae, most notably *Escherichia coli*, and *Klebsiella pneumoniae*, are among the most important causes of serious hospital-acquired and community-onset bacterial infections in humans [1]. The global spread of antimicrobial resistance was recently identified by the World Health Organization (WHO) as one of the three greatest threats to human health and is a public health threat [2]. Moreover, the WHO released a report in 2014 entitled: "Antimicrobial resistance: global report on surveillance" that focused on antibiotic resistance in seven different bacteria responsible for common, serious diseases such as bloodstream infections, diarrhea, pneumonia, urinary tract infections and gonorrhoea [3]. It states that antimicrobial resistance to common bacteria has reached alarming levels in many parts of the world and that in some settings, few, if any, of the available treatments options remain effective for common infections. [3].

The spread of multi-resistant bacteria is problematic for the medical community at large since it undermines empirical treatment regimens by delaying the administration of appropriate antibiotic therapy and by reducing the options for appropriate treatment. This contributes to increased patient mortality and morbidity [4]. The problem is so serious that it threatens the achievements of modern medicine.

One of the most urgent areas of antimicrobial drug resistance is the rapid evolution of carbapenem resistance in Enterobacteriaceae which has spread globally and rapidly during the last

decade [5]. Carbapenems are often the last line of effective therapy available for the treatment of serious infections due to multidrug resistant bacteria. Resistance to carbapenems involves multiple mechanisms, including alterations in outer membrane permeability mediated by the loss of porins, upregulation of efflux systems combined with high levels of AmpC cephalosporinases or other β-lactamases [6]. Enzymes that hydrolyze the carbapenems, referred to as carbapenemases, are the most important causes of carbapenem resistance among Gram negative bacteria [6].

The clinical diagnostic laboratory often 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 in hospitalised patients, is considered by most infection control practitioners as infection control emergencies [7]. Some clinical laboratories may not be fully aware of the importance and the methods for detecting bacteria with carbapenemases [8]. The consequences have been several treatment failures in patients who received inappropriate antibiotics and outbreaks of multidrug-resistant gram-negatives which required expensive control efforts. A recent example occurred in Alberta, Canada when the nosocomial outbreak of carbapenemase-producing Enterobacteriaceae and *Acinetobacter baumannii* resulted in death of a patient that was attributed to a different patient with recent foreign hospitalization [9].

Clinical microbiology laboratories should be able to rapidly detect carbapenemase-producing Enterobacteriaceae (CPE). This article will address the laboratory

detection of carbapenemases among isolates and provide a simplified approach for the accurate and rapid detection of CPEs that can easily be implemented in a clinical diagnostic laboratory. The detection of CPEs directly on patient specimens (e.g. the use of CHROMagar media etc.) is outside the scope of this article.

Carbapenemases

Carbapenemases belong to the molecular class A (i.e. KPC types), the class B, (or the metallo-β-lactamases) [i.e. VIM, IMP and NDM types] and the class D oxacilinases (i.e. OXA-48-like enzymes). The NDM, OXA-48-like, KPC, IMP and VIM types are the most common global carbapenemases among CPE [7]. Other types of carbapenemases (e.g. GES, SME, IMI, and NMC) that are far less commonly encountered in the clinical laboratory and will not be addressed in this article.

The class A KPC-type ß-lactamases have been extensively and almost exclusively reported in *K. pneumoniae* [10]. To date more than 20 different KPC variants have been described even though KPC-2 and -3 remains the most dominant variants [11]. These enzymes provide resistance to the penicillins, carbapenems, cephalosporins, cephamycins and monobactams and are inhibited by β-lactamase inhibitors such as clavulanic acid (weakly), tazobactam (weakly), boronic acid and avibactam. KPC β-lactamases (especially KPC-2 and –3) have been described in several enterobacterial species, especially *Klebsiella* spp. and to a lesser extend in *Enterobacter spp* and *E.*

coli. [12]. Several nosocomial outbreaks most often due to *K. pneumoniae*, have been reported from North America (especially the USA), South America (Colombia, Argentina), Europe (Greece, Italy, Poland), Asia (China) and Middle East (Israel) [11,13,14]. KPC-producing bacteria are endemic in these regions [14]. *K. pneumoniae* ST258 with KPC-2 and KPC-3 had significantly contributed to the world-wide distribution of this resistance trait [14].

The class B ß-lactamases or metallo-ß-lactamases (MBLs) had been identified in various enterobacterial species including *K. pneumoniae, E. coli* and *Enterobacter spp.* [7]. They mainly consist of NDM-, VIM-, and IMP-type enzymes, with the first type are endemic in certain regions such as South Asia. Although IMP producers are mainly identified in China, Japan, and Australia, VIM-producing *K. pneumoniae* isolates are mainly found in Italy and Greece [7]. Since the first description of NDM-1, more than 10 variants of this enzyme has been described, the majority of them originated from Asia [8]. The majority of NDM-producing bacteria are broadly resistant to various drug classes and also carry a diversity of additional resistance mechanisms [7]. These include plasmid-mediated AmpC β-lactamases (especially CMY types), ESBLs (especially CTX-M-15), different carbapenemases (e.g. OXA-48-, VIM-, KPC-types), 16S ribosomal RNA methyltransferases, plasmid-mediated quinolone resistance determinants, macrolide modifying esterases, and rifampicin-modifying enzymes. Consequently, Enterobacteriaceae with NDMs remain only susceptible to agents such colistin, fosfomycin and tigecycline [8].

