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Phenotypic Detection of Carbapenemase-Producing Organisms from Clinical Isolates

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ABSTRACT The rapid spread of multidrug-resistant Gram-negative organisms constitutes one of the greatest challenges to global health. While Gram-negative organisms have developed several mechanisms to avert the bactericidal effects of commonly prescribed antibiotic agents, the increasing prevalence of carbapenemase-producing organisms (CPO) is particularly concerning given the rapid spread of mobile genetic elements containing carbapenemase genes, the limited treatment options for infections caused by these organisms, and the high mortality rates associated with CPO infections. Understanding if an organism is carbapenemase producing and, if so, the class of carbapenemase(s) produced has treatment implications, as some agents preferentially have activity against specific carbapenemases. Furthermore, CPO disseminate between patients with greater ease than non-CP-carbapenem-resistant organisms and warrant more intensive infection control measures than would be employed in the absence of carbapenemase production. Phenotypic assays currently used in clinical practice to detect CPO consist of the following: (i) growth-based assays which measure carbapenem resistance based on organism growth in the presence of a carbapenem antibiotic (e.g., modified Hodge test and modified carbapenem inactivation method), (ii) hydrolysis methods which detect carbapenem degradation products (e.g., Carba NP test and matrix-assisted laser desorption–ionization time of flight mass spectrometry), and (iii) lateral flow immunoassays which detect carbapenemase enzymes through the use of specific antibodies. Although there is no single phenotypic test that meets all specifications of the ideal test, as we describe in this review, there are a number of tests that are user-friendly, affordable, accurate, and feasible for implementation in clinical microbiology laboratories of all sizes.

KEYWORDS carbapenemase-producing *Enterobacteriaceae*, modified Hodge test, Carba NP test, modified carbapenem inactivation method, lateral flow assay, Carba NP, carbapenemase, carbapenemase-producing organism, lateral flow antigen, phenotypic

Infections due to carbapenemase-producing organisms (CPO) are associated with alarming rates of mortality (1). Carbapenemase genes offer a stable and transferable form of resistance, enabling spread via clonal expansion or by horizontal transfer of genes to naive bacteria (2). Carbapenemases defy geographic boundaries, making the prevention of CPO a significant public health concern that requires international coordination to contain (1). Distinguishing CPO from Gram-negative organisms that are carbapenem resistant due to non-carbapenemase-mediated mechanisms is important, as CPO disseminate between patients more readily than non-CPO and warrant implementation of more intensive infection control measures than would be employed in the absence of carbapenemase production (2). The U.S. Centers for Disease Control and Prevention (CDC) recommends that clinical laboratories consider actively screening isolates for carbapenemase production that

meet the CDC surveillance definition for carbapenem-resistant *Enterobacteriaceae* (http://www.cdc.gov/hai/organisms/cre/definition.html).

Currently, characterization of the underlying mechanism of carbapenem resistance is not undertaken by most clinical microbiology laboratories for therapeutic decision-making. However, understanding if an organism is carbapenemase producing and, if so, the class of carbapenemase(s) produced has treatment implications. Although antimicrobial susceptibility testing (AST) results alone are frequently sufficient for the selection of appropriate antibiotic therapy, the availability of antibiotics, like ceftazidime-avibactam or meropenem-vaborbactam, which have activity against some carbapenemases (e.g., *Klebsiella pneumoniae* carbapenemases [KPCs]) but not others (e.g., metallo-β-lactamases [MBLs], such as New Delhi metallo-β-lactamases [NDMs]), makes carbapenemase mechanism identification important, particularly when access to susceptibility testing for newer agents is limited. Unfortunately, there is no constellation of AST results that reliably distinguishes carbapenemase producers from noncarbapenemase producers.

CLASSIFICATION OF CARBAPENEMASES

Carbapenemases are commonly categorized using the Ambler classification scheme (3). KPCs are the most common carbapenemases identified in the United States and are members of the class A carbapenemases. Although commonly found in *Enterobacteriaceae*, they are not exclusive to *Enterobacteriaceae* and can be occasionally identified in *Pseudomonas aeruginosa* or *Acinetobacter baumannii* (4). Class B β-lactamases (MBLs) are characterized by the requirement for zinc ions at their active site, which can be useful diagnostically, as chelators, like EDTA or dipicolinic acid (DPA) inhibit MBL activity by binding zinc (5). Common MBLs include NDM, Verona integron-encoded metallo-β-lactamase (VIM), and IMP (named for its imipenem-resistant phenotype) enzymes. Common class D carbapenemases include OXA-48-like enzymes generally produced by *Enterobacteriaceae* (6) and OXA-23-like, OXA-40-like, OXA-58-like, and OXA-143-like enzymes, which are frequently produced by *A. baumannii* (4). Other carbapenemases that belong to a variety of molecular classes (e.g., *Serratia marcescens* enzymes [SME] and Guiana extended-spectrum β-lactamases [GES]) are reported sporadically (1). These are generally species specific, likely because their corresponding genes are either chromosomal or located on narrow-host-range plasmids, limiting large-scale dissemination (1). The diverse range of carbapenemase enzymes contributes to difficulty in their detection.