The class D carbapenem-hydrolyzing ß-lactamase found in *K. pneumoniae* isolates is OXA-48 (and derivatives) that was firstly reported from a *K. pneumoniae* isolate from Turkey [15]. OXA-48 hydrolyses efficiently narrow-spectrum ß-lactams such as penicillins, weakly hydrolyses carbapenems, and spares broad-spectrum cephalosporins [16]. It has been found among all Enterobacteriaceae however it is mostly identified in *K. pneumoniae* (mostly from nosocomial origin) and *E. coli* (mostly from community origin) isolates. OXA-48-producing *K. pneumoniae* is endemic in Turkey and certain North African countries (e.g. Morocco, Tunisia) showing a wide range of susceptibility profiles [15]. Indeed MICs of carbapenems may significantly vary from isolate to isolate, depending on the host permeability background. Similarly, susceptibilities to broad-spectrum cephalosporins can also significantly vary, depending on the co-production of other ß-lactamases such as the ESBLs. Some OXA-48 derivatives have also been identified in *K. pneumoniae*, being OXA-181, OXA-204, and OXA-232, all sharing similar hydrolytic properties [17]. These enzymes have been identified in North Africa, Australia, New Zealand, but one of the main sources of OXA-181 (which is the second most common OXA-48 derivative) is the Indian subcontinent.

K. pneumoniae has been the most common species among CPE, followed by *E. coli* but carbapenemase producers has been found in various other Enterobacteriaceae, such as *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp, and *Proteus* spp. A summary of the characteristics and distribution of the five major carbapenemases are shown in Table 1. It is important to remember that

Table 1. Characteristics of carbapenemases among Enterobacteriaceae.

Amber class	Enzyme	Prevalent subtype	Spectrum	Endemic area
A	KPC	KPC-2, KPC-3	Penicillins, cephalosporins, cephamycins, aztreonam, carbapenems	United States, Greece, Italy, Poland, Israel, Brazil, Colombia, Argentina, China, Taiwan
B	NDM	NDM-1	Penicillins, cephalosporins, cephamycins, carbapenems	India, Pakistan, Sri Lanka, United Kingdom, France, Balkan states, Arabian peninsula, North African countries
B	IMP	IMP-1-like, IMP-2-like	Penicillins, cephalosporins, cephamycins, carbapenems	Japan, Taiwan, China
B	VIM	VIM-1-like	Penicillins, cephalosporins, cephamycins, carbapenems	Greece, Spain, Italy, South Korea, Taiwan

D	OXA	OXA-48, OXA-181 others	Penicillins, cephalosporins, cephamycins, carbapenems (weak)	Turkey, Morocco, Tunisia, Europe (Spain, Belgium)

carbapenemases have different features which will affect the diagnostic performance of laboratory tests designed to detect CPE [8].

Detection of Enterobacteriaceae with carbapenemases

The detection of carbapenemases in Enterobacteriaceae consists of a two-step approach namely a screening process using the carbapenems followed by a confirmation test to detect the presence of a carbapenemase in isolates that testes non-susceptible to the carbapenems. As mentioned before, Enterobacteriaceae with OXA-48-like enzymes can test susceptible to the broad-spectrum cephalosporins (e.g. 3rd and 4th generation cephalosporins) and the carbapenems and the laboratory detection of such bacteria remains a challenge to most clinical laboratories.

Screening for non-susceptibility (NS) to the carbapenems

CPE are often NS to carbapenems and this remains a simple initial screen for the presence of carbapenemases among Enterobacteriaceae. However, the choice which carbapenems to use for screening with subsequent break points remains a controversial issue. Some CPEs, especially the OXA-48 producers, show only slight increases in carbapenem MICs [18,19]. For the detection of OXA-48-like producing isolates, a recent Belgium study advocates the use of NS to piperacillin-tazobactam and temocillin. This combination shows the best sensitivity (i.e. up to 98%) for *K. pneumoniae* but low specificity for the detection of OXA-48-like producers in especially in non- *K. pneumoniae* Enterobacteriaceae [19].

The Clinical and Laboratory Standards Institute (CLSI) in 2010 revised their breakpoints for the carbapenems and do not recommend routine confirmation tests for carbapenemases if the current interpretive criteria are used. CLSI do advocate the detection of carbapenemases in Enterobacteriaceae for infection control purposes [20]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) uses different carbapenem screening breakpoints from clinical breakpoints [21]. In the EUCAST guidelines, the meropenem MIC of > 0.12 µg/ml or disk diameter ≤ 25 mm are recommended for the screening of carbapenemases in Enterobacteriaceae. The EUCAST meropenem screening criteria have difficulty to detect some OXA-48-producers [19]. Only 80% of CPE clinical isolates from Belgium and France were detected using the EUCAST meropenem criteria; 25% of OXA-48-producers and 19% of VIM-1-producers tested susceptible to meropenem.

Ertapenem non-susceptibility has excellent sensitivity but poor specificity for CPE, especially in species such as *Enterobacter* spp. due to the presence of high-level production of AmpC β-lactamases in combination with porin loss [8]. Imipenem MICs breakpoints cannot reliably separate wild-type isolates from carbapenemase producers in species such as *Proteus* spp., *Providencia* spp., and *Morganella morganii*. Faropenem is an oral penem antibiotic and one UK study evaluated its use as a potential screening agent for CPE [22]. Using clinical isolates with several types of carbapenemases, including OXA-48 producers, growth up to the edge of a 10 µg

faropenem disc showed 99% sensitivity and 94% specificity for the detection of CPE [22].

The most current approach to screen for CPE should at least include ertapenem in combination with imipenem or meropenem [23]. The combination of piperacillin-tazobactam in temocillin is an option to screen for OXA-48-like producers [19]. Faropenem is an attractive option but additional evaluation of this agent is needed.