DEFINING CARBAPENEM-RESISTANT GRAM-NEGATIVE ORGANISMS

The Clinical and Laboratory Standards Institute (CLSI) lowered the carbapenem breakpoints for the *Enterobacteriaceae* in 2010 (7). The revised breakpoints recommend meropenem or imipenem susceptibility with MICs of $\leq$1 μg/ml and ertapenem susceptibility with MICs of $\leq$0.5 μg/ml (7). Furthermore, updated CLSI recommendations remove the recommendation for the routine identification of carbapenemase production among isolates with elevated carbapenem MICs (7). Therefore, providing that the 2010 carbapenem breakpoints have been implemented, it is recommended that AST results should be reported as tested, regardless of any mechanisms of resistance that have been identified. However, laboratories may choose to continue to screen for carbapenemase production for epidemiologic, infection control, or antibiotic stewardship initiatives. Over the past 10 years, a number of phenotypic methods for the detection of carbapenemase producers among CRO have been developed.

OVERVIEW OF PHENOTYPIC ASSAYS

Both phenotypic and molecular-based assays are available for the detection of carbapenemase producers from cultured isolates. Phenotypic assays currently used in clinical practice consist of the following: (i) growth-based assays which measure resistance based on growth in the presence of an antibiotic (e.g., modified Hodge test [MHT]) and modified carbapenem inactivation method [mCIM]); (ii) hydrolysis methods which detect the product of hydrolysis that is catalyzed by carbapenemase enzymes

(e.g., Carba NP and matrix-assisted laser desorption–ionization time of flight mass spectrometry [MALDI-TOF MS] methods); and (iii) lateral flow immunoassays which detect carbapenemase enzymes through the use of specific antibodies. Nucleic acid-based carbapenemase detection directly identifies the molecular determinants of carbapenemase production. This review is limited to phenotypic methods of carbapenemase detection and will not address nucleic acid-based approaches, including PCR, microarray, and whole-genome sequencing. We refer the reader to existing comprehensive reviews to learn more about the utility of nucleic acid-based techniques for carbapenemase gene identification (8, 9).

The selection of a carbapenemase detection test is contingent upon several factors, including local carbapenemase prevalence, regional molecular epidemiology, diagnostic performance characteristics, labor intensity, cost, and turnaround time (TAT) of the test (10). The TAT is important both for therapeutic decision-making and infection control purposes, with same-day results being ideal. Other considerations include the organisms to be tested (i.e., *Enterobacteriaceae* and/or glucose-nonfermenting Gram negatives), ease of use, workflow, regulatory status, necessary equipment, and reagent preparation requirements. Unfortunately, no currently available assay has a favorable profile for all of the criteria listed above. However, several options are available, allowing labs to choose a method that best suits their needs.

Common phenotypic tests employed by clinical microbiology laboratories include the MHT, the Carba NP test and its variants, and most recently, the mCIM. These tests identify carbapenemase production but in their traditional forms lack guidance regarding the specific carbapenemase being produced. Some modifications to these assays can provide more information on the specific carbapenemase groups being produced. For example, although the mCIM is indifferent to the carbapenemase class being expressed, setting up the test in parallel with the addition of the divalent cation chelator EDTA-mCIM (eCIM) enables the differentiation of serine and metallo-carbapenemases.

Multiplex lateral flow immunoassays (LFIAs) are actively being investigated as an approach to identify the specific carbapenemase group being produced, even when multiple carbapenemases are present simultaneously (11–17). Inhibitor-based methods are alternative phenotypic approaches that can be used to differentiate between carbapenemases by applying class-specific inhibitors. For example, the MBL Etest (AB Biodisk, Solna, Sweden) and the combined EDTA disk test rely on decreased MBL activity as a consequence of EDTA binding to zinc. Although MALDI-TOF MS is commonly used by clinical microbiology laboratories for bacterial and fungal genus and species identification, its role for carbapenemase detection is still evolving. The relative pros and cons of each of these phenotypic approaches for carbapenemase detection are described in more detail below and in Table 1.

Modified Hodge test. The MHT is probably the most well-known approach for carbapenemase detection. It involves the streaking of a clinical isolate in a line away from a disk impregnated with either ertapenem or meropenem which was previously placed on an agar plate inoculated with a lawn of a carbapenem-susceptible *Escherichia coli* strain. The MHT relies on the ability of carbapenemase producers to decrease the local concentration of carbapenem antibiotics, enabling the carbapenem-susceptible *E. coli* isolate to grow uninhibited around the streak line near the carbapenem disk, producing a cloverleaf appearance (Fig. 1A). This assay demonstrates acceptable sensitivity for most carbapenemases, particularly KPC enzymes, but low sensitivity for MBLs (18–20). In one study, the MHT was able to correctly detect carbapenemase activity in only 50% of NDM-producing isolates (18). For U.S. collections of *Enterobacteriaceae*, where KPC producers comprise greater than 95% of carbapenemases (21, 22), the sensitivity of the MHT has been reported to be between 93% and 98% (23, 24). However, with the rapid spread of NDM-producing *Enterobacteriaceae*, limitations in detecting this epidemiologically important resistance mechanism may have significant consequences. As isolates producing extended-spectrum β-lactamases (ESBLs) or AmpC cephalosporinases in conjunction with porin mutations often yield false-positive

TABLE 1 Select phenotypic tests for carbapenemase detection in clinical isolates[a]

Test parameter	Modified Hodge test (10,18–24)	Carba NP test and variants (10, 24–29, 32)	mCIM (7, 10, 32, 43)	Lateral flow immunoassays (11–16)	Targeted carbapenemase assays (47–53)	MALDI-TOF MS hydrolysis approach (28, 54–61)
Carbapenem-resistant organisms	Enterobacteriaceae	Enterobacteriaceae, Pseudomonas aeruginosa	Enterobacteriaceae, P. aeruginosa	Enterobacteriaceae	Generally Enterobacteriaceae	Enterobacteriaceae, P. aeruginosa, A. baumannii
Accuracy	For Enterobacteriaceae, sensitivity, ~95% and specificity, ~91%; false-positive results with ESBL- or AmpC-positive isolates with porin defects; false-negative results with MBLs	For Enterobacteriaceae, sensitivity, ~84% and specificity, ~100%; for P. aeruginosa, sensitivity, 98% and specificity, 98%; with change in lysis buffer and starting pH, sensitivity increases to close to 99%; false-negative results with OXA-48-like producers or mucoid isolates	For Enterobacteriaceae, sensitivity, 97% and specificity, 99%; for P. aeruginosa, sensitivity, 98% and specificity, 95%; mCIM improves detection of OXA-48-like producers over CIM; false-positive results may occur with AmpC-hyperproducing E. cloacae; addition of eCIM identifies MBLs in Enterobacteriaceae	For Enterobacteriaceae, sensitivity, 100% and specificity, ≥95%; false-positive results with some noncarbapenemase OXA-enzymes	Accuracy varies by test, e.g., OXA-48 disk test sensitivity, 96% and specificity, 98%; KPC gradient strips, sensitivity, 92% and specificity, 100%; MBL gradient strips, sensitivity, 94% and specificity, 95%; BD Phoenix CPO Detect, sensitivity, 97% and specificity, 69%	For Enterobacteriaceae, sensitivities, 77%–100% and specificities, 94%–100%
Ease of use	Easy to perform, no special reagents or media necessary	For manual versions, fresh reagents need to be prepared frequently due to limited shelf-life of imipenem solution; pH meter needed; commercially available tests remove the requirements for reagent preparation and need for a pH meter	Easy to perform, no special reagents or media necessary	Easy to perform, no special reagents or media necessary besides the test kit	All supplies generally readily available except gradient strips or disk	Complexity varies based on approach
Interpretation of results	Indentation of zone diam of E. coli toward carbapenem disk along streaked isolate; reading of zone indentation can be subjective	Color change from red to yellow or blue to yellow depending on the pH indicator; color change can be subjective for intermediate results	Positive, zone diam of 6–15 mm; indeterminate, 16–18 mm; negative, ≥19 mm; requires initial setup and then plating of disk onto lawn of E. coli following 4 h of incubation	Positive results based on the presence of visible lines specific for carbapenemase-type	Varies by test; for MBL Etest, only used for MBL detection; ratio of the IP/IPI MIC of ≥8 or presence of phantom zone or deformation of ellipse	Complex to interpret; requires MALDI-TOF instrument settings different from those traditionally used for FDA-approved microbial identification
Total time to perform test	15 min for setup and 2 min to read the following day	40 to 170 min (varies based on test)	5 min for initial setup, 10 min to inoculate plate, and 2 min to read the following day	5 min for initial setup and 2 min to read	Varies by test; for MBL Etest, 10-min setup and <5 min to read the following day	3–4 h
Turnaround time	18–24 h	Same-day result; 30 min to 2 h	18–24 h; shorter TAT has been described with shorter incubation of the disk with the indicator strain	Same-day result; 15 min	18–24 h	Same-day result; 4 h
Estimated cost	<$1.00	For manual versions, ≥$2.00; cost increases if imipenem-based reagents are not used within 3 days; commercial versions are more expensive, at ~$2.50–$15.00 (varies based on test)	<$1.00	Prices have not yet been established	Varies by test; ranges from <$1.00–$6.00; MBL gradient strips, ~$6.00	<$1.00 if MALDI-TOF MS instrument already available
Regulatory status	LDT; no longer CLSI endorsed	LDT; except the Rapidec Carba NP is FDA cleared for use with Enterobacteriaceae and P. aeruginosa; manual Carba NP is CLSI endorsed	LDT; CLSI endorsed	RUO	RUO	RUO

[a]IP, imipenem; IPI, imipenem inhibitor (imipenem plus EDTA); RUO, research use only; LDT, laboratory-developed test. Table modified from reference 10.

Modified Hodge Test

A.

A lawn of carbapenem susceptible *E. coli* ATCC 25922

Zone of inhibition of *E. coli* ATCC 25922 by ertapenem

Indentation of *E. coli* ATCC 25922 growth (clover leaf appearance) around the streak line of the carbapenemase-producing *K. pneumoniae* ATCC BAA-1705.