Confirmation tests: Phenotypic methods

Phenotypic methods that detect the enzyme activity of carbapenemases will be able (in theory at least) to identify all types of carbapenemases, including novel enzymes.

a. Inhibitor-based synergy tests

Inhibitor-based synergy tests are based on the ability of certain substrates to inhibit the action of carbapenemases [24]. This process involves the testing of a carbapenem with and without the addition of an inhibitor that is specific to the type of carbapenemase. The inhibitor-based methods are often based on disk susceptibility testing such as the double-disk synergy test (DDST) and combined disk test (CDT). In the DDST, an inhibitor disk is placed near a carbapenem disk and presence of carbapenemase expands the growth-inhibitory zone between the two disks. This is often referred to the “keyhole” or “champagne cork” sign. In the CDT, a carbapenem disk with and without an inhibitor are used and an increase in inhibitory zone diameters indicates the presence of carbapenemase. The interpretation tends to be subjective with the DDST but is standardized with the

CDT (e.g. zone diameter difference of Xmm is indicative of a positive test).

Boronic acid derivatives (phenylboronic acid [PBA] and 3-aminophenylboronic acid [ABPA]) are often used for the inhibition of KPCs [24]. These agents have a broad-spectrum of inhibition and also inhibit other β-lactamases such as AmpC β-lactamases. Cloxacillin, an AmpC inhibitor without activity against KPC, can be used to discriminate between KPCs and AmpCs. Inhibitors for MBLs (i.e. NDM, IMP, and VIM) include metal chelators such as EDTA, dipicolinic acid (DPA), 2-mercaptopropionic acid, and sodium mercaptoacetic acid [24]. Chemical compounds with specific inhibitor properties for OXA-48-like enzymes are not been yet available for the identification of these carbapenemases.

For the reliable detection of the most common carbapenemases among CPE using an inhibitor-based approach, clinical laboratories should use a combination of inhibitors for the detection of KPC (i.e. boronic acid) and MBLs (i.e. metal chelator). Other supplementary tests (i.e. NS to piperacillin-tazobactam and temocillin) are necessary to screen for the presence of OXA-48-like producers [19]. Several investigators have evaluated synergy testing using boronic acid (for KPCs), boronic acid with cloxacillin (to distingisuh between KPCs and AmpCs), and DPA or EDTA (for MBLs) [25-27]. Commercial disk kits have been available and include the KPC/MBL Confirm Kit (Rosco Diagnostica, Taastrup, Denmark) and the Mastdiscs™ Carbapenemase Detection Set (MAST GROUP, Merseyside, UK). Both assays were evaluated and showed > 97% sensitivity

and 93% specificity for KPC and NDM-producers [28]. However, about a half of IMP and VIM producers were not detected. Giske et al described an inhibitor-based method that performed better with IMP and VIM CPE [26]. The overall sensitivities for detection of all clinical CPEs isolates remained 78%–80% due to inability for the commercial tests to detect OXA-48-like producers. Recently, Rosco Diagnostica and Liofilchem (Roseto degli Abruzzi, Italy) launched new disk kits which includes the addition of temocillin for the screening of OXA-48-like producers (KPC/MBL & OXA-48 Confirm kit, Rosco Diagnostica, and KPC&MBL&OXA-48 disks kit, Liofilchem®). These approaches have not yet officially been evaluated.

Miriagou et al. used two types of inhibitors (i.e. DPA and PBA) in combination with meropenem for the identification of CPE that simultaneously produce KPCs and VIMs [29]. Several other investigators used similar approaches to accurately detect CPE that produce both class A and class B carbapenemases [30,31]. Maurer et al. recently published an extensive algorithm that include synergy tests using cloxacillin supplemented agar with ABPA, EDTA, and temocillin disks which resulted in 100% sensitivity and specificity for detecting CPE that contain the most common types of carbapenemases [32]. Tsakris et al. described an OXA-48 disk confirmation test that used an imipenem disk impregnated with EDTA and EDTA + PBA [33]. The interpretation of the test is based on the distortion of zone sizes and is extremely subjective and very difficult to interpret.

Inhibitor-based disk tests are very popular with certain clinical laboratories due to their user

friendliness, cost-effectives and the availably of commercial tests. These approaches should at least include 2 types of inhibitors (e.g. boronic acid derivatives for KPCs and metal chelators for MBLs) and are especially reliable for the detection of KPCs and NDMs. The prolonged turn-around times and lack of inhibitors specific for OXA-48-like carbapenemases curtail the use of inhibitors as a stand-alone approach for the detection of CPE.

b. Modified Hodge test (MHT)

The modified Hodge test (MHT) or clover leaf technique is a phenotypic confirmation test for carbapenemases that is currently proposed by the CLSI) [20]. This test is based on the inactivation of a carbapenem by CPEs that enable a carbapenem-susceptible indicator strain to extend growth toward a carbapenem-containing disk, along the streak of inoculum of the tested isolate. The MHT is cost-effective, easy to perform but unfortunately difficult to interpret in some instances and false-positive results are a concern especially among non-*K. pneumoniae* [34-36]. Moreover, the turn-around time is around 16-18 hours. The MHT has an excellent sensitivity for detecting CPE with bla_{KPCs} and $bla_{\text{OXA-48-like}}$ but performs poorly in detecting those with bla_{NDMs} [28,35]. However it remains a valuable option in clinical laboratories with for the detection of CPE especially in KPC and OXA-48-like endemic regions.

c. Carbapenem Inactivation Method

Carbapenem Inactivation Method (CIM) is a new method described in 2015 [37]. This

method involves a suspension of the test isolate in 400 µl water to which a meropenem disk is added and incubated at 35°C for approximately two hours. The meropenem disk is then placed on an agar plate inoculated with a susceptible *E. coli* indicator strain and subsequently incubated at 35°C for another 12 -18 hours. If the test isolate produced a carbapenemase, the meropenem will be inactivated allowing uninhibited growth of the susceptible indicator strain (i.e. no zone of inhibition). Isolates without carbapenemases showed inhibition zones. The test can be read after 6 hours but the best results are obtained after overnight incubation. This method showed high concordance with results obtained by PCR to detect genes coding for the following carbapenemases: KPC, NDM, OXA-48, VIM, IMP and OXA-23 [37].