Carba NP and variants

B. C. D. E.

Modified Carbapenem Inactivation Method (mCIM) & EDTA- mCIM (eCIM)

F. G.

H. I.

mCIM   eCIM   mCIM   eCIM

Lateral Flow Immunoassay
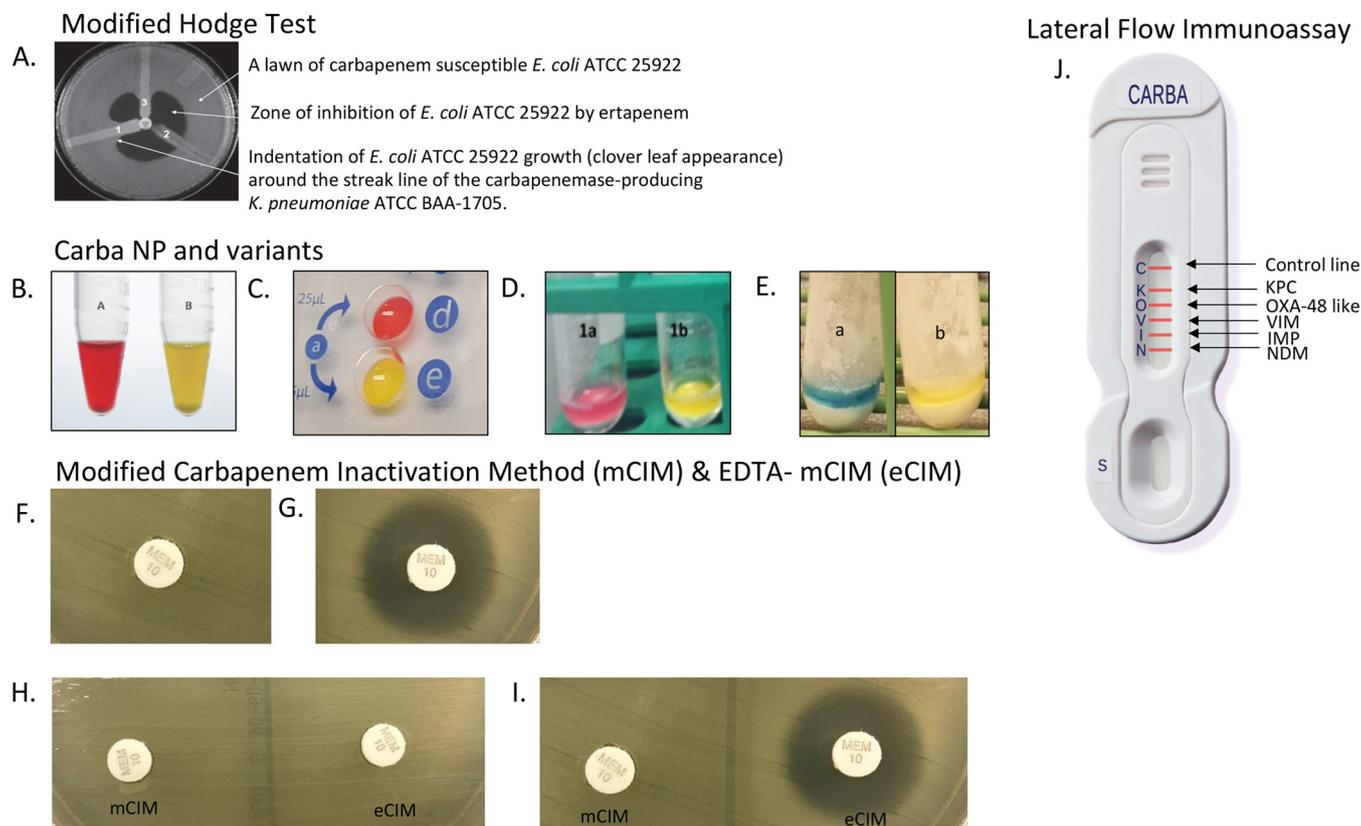
J.

CARBA

Control line
KPC
OXA-48 like
VIM
IMP
NDM

FIG 1 (A) Modified Hodge test. 1, *Klebsiella pneumoniae* ATCC BAA-1705, positive result; 2, *K. pneumoniae* ATCC BAA-1706, negative result; 3, clinical isolate, positive result. (B) CLSI Carba NP positive result. Tube A, no imipenem added, red; tube B, imipenem added, yellow. (C) Rapidec Carba NP (bioMérieux, Inc.) positive result. Well d, no imipenem added, red; well e, imipenem added, yellow. (D) Neo-Rapid Carba screen (Rosco Diagnostica) positive result. Tube 1a, no imipenem added, red; tube 1b, imipenem added, yellow (67). (E) Rapid Carb Blue Screen (Rosco Diagnostica) positive result. Tube a, no imipenem added, blue; tube b, imipenem added, yellow. (F) Modified carbapenem inactivation method (mCIM) positive result. (G) mCIM negative result. (H) mCIM and EDTA-mCIM (eCIM) results that are positive for a serine carbapenemase producer, as there is no inhibition of carbapenemase activity in the presence of EDTA. (I) mCIM and eCIM results that are positive for a metallo-beta-lactamase producer, as there is inhibition of carbapenemase activity in the presence of EDTA. (J) NG-Test Carba 5 (NG Biotech) lateral flow immunoassay results for the different carbapenemases detected. Panel A is republished from reference 45 with permission from the publisher. Panels B and F to I are republished from reference 7 with permission from the publisher. The image from panel J was provided by NG Biotech.

MHT results, the MHT has limited specificity, reported at approximately 91% (10). Although the MHT is inexpensive, relatively straightforward to perform, and uses reagents readily available in most clinical laboratories, it is occasionally challenging to interpret and time-consuming, as it requires an additional 24-h growth step after AST results become available (20). Due to these limitations, the MHT was removed from the CLSI M100 document in 2018, as newer phenotypic approaches with improved accuracy have become available (7).

Carba NP test and variants. The Carba NP test detects carbapenemases by measuring the *in vitro* hydrolysis of imipenem in bacterial extracts and produces color changes within approximately 2 h (24). Imipenem hydrolysis results in a carboxylic derivative, which in turn decreases the pH, producing a resultant color shift of a phenol red indicator from red to yellow (Fig. 1B) (25). It has a high sensitivity for detecting most carbapenemases, with a reported range of 73 to 100% (10, 24–27). Its sensitivity for the OXA-48-like carbapenemases, however, is significantly lower and in one study was only 6% (28). Furthermore, the Carba NP test requires the preparation of reagents with a short shelf-life, and there may be various interpretations of the results (10). Many variants of the Carba NP test have since been described with modifications to the inoculum, extraction regents, starting pH, pH indicators, and reading times, such as the Blue Carba test (29), which utilizes the pH indicator bromothymol blue. The modified Carba NP test is another variant that uses 0.02% cetyltrimethylammonium bromide lysis buffer and a starting pH of 7.5 instead of 7.8, enabling the improved identification of

carbapenemase production (30). In an investigation comparing 191 retrospective *Enterobacteriaceae* isolates, the CLSI Carba NP method had a sensitivity of 84% (95% confidence interval [CI], 76 to 90%) and a specificity of 100% (95% CI, 93 to 100%), whereas the modified Carba NP test had an increased sensitivity of 99% (95% CI, 95 to 100), with the specificity remaining at 100% (95% CI, 93 to 100%) (10). Others have obtained more modest results with the modified Carba NP test. Tijet and colleagues demonstrated that the sensitivity of OXA-48-like detection increased from 56% to 71% with the original Carba NP versus the modified Carba NP test and found that mucoid isolates were particularly prone to false-negative results (26, 31). The Carba NP test II was developed to both identify carbapenemase production and also to discriminate carbapenemase classes (32). It relies on pH changes due to imipenem hydrolysis in conjunction with specific enzyme inhibition through the use of tazobactam for KPC detection and EDTA for MBL detection.

The Carba NP test was also investigated for identifying carbapenemase production in non-glucose-fermenting organisms. The CLSI Carba NP method was used to evaluate 30 *P. aeruginosa* isolates and 30 *A. baumannii* isolates at three sites and included representation from Ambler class A, B, and D carbapenemases (33). The mean sensitivity and specificity for carbapenemase detection in *P. aeruginosa* were 98% (range, 93% to 100%) and 98% (range, 93% to 100%), respectively, whereas the mean sensitivity and specificity for carbapenemase detection in *A. baumannii* were 19% (range, 9% to 26%) and 100% (range, 100% to 100%), respectively (33). The modified Carba NP test increases the ability to detect carbapenemase production in *A. baumannii* but remains suboptimal. In one study, the sensitivities of the CLSI Carba NP and modified Carba NP test were 21% (95% CI, 6 to 51%) and 79% (95% CI, 49 to 94%), respectively (34).

The poor sensitivity of the Carba NP for carbapenemase detection in *A. baumannii* is concerning but not entirely surprising. OXA-type carbapenemases common to carbapenem-resistant *A. baumannii* strains are known to be inefficient at hydrolyzing the β-lactam ring of carbapenem antibiotics. The elevated carbapenem MICs observed in carbapenem-resistant *A. baumannii* strains are largely attributable to additional manifestations of resistance, such as reduced porin expression or upregulation of efflux pumps (4). Furthermore, the low-level outer membrane permeability of these organisms encumbers the activity of the Carba NP test that relies on cell lysis and the subsequent release of carbapenemases to detect color changes (4).

The CarbAcineto NP test was designed to overcome some of the impediments associated with detecting carbapenemases produced by *A. baumannii* (35). The CarbAcineto NP test requires modified lysis conditions and an increased bacterial inoculum compared to the original Carba NP test. More specifically, the lysis buffer (bacterial protein extraction reagent [B-PER II]; Thermo Scientific Pierce, Villebon-sur-Yvette, France) was replaced by a hyperosmotic solution of 5 M NaCl solution in response to the low permeability of the outer membrane, and the inoculum was increased from approximately half a 10-μl loop to a full 10-μl loop (35). The sensitivity of the Carba NP test in detecting acquired carbapenemase subgroups common to *A. baumannii*, most notably OXA-23-like, OXA-40-like, OXA-58-like, and OXA-143-like subgroups, was 12% in a collection of 151 carbapenemase-producing *A. baumannii* isolates (35), whereas the CarbAcineto test was able to recognize all of these acquired carbapenemases. Inconsistencies were observed with the CarbAcineto assay's ability to detect OXA-51-like carbapenemases. bla_{OXA-51}-like genes are chromosomally integrated, and OXA-51-like enzymes are either inactive or weakly active at hydrolyzing carbapenem antibiotics in the absence of an IS*Aba1* insertion sequence at the 5′ end of the bla_{OXA-51}-like gene (36). Overall, the CarbAcineto test had a sensitivity of 95% and a specificity of 100% for detecting carbapenemases commonly associated with *A. baumannii* (35).