A different group evaluated the CIM and found it be 98.8% sensitive and 100% specific to detect CPE with OXA-48-like, NDM and KPC that tested previously negative with the Carba NP test [38]. One IMP-producing mucoid isolate was negative for both the CIM and the Carba NP test. The advantages of CIM included cost-effectives, reagents that are readily available in most clinical laboratories and it is easy to perform. Unfortunately this test is time consuming since the best results are obtained when agar plates are incubated for 12-18 hours.

d. The Carba-NP test

The Carba-NP test was developed by Nordmann and Poirel in 2012 and the CLSI recommends its use as a confirmation test since 2015 [39]. This method involves the incubation of a

test bacterium (the lysate) with a solution containing imipenem, zinc sulfate, and phenol red. The production of carbapenemases is indicated detected by pH change due to the hydrolysis of imipenem (i.e. a color change from red to orange or yellow). The initial study from Nordmann and Poirel reported 100% sensitivity and 100% specificity for CPE with bla_{KPCs}, bla_{NDMs} bla_{IMPs}, bla_{VIMs} and $bla_{OXA-48-like}$. Most of the positive samples reacted within 30 min but the authors recommended incubation for up to 2 h. The Carba-NP test has been validated with colonies grown on Mueller-Hinton, blood, and trypticase soy agar plates but it cannot be performed with colonies obtained from Drigalski or McConkey agar plates.

During various validation and verification studies, some detection issues with the carba NP test have been found and improved on to some extent. Mucoid *K. pneumoniae* and some CPE with with OXA-48-like often give false negative results and remain problematic for the Carba-NP test. Tijet et al. reported a sensitivity of 21% for OXA-48-like producers and poor results were obtained especially with mucoid *K. pneumoniae* isolates [40]. They reported that increasing the bacterial inoculum improved the sensitivity of the Carba-NP test for OXA-48-like producers to 59%. The Carba-NP methodology was recently standardized by the CLSI and incorporated into the 2015 CLSI guidelines [20]. That guideline shows a detailed protocol with appropriate control reactions and isolates. This CLSI standardized method demonstrated an excellent sensitivity and specificity, and positive results were obtained within 15 min in 94% of CPE with various carbapenemases [41]. One

of the major drawbacks of the Carba-NP test, is that laboratories need to prepare most of reagents. The cost of imipenem powder can be as high as $317 for 100 mg. At least 10 mg is needed to make solution which can be used for 13 samples and stored for up to 3 days. However, a cheaper alternative, intravenous imipenem/cilastatin powder (approximately $4 per 100 mg of imipenem) provided similar performance than pure imipenem powder [42]. Commercial versions have recently been launched for clinical use and include the RAPIDEC® CARBA NP (bioMérieux), Rapid CARB Screen (Rosco Diagnostica), and Neo-Rapid CARB (updated version of Rapid CARB Screen; Rosco Diagnostica) kits. These kits are convenient and RAPIDEC® CARBA NP and Neo-Rapid CARB had similar performances to the manual method [42,43].

Additional developments or applications of the carbaNP tests have recently been reported. A modification called the Carba NP test II has the ability to identify which type of carbapenemase is present among CPE. The original version is combined with β-lactamase inhibitors and this medication has the ability to distinguish between class A, B, and D β-lactamases [44]. The presence of class D β-lactamases is deduced from a lack of inhibition by both class A and B inhibitors. A shorter turn-around time of the original carba NP test was reported by Lee et al. when they performed this method on five hour old bacterial cultures [45]. Nordmann and Poirel also described the direct detection of CPE on blood culture bottles that flagged positive and were Gram negative on stains [46]. This assay showed a 100% sensitivity and specificity for all types of CPE, excluding for

OXA-48-like producers (91.3% sensitivity and 100% specificity). This method enables the rapid reporting of carbapenemases from blood cultures up to 24 hours earlier than from conventional growth on agar plates

The Carba NP test and its modifications are relatively easy to perform and provide rapid results, especially for CPE with *bla*$_{\mathrm{KPCs}}$, and *bla*$_{\mathrm{NDMs}}$. However, this test can be challenging for some technologists to interpret (due to various ranges of the orange colour) and OXA-48-like producers remain a problem. Moreover, the commercial versions are expensive and showed a sensitivity of 98% and specificity of 99% for detecting different types of CPE [47].

e. The Blue Carba test

The Blue-Carba test is another modification of the Carba-NP test developed in Portugal by Peixe and colleagues. This method uses bromothymol blue as the indicator, imipenem/cilastatin as the antibiotic/substrate and can be performed directly on bacterial colonies (as opposed to bacterial extracts in the original Carba NP test) [48]. The Blue-Carba test performs very well for KPC and MBL CPE with low MICs to the imipenem but gives similar results and has the same issues as the CarbaNP test regarding OXA-48-like CPE [49-51]. The commercial version has been recently launched as the Rapid Carb Blue kit (Rosco Diagnostica) [52].

f. Starch-iodine assay

The starch-iodine assay is based on color changes of a starch-iodine compound (i.e. from

dark pink to clear) due to the release of hydrogen ions that occurs during the hydrolysis of the β-lactam ring. A commercial version, the Carbapenembac assay, comprised of a strip that contains imipenem and starch [53]. A bacterial suspension is deposited onto the strip and incubated for 10 min, followed by the addition of iodine. A change in color from dark pink to clear within 30 min is indicative for the presence of carbapenemases. CPE with $\textit{bla}_{\text{KPCs}}$ were detected with 100% sensitivity and specificity. The Carbapenembac assay is cost effective and easy to perform but further validation using CPEs with different carbapenemases need to be performed.

g. Immunochromatography

Immunochromatography is based on an antigen-antibody reaction performed on chromatographic paper. A commercial version named Quick Chaser® IMP (Mizuho Medy, Saga, Japan) was designated to detect all IMP-type carbapenemases. This test detects the presence of IMPs in Enterobacteriaceae and non-glucose-fermenting gram-negative rods with 100% sensitivity and 100% specificity [54]. Results are obtained within 15 min after three drops of bacterial colonies suspended in an extract solution are applied to a test cartridge. The assay is easy to perform and provides rapid results but it not relevant in regions non-endemic for CPE with $\textit{bla}_{\text{IMPs.}}$ Glupczynski et al. recently evaluated 2 new commercial immunochromatograhic assays (OXA-48 K-SeT® and KPC K-SeT®, Coris Bioconcept, Gembloux, Belgium) that showed 100% sensitivity and specificity for the detection of OXA-48-like and KPC CPE respectively [55]. These tests were easy to perform with

very short turn-around time (15 min).

f. Electrochemical Assay

Another innovative method is the electrochemical detection of carbapenem hydrolysis (BYG Carba test from Belgium) whicthe change of pH and redox activity following imipenem hydrolysis and that detects the presence of carbapenemase within 30 minutes [56]. The BYG Carba tests in comparison with PCR results, displayed 95% sensitivity and 100% specificity. This technique is novel, rapid and efficient based on an electro-active polymer biosensing technology discriminating between CPE and non-CPE. The precise electrochemical signal (i.e. electrochemical impedance variations) allowed for real time objective measurement and interpretation criteria which should facilitate the accreditation process of this technology.

g. Spectrophotometry

A clinical application for using ultraviolet spectrophotometry to determine imipenem hydrolysis was developed in 2012 and has the ability to detect the most clinically relevant CPEs with 100% sensitivity and 98.5% specificity [57]. Bacterial proteins are extracted after sonication and mixed with imipenem and buffer. UV absorbance was recorded at 297 nm for 10 min and slope per minute was used to distinguish hydrolysis from the self-degradation of imipenem. A follow-up study [58] report a sensitivity of 100% and specificity of 76.7% [58]. The UV spectrophotometry assay is labor intensive and not routinely available in most diagnostic clinical laboratories.

h. Mass spectrometry

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) has been recently introduced into clinical microbiology laboratories for identification of bacterial species. Two MALDI-TOF MS systems (Microflex LT [Bruker Daltonics, Germany] and VITEK MS [bioMérieux, France]) are in clinical use and provide a rapid, inexpensive (after the initial financial outlay), and accurate identification of most bacterial species. The Bruker system includes software for analysis of raw spectra data (i.e. FlexAnalysis) and has been used to detect the presence of carbapenemases.

Carbapenemase-hydrolyzed degradation products were measured by MALDI-TOF after the 2-3 hour incubation of bacterial cultures with carbapenems (i.e. meropenem or ertapenem) solutions [59,60]. Excellent sensitivities and specificities of MALDI-TOF were reported. Unfortunately, false-negatives were reported mostly due to the weak carbapenemase activities of OXA-48-like producers as well as the interactions of polysaccharides present in highly mucoid isolates [61]. Recently Sauget et al. reported a sensitivity of 98.9% and a specificity of 97.8% for the detection of OXA-48-producers using imipenem (as opposed to meropenem or ertapenem) [62]. Studentova et al. reported modified MALDI-TOF method which adds ammonium bicarbonate to the solution that enhances activity of OXA enzymes [63]. Wang et al. successfully used an automated statistical peak analysis software program called ClinProTools in the Bruker system to detect carbapenem

degradation products that negated the need of manual inspection of raw spectra. [64]. The MBT STAR-BL prototype software developed by Bruker also succeeded in the automated detect of carbapenem degradation products [65].

Another approach is to use MALDI-TOF for the detection of antimicrobial resistance determinants associated with carbapenemases. An example is the detection of pKpQIL_p019 protein present in some bla_{KPC} containing plasmids [66]. The follow-up validation study using FlexAnalysis showed a 96% sensitivity and a 99% specificity for the presence of KPC-producing CPE [67]. However, this approach is really only valid for molecular epidemiology studies.

The more advanced and expensive mass spectrometry systems, such as liquid chromatography-MS (LC-MS), tandem-MS (MS-MS), capillary electrophoresis-electrospray ionization-tandem-MS (CE-ESI-MS), and PCR-electrospray ionization-MS (PCR-ESI-MS) have also been evaluated to detect carbapenemases. However, these systems are utilized within the research setting and are rarely available in clinical laboratories.

The use of MALDI-TOF for the detection of CPE is currently in investigative stages and is not available for routine use. One of the drawback is the need of time-consuming preparation of reagents. However, this technology has the potential to rapidly and cost-effectively detect CPE. The initial financial outlay for acquiring MALDI-TOFF will curb the wide-spread use of this technology, especially in developing countries.

Molecular methods

Molecular methods are excellent options for the confirmation of carbapenemases among Enterobacteriaceae. These tests are highly sensitive and specific for detecting different carbapenemase genes and are considered by some microbiologists to be the gold standard. Some assays have been validated for the identification of carbapenemase genes directly on clinical samples. Several in-house multiplex PCR's, commercial real time PCR and DNA microarray methods are available for routine use and should at least include the most clinically relevant carbapanemase genes (i.e. bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP} and bla_{VIM}). Disadvantage of molecular methods includes the relative high costs (as compared to most phenotypic tests), the ability to only detect carbapenemase genes included in the assay (as opposed to hydrolysis assays) and longer turnaround times (as opposed to the rapid phenotypic tests). To negate the cost, some clinical laboratories will run the test in batches that can lead to delayed turnaround times. The use of automated commercial systems is easier to incorporate into clinical laboratories and may help to reduce the work load, turn-around-time and errors but such tests are often more expensive than in-house methods.