The manual versions of the Carba NP test and its variants require frequent reagent preparation because the imipenem-containing solution has a maximum shelf-life of 72 h. Other commercially available assays using principles similar to those of the Carba NP test have been developed that generally simplify testing, including the Rapidec Carba NP test (37) (bioMérieux, Marcy-l'Etoile, France; Fig. 1C), the Neo-Rapid Carb

screen (38) (Rosco Diagnostica, Taastrup, Denmark; Fig. 1D), the β-Carba NP test (Bio-Rad, Marnes-la-Coquette, France), and the Rapid Carb Blue screen (39) (Rosco Diagnostica; Fig. 1E). These commercial tests simplify testing by including all reagents required for testing without the need for reagent preparation. Overall, these tests (not including the β-Carba NP) have sensitivities ranging from 89% to 98% and specificities approaching 100% (10). A recent comparison of the Rapidec Carba NP and the β-Carba NP tests demonstrated that the Rapidec Carba NP test exhibited better performance characteristics (sensitivity, 94%; specificity, 100%) than the β-Carba NP test (sensitivity, 65%; specificity, 90%) (40). Similar to the Carba NP test, the same limitations exist for these commercial assays, as false-negative results occur with OXA-48-like enzymes, and the interpretation of results can be subjective due to minor color changes. More detailed characteristics specific to these assays are presented in Table 1.

Carbapenem inactivation method. The CIM was first described in 2015 (41). This test is based on the premise that when a 10-μg meropenem disk is incubated for 2 h in water with a 10-μl loop of carbapenemase-producing isolate, the meropenem will be hydrolyzed. Alternatively, if the organism does not produce a carbapenemase, the meropenem disk retains its activity. The meropenem disk is then removed and placed on a Mueller-Hinton agar plate streaked with a susceptible laboratory strain of *E. coli*. Following overnight incubation, the zone of inhibition is measured. The absence of an inhibition zone indicates the presence of a carbapenemase. In contrast, a zone of inhibition indicates that the meropenem in the disk has preserved its activity and the isolate is not producing a carbapenemase. The CIM yields high sensitivities and specificities, at approximately 91% to 94% and 99% to 100%, respectively (10, 42). It is straightforward both to perform and interpret (10, 41). It requires inexpensive materials readily available in clinical microbiology laboratories at a cost of less than $1 per test, similar to the MHT (10). Both the MHT and CIM are less costly than the Carba NP test ($2 to $10 per test based on the variant), and all of these phenotypic tests are substantially more affordable than molecular assays.

Some concerns based on initial investigations suggested that the CIM may have limitations with the detection of carbapenemases with reduced hydrolytic activity (e.g., OXA-type carbapenemases), MBL enzymes that require divalent cations for activity, or in settings of lower levels of carbapenemase expression (43). Data from a CLSI working group suggested that modifying the carbapenem inactivation step by preparing the bacterial suspension (1-μl loopful of *Enterobacteriaceae*) in tryptic soy broth (TSB) and extending the time of incubation to 4 h could further improve carbapenemase detection (43). They conducted a multicenter study using the modified CIM (mCIM) approach for 61 isolates provided by the CDC-FDA Antimicrobial Resistance Isolate Bank and identified the mean sensitivity and specificity of the mCIM across the nine testing sites to be 97% (range, 93% to 100%) and 99% (range, 97% to 100%), respectively (43). Excellent reproducibility was shown across laboratories for representative carbapenemases from Ambler classes A, B, and D (43). One of the participating sites compared the CIM to the mCIM using the same set of isolates and demonstrated that the sensitivity increased from 82% to 93% and the specificity remained at 100% (43). Furthermore, at test sites where microbiologists had experience performing the MHT and/or the Carba NP test, interpretation of mCIM results was regarded as less subjective and easier to perform. A major drawback with the mCIM, similar to the MHT, is that it requires overnight incubation, so results will not be available within a single work shift, as is the case with the Carba NP test. Some investigators have implemented shorter incubation periods of the disk with the carbapenem-susceptible indicator strain (6 h) to report CIM or mCIM results within the same day (44). Based on our experience from unpublished work, the specificity of the mCIM might be slightly lower than initially reported, as we have encountered false-positive results with *Enterobacter cloacae* isolates lacking known carbapenemases.