It should be noted that molecular assays will not be able to discover novel carbapenemase genes and some methods fail to detect all the variants. It is important to keep this in mind when deciding on implementing molecular tests for routine diagnostic testing.

a. PCR-based methods

The strategy for the identification of carbapenemase gene is to first amplify the specific gene (e.g. *bla*$_{NDM}$) using simplex PCR and then to sequence the amplicon for the identification of the subtype (e.g. *bla*$_{NDM-1}$ etc.). For the screening and detection of carbapenemases among clinical isolates, multiplex PCR assays that detect different types of genes (e.g. *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{IMP}$ etc) at the same time are useful and commonly used. A conventional in-house PCR method includes DNA extraction, PCR amplification, and gel electrophoresis steps. Table 2 shows an up-to-date list of in-house and commercial multiplex PCR assays currently available for clinical utilization. The most extensive multiplex assay available has the ability to identify 11 different carbapenemase genes in three separate reactions [68]. However, for the routine use in clinical laboratories, one-reaction multiplex PCR assay that includes the most clinical relevant genes (i.e. *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48-like}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ are most often implemented [28].

The reagents and instruments used in conventional in house methods are cost-effective but time consuming (i.e. it takes 2 to 6 hours to get results) and relatively laborious. Real-time PCR method is superior to conventional method in terms of specificity, speed, and less labor intensive but is more expensive. Real time PCR uses melting curve analysis to confirm specific melting temperature (Tm) value of the target amplicon, or uses specific oligonucleotide probes to the target amplicon that detects an amplification signal. The probe assays are more specific than the melting curve assays. The amplification is monitored in a real-time manner negating the need for gel

Table 2. PCR-based methods for the detection of carbapenemase genes.

PCR (details)	Assay name	Target carbapenemase genes			Other genes	No. of reactions	Comment	Reference
		KPC, NDM, OXA-48-like	IMP, VIM	Others				
Conventional	In-house	All	Both	SPM, BIC, AIM, GIM, SIM, DIM	–	3		[1]
Conventional	In-house	All	Both	–	–	1		[2]
Real-time (evagreen, melting analysis)	In-house	All	Both	GES	–	1		[3]
Real-time (SYBR green, melting analysis)	In-house	All	Both	GES, OXA-23	–	2	Tested on BD MAX system. VIM-1 and VIM-2 groups can be discriminated.	[4]
Real-time (molecular beacon probe)	Check-Direct CPE (Checkpoints, Wageningen, Netherlands)	All	VIM	–	–	1	NDM/VIM signal cannot be differentiated (BD MAX can)	[5]

electrophoresis detection step. Monteiro et al. developed a one-reaction assay for *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA\text{-}48\text{-}like}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{GES}$ using melting curve analysis [69]. A probe-based assay was developed by Lee et al. [70] but it targets only three carbapenemase genes. Increasing numbers of commercial real-time probe-based PCR assays are available for clinical laboratories. These include Check-Direct CPE (Checkpoints, Netherlands) [71].

To further reduce turn-around-time and decrease labor, fully automated real-time PCR systems that incorporate the DNA extraction step. Recently released tests available for clinical laboratory use included Unyvero, Eplex (GenMark), and PCR-ESI MS (IRIDICA, Abott).

b. Non-PCR methods

A non-PCR molecular rapid commercial confirmation test (LAMP; Easyplex superbug CRE from Amplex Diagnostics [Bahnhof, Germany]), uses loop-mediated isothermal amplification, for the detection of CPE (KPC, NDM, VIM, OXA-48-like) from a plate or directly on positive blood cultures [72]. The system gives results within 15 minutes.

b. Microarrays

The advantage of microarray-based assays is the ability to simultaneously detection a large number of carbapenemase genes. An example of a commercial microarray is the Check-MDR CT103 (Checkpoints, Wageningen, Netherlands) assay that has the ability to detect 11 different β-lactamase genes (including (i.e. *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA\text{-}48\text{-}like}$, *bla*$_{IMP}$ and *bla*$_{VIM}$) that takes about 6 hours to

Table 3. Microarray-based methods for the detection of carbapenemase genes.

Assay name	Target carbapenemase genes		Other genes	Comment	Reference
	KPC, NDM, OXA-48-like, IMP, VIM	Others			
Check-MDR CT103 (Checkpoints)	All	GES, GIM, SPM, OXA-23, OXA-24, OXA-58	ESBLs, AmpCs	Run time: 6 h	[6]
Verigene® BC-GN (Nanoshpere, Illinois, USA)	All	CTX-M group, OXA-23, OXA-24, OXA-58	Genes for identification of 9 gram-negative bacteria	Proprietary, automated system for blood culture. Run time: 2 h.	[7]

complete the procedure [73] (Table 3). The major limitations are the high cost, turn-around time and labor associated with the procedure. Verigene® system (Nanoshere, Illinois, USA) is an automated microarray-based gene detection system and developed the Verigene® BC-GN test that has the ability to identify Gram negative bacteria and detects several genetic resistance determinants (including bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP}, bla_{VIM} and $bla_{CTX\text{-}M}$). This assay is expensive but can be performed directly on positive blood culture bottles with quick turn-around time of 2 hours [74].

Electrochemical DNA biosensor is a new device that detects hybridization of DNA to a biosensor component (usually complementary single-stranded DNA) by electrical transducer or optical detector. One recent report described application of this technology for the detection of a partial KPC gene [75].

c. Next generation sequencing

Next generation sequencing (NGS) is a new high-throughput DNA sequencing technology that can read large amounts of DNA sequences in a rapid fashion. After a launch of the first NGS instrument in 2005, this technology (also referred to as second generation sequencing) has continuously evolved to become more accurate, faster, easier to perform, and more cost effective. Recent NGS systems can determine the sequences of over 100 whole bacterial genomes during a single run. NGS systems provide sufficient data for the assessment of target genes and genetic relatedness between isolates. In fact, WGS has been used in the detailed analysis of CPE, especially

during outbreak investigations [76] and large-scale surveillance [77].

Advantages of NGS for the detection of resistance genes include the following: 1) Predefined target genes are not required and novel elements can be discovered. 2) NGS provides comprehensive gene characteristics including antimicrobial resistance genes, clonal relatedness, plasmid replicon types, virulence genes, mobile genetic elements, and phage types. 3) NGS is becoming cost-effective and less time consuming than standard sequencing techniques.

The major disadvantage of WGS is data analysis. Bioinformatics and high-performance computing environment are required to process and analyze such a large amount of raw sequence data. Easy-to-use software packages or public web-based systems are available: for example, iMetAMOS (genome assembly and annotation pipeline; http://www.cbcb.umd.edu/software/imetamos), CLC genomics workbench (commercial NGS tools suite; CLC bio, Denmark), Galaxy (web-based NGS tools suite; https://galaxyproject.org/). After the assembly of a draft genome, several resistance gene databases such as ResFinder [78] and ARG-ANNOT [79], can be utilized for the discovery of resistance genes. ResFinder has a web-based interface and can accept raw sequence data before genome assembly has taken place. The ResFinder system was able to predict antimicrobial susceptibility with 99.7% concordance [77]. The ARG-ANNOT system includes a free sequence editing software named BioEdit. Both systems uses sequence similarity searching software such as BLAST [80].

The availability of cost effective NGS combined with more user friendly and rapid bioinformatics, has the potential to replace other molecular methods in the near future for the identification of carbapenemase genes.

Expert commentary

Why and when should clinical laboratories test for carbapenemases?

The emerging of resistance to the carbapenems among the Enterobacteriaceae is of special concern to the medical community at large since these agents are often the last line of effective therapy available for the treatment of infections caused by multidrug-resistant isolates [81]. Resistance to carbapenems involves multiple mechanisms, including alterations in outer membrane permeability mediated by the loss of porins, upregulation of efflux systems combined with high levels of AmpC cephalosporinases or other β-lactamases, however, the production of carbapenemases remains the most important mediators [1]. Carbapenemases are often part of mobile genetic elements such as plasmids that has the ability to easily move between different bacteria (i.e. horizontal transfer) (3). Controlling the spread of antibiotic resistance per say is a global public health problem.

The clinical laboratory acts as an early warning system, alerting the medical community to new resistance mechanisms present in clinically important bacteria. The presence of CPE in some instances, can be infection control emergencies and clinical laboratories should be able to rapidly

detect carbapenemases among members of the Enterobacteriaceae; especially when these enzymes are first introduced into the local bacterial population [8]. CLSI and EUCAST do not recommend routine patient confirmation tests for carbapenemases if the current carbapenem interpretive criteria are used. This issue remains debatable since some observations had shown the importance of carbapenemases for the choice of therapeutic regimens [81]. However, the detection of CPE is a critical initial step required for appropriate management of patients during infection prevention and control efforts. Moreover, CPE testing is also appropriate for surveillance and epidemiological studies.

How do methods perform for detecting different carbapenemases?

Phenotypic methods show wide ranges in sensitivities and specificities for their abilities to detect different carbapenemases. As a general rule of thumb, the MHT is good for identifying CPEs with bla_{KPC}, and $bla_{\text{OXA-48-like}}$ enzymes [35]; Inhibitor-based tests are good for CPEs with bla_{KPC}, bla_{NDM} and when combined with temocillin are sufficient for those with $bla_{\text{OXA-48-like}}$ [32]; CarbaNP test (and modifications) is excellent for CPEs with bla_{KPC}, bla_{NDM}, bla_{IMP}, and bla_{VIM} [82] while the CIM test are good for CPEs with bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48-like}}$, bla_{IMP}, bla_{VIM} [38]. Please refer to Table 4 for details on the performance of phenotypic tests [83,84].

Genotypic methods (including in-house and commercial tests) show the best sensitivities and specificities (as compared to phenotypic tests) for detecting different carbapenemase genes

Table 4. Sensitivities of Phenotypic methods for the detection of Enterobacteriaceae with carbapenemases

Carbapenemase	Modified Hodge test	Inhibitor based tests with temocillin	CarbaNP	CIM
KPC	98-99%	93-95%	100%	100%
NDM	34-99%	90-95%	100%	100%
VIM, IMP	12-85%	85-90%	100%	100%
OXA-48-like	93-100%	100%	38.5-90%	96-100%

References: [28], [31], [32], [34], [36], [37], [39], [40], [80] [83], [84]

among CPEs. However, the ability of some commercial PCR assays to detect different carbapenemase genes significantly differ between assays [71]. Both phenotypic and same molecular methods have problems in the detection of OXA-48-like producer. Therefore, special attention should be given to the detection of OXA-48-like carbapenemases in endemic regions and should also be a priority in patients that had recently visit such an endemic region.

A practical approach for the detection of carbapenemases

We recommend that laboratory methods suitable for testing of CPE should at least be able to reliably detect the most clinical relevant carbapanemases (i.e. KPC, NDM, OXA-48-like, IMP and VIM) in a reasonably rapid fashion. We want to provide an approach for those clinical laboratories that do not have the necessary expertise for the detection of the most common types of CPEs. Our approach is practical and easy to introduce into the work flow of a clinical laboratory and will ensure that the most common types of CPEs are detected on a rapid fashion. It is important to remember that knowledge regarding the local epidemiology of carbapenemase types will always play an important role on deciding which laboratory methods are best suited to that specific region. We do acknowledge that rapid turnaround time is not required for CPE testing for surveillance and epidemiological surveys.

A simple approach for infection prevention and control efforts consists of two-steps namely a screening process (i.e. susceptibility testing with the carbapenems [i.e. ertapenem with meropenem,

or imipenem using CLSI or EUCAST breakpoints]), followed by a confirmation test (i.e. phenotypic or genotypic) for the presence of a carbapenemase in isolates that test non-susceptibility to one or more of the carbapenems.