The CLSI working group recommended a zone diameter of 6 to 15 mm for a result to be identified as positive (Fig. 1F), including when multiple small bacterial colonies

are observed within the zone of inhibition around the disk, and a zone diameter of ≥19 mm for a result to be categorized as negative (Fig. 1G). Zone diameters between 16 and 18 mm should be considered indeterminate and require additional testing to establish the presence or absence of carbapenemase production. In 2017, the mCIM was added to the 27th edition of the CLSI M100 supplement document as a reliable method for the detection of carbapenemase production in *Enterobacteriaceae* (45).

As the mCIM can accurately identify the presence of carbapenemases but cannot distinguish between serine and metallo-β-lactamases, a further modification to this assay with the addition of EDTA has been proposed. The basic premise of this assay is that if the test isolate produces an MBL, EDTA will inhibit carbapenemase production, resulting in the meropenem disk not being hydrolyzed as efficiently as expected in the absence of EDTA (7). Briefly, this method involves adding 20 μl of 0.5 M EDTA to a second 2-ml TSB tube. Then, the mCIM and eCIM tubes are processed in parallel (7). The meropenem disks from the mCIM and eCIM tubes are placed on the same Mueller-Hinton agar plates, inoculated with the *E. coli* ATCC 25922 indicator strain. The eCIM results should only be interpreted if the mCIM result indicates the presence of a carbapenemase. A ≥5-mm increase in the zone diameter for the eCIM compared to the zone diameter for the mCIM suggests the likelihood of an MBL-producing strain (Fig. 1H and I) (7). Unlike the mCIM, pinpoint colonies surrounding the meropenem disk within the zone of inhibition should be ignored when interpreting eCIM results (7). The eCIM is endorsed in the CLSI M100 supplement as an approach to distinguish MBL carbapenemases from serine carbapenemases, as a CLSI working group determined the eCIM's sensitivity and specificity to be greater than 95% and greater than 92%, respectively (7). Of note, if both serine and metallo-carbapenemases are coproduced by a single isolate, false-negative results may occur. This is important, as the coproduction of both OXA-48-like and NDM enzymes have been reported (10).

The mCIM has also been investigated for the detection of carbapenemases in *P. aeruginosa* and *A. baumannii*, using criteria similar to those established with the mCIM for *Enterobacteriaceae*. A 10-site study evaluating the performance of the mCIM against 30 *P. aeruginosa* and 30 *A. baumannii* isolates from the CDC-FDA Antibiotic Resistance Isolate Bank demonstrated a mean sensitivity and specificity of the mCIM for the detection of carbapenemase-producing *P. aeruginosa* of 98% (range, 87% to 100%) and 95% (range, 93% to 100%) (33). The accuracy of the mCIM for carbapenemase production by *A. baumannii* proved to be much less reliable. The mean sensitivity and specificity for *A. baumannii* were 80% (range, 36% to 96%) and 53% (range, 29% to 100%), respectively (33). Significant site-to-site variability in results was identified. Moreover, it was recognized that a larger inoculum (i.e., a 10-μl loopful of organism) was required for reliable carbapenemase detection for non-glucose-fermenting organisms, compared with the *Enterobacteriaceae*. This was most apparent with VIM-producing *P. aeruginosa* and OXA-producing *A. baumannii* isolates. However, the increased sensitivity (60% to 93%) was at the expense of specificity, as was observed with *A. baumannii* isolates, where the specificity decreased from 100% using a 1-μl loop to 63% using a 10-μl loop (33). Others have found similar limitations with the performance of the mCIM in detecting carbapenemase-producing *A. baumannii* strains (34). The 28th edition of the CLSI M100 supplement document endorses the use of the mCIM using a 10-μl inoculum for the detection of carbapenemase production among carbapenem-resistant *P. aeruginosa* strains (7). The mCIM is not currently recommended for the detection of carbapenemase activity in *A. baumannii*. A modification to the mCIM using 0.5 M Tris-HCl buffer for extraction (CIMTris) detected carbapenemase production in *Acinetobacter* and *Pseudomonas* species with a sensitivity of 98% and specificity of 93% (46).

Lateral flow immunoassays. LFIAs are antibody-based methods to identify the presence of carbapenemases. A number of LFIAs have been recently developed but generally enable the detection of one or a few of the most epidemiologically important carbapenemases. LFIAs have been developed for NDM production (14), IMP-production

(15), OXA-48-like production (14), KPC and OXA-48-like production (16), and KPC, NDM, and OXA-48-like production (12). Available data suggest that LFIAs produce accurate results from cultured isolates within 15 min. Recently, an LFIA targeting the five main carbapenemase families (i.e., KPC, NDM, VIM, IMP, and OXA-48-like carbapenemases) was evaluated (11). Investigators both retrospectively and prospectively evaluated this LFIA, named the NG-Test Carba 5, using 296 *Enterobacteriaceae* isolates (11). Briefly, they collected a single bacterial colony from a Mueller-Hinton agar plate and suspended it in 150 μl of extraction buffer. Subsequently, 100 μl of this extract was loaded on a cassette, and results were read within 15 min of migration, based on the presence of visible lines indicating a positive test, not unlike the interpretation of a home pregnancy test (Fig. 1J). In both the retrospective and prospective studies, the sensitivity of the NG-Test Carba 5 was 100% (11). The specificity was 95% for the retrospective cohort and 100% for the prospective cohort, with two noncarbapenemase OXA enzymes (an OXA-405 and two OXA-163 enzymes) misclassified as OXA-48-like enzymes (11). Importantly, the Carba5 was accurately able to detect isolates producing two carbapenemases (e.g., NDM-type and OXA-48-like carbapenemases), and cross-reactivity was not observed with nontargeted carbapenemases (e.g., GES, SME, etc.). In the future, it is plausible that the NG-Test Carba 5 or similar LFIAs could offer easy-to-use, accurate, rapid, and cost-efficient approaches for clinical microbiology laboratories to identify the presence of specific carbapenemases similar to molecular-based approaches.