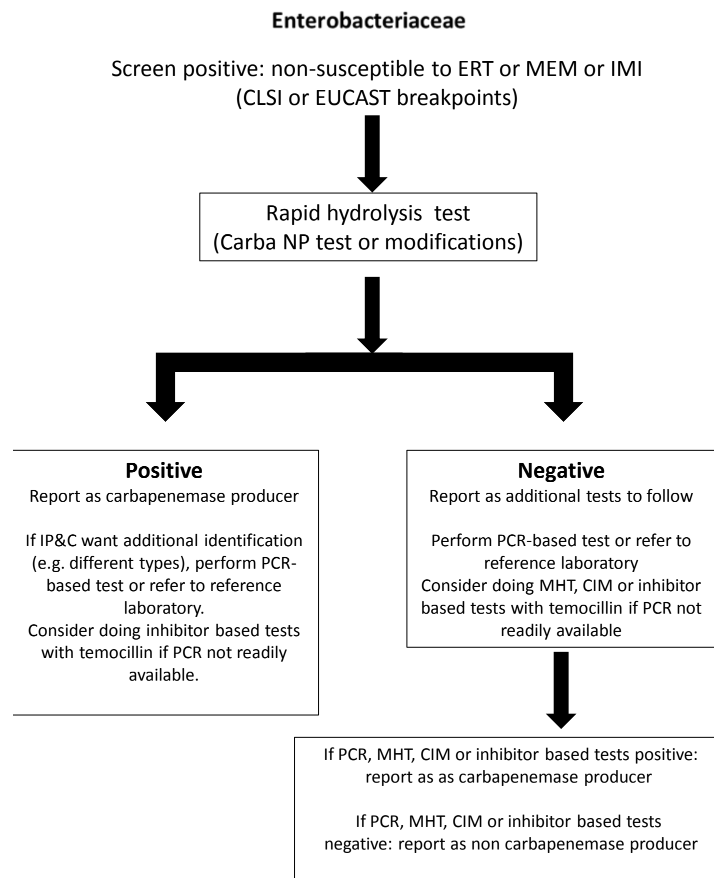
Phenotypic tests, in general terms, are simple to perform, interpret and can easily be introduced into the workflow of a clinical laboratory. For the rapid phenotype confirmation of CPE, the standardized Carba NP test as depicted in the CLSI 2015 guidelines [20] is most likely the best choice considering their performances in isolates with KPC and MBLs [82]. This rapid tests can easily be implemented in routine workflow of a clinical laboratory [43]. An inhibitor-based approach combined with temocillin susceptibility, the CIM or the MHT with an easy-to-follow algorithms, also performs adequately [32]. However, these assays are time consuming and add another 18-24 hours to the reporting of the final result.

PCR-based molecular confirmation methods (in-house and commercial assays) have excellent sensitivities and specificities but are unfortunately rather expensive and time consuming (as compared to most phenotypic tests). Figure 1 provides an easy-to-follow algorithm for the detection of carbapenemase among Enterobacteriaceae.

Five-year review

Rapid phenotypic methods, most likely the Carba NP-based tests, will be used as routine testing in most clinical laboratories. The developing countries, few clinical laboratories will

Figure 1. Detection of carbapenemases in Enterobacteriaceae



implement automated PCR-based detection methods. Such convenient molecular methods will also be used for the direct detection of CPE from clinical specimen, which will shorten current turn-around time (usually several days to hours) and impact infection control measures of patients with CPE. However, the nightmare of CPE spread will continue across hospitals, healthcare-associated facilities, and countries, despite implementation of infection control prevention measures [85,86].

The antibiotic pressure in healthcare settings and other environments will continue select for variants of carbapenemases and CPE with these enzymes will continue to spread globally. The strong need to control CPE will further promote research on development and application of diagnostic technologies. NGS and MALDI-TOF applications are the most promising methods and will advance further for the detection of CPEs.

Key issues

1. CPE is an emerging global public health threat that can easily spread among patients.
2. Carbapenemases are versatile β-lactamases, which complicates their detection. The five clinical relevant carbapenemases are KPC, NDM, OXA-48-like, IMP, and VIM enzymes.
3. CLSI and EUCAST do not recommend routine patient confirmation tests for carbapenemases if the current carbapenem interpretive criteria are used.
4. The detection of CPE is a critical initial step required for appropriate management of patients

during infection prevention and control efforts. Moreover, CPE testing is also appropriate for surveillance and epidemiological studies.

5. Screening for non-susceptibility to the carbapenems is the important initial step to successfully detect CPE and should be followed by phenotypic or genotypic confirmation tests.
6. For the rapid phenotype confirmation of CPE, the standardized CLSI version of Carba NP test or the use of commercial versions is most likely the best choice for laboratories that have limited access to molecular methods.
7. An inhibitor-based approach combined with temocillin susceptibility, the carbapenem inactivation method or the MHT with an easy-to-follow algorithms, also performs adequately.
8. The molecular procedures to detect CPE have the best sensitivities and specificities. Multiplex real-time PCR assays allow reliable detection in most clinical laboratories.
9. The detection of certain carbapenemases (e.g. OXA-48-like) with phenotypic methods can be challenging due to their weak carbapenemase activities.
10. Recent advances in NGS and MALDI-TOF MS applications are promising for the detection of CPE.

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Highlighted References

[28] 114 CPE including all of the five major carbapenemases and 28 non-CPE clinical isolates were used to compare detection performance of MHT and two commercial inhibitor-based disk combination kits. One-reaction multiplex PCR assay was also described.

[77] ResFinder, web-based comprehensive detection system of all the known resistance genes using WGS data.

[39] The first paper to describe the Carba NP test for the detection of carbapenemase. The original concept, protocol and interpretation (color change) are described.

[58] Detection performance of the Carba NP test, UV spectrophotometry, and a commercial DNA microarray were compared prospectively for 862 *Enterobacteriaceae* isolates. The authors also proposed a detection algorithm.

[59] MALDI-TOF MS-based detection of meropenem degradation product. Its concept and changes in spectra are shown.