Targeted carbapenemase assays. Targeted phenotypic carbapenemase assays compare carbapenem activity with and without the presence of inhibitors (e.g., phenylboronic acid [PBA] for KPC and EDTA for MBL). There are both commercially available and laboratory-developed options to evaluate for multiple carbapenemase resistance mechanisms (e.g., KPC + MBL Confirm ID kit [Rosco Diagnostica] and Mastdiscs combi Carba Plus [MAST Diagnostics] to evaluate for KPC, MBL, and OXA-48-like producers) (47–49). As an example, a laboratory-developed OXA-48 disk test was developed relying on an imipenem disk, a disk impregnated with EDTA, and another disk containing EDTA and PBA. Evaluating 254 isolates, the OXA-48 disk test had a sensitivity of 96% and a specificity of 98% (48). Targeted carbapenemase tests offer straightforward, affordable, and accurate options for the detection of specific carbapenemases. When used more broadly to identify the production of multiple carbapenemases, as can be commonly observed in some regions of the world (1), however, algorithms and workflows can become complex. To simplify the manual interpretation of these algorithms, the BD Phoenix CPO Detect test was designed to be incorporated into automated AST panels with computer-assisted algorithm-based detection (50). The test consists of nine wells on the BD Phoenix AST panel, each containing a β-lactam antibiotic, alone and in combination with various β-lactamase inhibitors for the detection and classification of carbapenemases, analogous to ESBL tests on automated AST instruments. One high-stringency evaluation of the assay reported a sensitivity of 97% and specificity of 69% to detect carbapenemase producers. Furthermore, it classified carbapenemases appropriately for 85% of class A, 72% of class B, and 89% of class D carbapenemases (50).

Gradient diffusion strips can also be reliable easy-to-use options for identifying specific resistance mechanisms and are growth-based approaches for carbapenemase detection. They rely on a combination of a β-lactam and a β-lactam–β-lactamase inhibitor (generally either boronic acid for KPC or EDTA for MBL) to detect these two carbapenemase types. KPC gradient MIC strips have a reported sensitivity of 92% and specificity of 100% (51). MBL gradient MIC strips have a reported sensitivity of 94% and a specificity of 95% (52).

Notably, there have been reports of false-positive results using MBL Etest strips (AB Biodisk, Solna, Sweden) in the presence of select OXA-type carbapenemases. Certain OXA-type carbapenemases (e.g., OXA-10 and OXA-14 in *Enterobacteriaceae* or OXA-23-like in *A. baumannii*) exist in highly active dimeric forms and less-active monomeric forms (53, 54). Metal chelators stabilize the active dimeric forms, converting the

enzymes to their less-active monomeric states, reducing carbapenemase activity, yielding false-positive MBL Etest results. Targeted carbapenemase assays can be valuable during outbreaks or when considering treatment options with activity against select carbapenemases.

MALDI-TOF MS. MALDI-TOF MS platforms are well established for microbial genus and species identification and are increasingly commonplace in clinical microbiology laboratories. Two major applications of MALDI-TOF MS for the rapid identification of carbapenemase production are being pursued. The first ("hydrolysis approach") detects carbapenem degradation products when bacterial protein extracts are incubated with a carbapenem substrate. The second approach ("plasmid-associated peak approach") involves the detection of a known carbapenemase-bearing plasmid-associated protein peak.

Regarding the hydrolysis method, a number of protocols have been described; however, a standardized approach is not yet available (28, 55–62). In short, carbapenem antibiotics are incubated with bacterial cultures, the cultures are centrifuged, and the supernatants are analyzed for β-lactam hydrolysis by MALDI-TOF MS. Reported sensitivities and specificities range from 77% to 100% and 94% to 100%, respectively, with a turnaround time of within 4 h. Similar to the Carba NP test, MALDI-TOF MS has difficulty detecting OXA-48-like enzymes. One study reported a sensitivity of 77% and a specificity of 100%, with the lower sensitivity due to missing 16 of 19 OXA-48 producers (28). However, this report suggested that the addition of bicarbonate to the reaction buffer could enhance the detection of OXA-48-like producers, increasing the overall sensitivity to 98%, without compromising the detection of other enzymes (28). Similarly, this modified approach was reported to have both a sensitivity and specificity of 100% in identifying carbapenemase activity in *A. baumannii* (63). Recent investigations suggest that the inclusion of temocillin, for which high-level resistance is suggestive of OXA-48 activity, may effectively detect this specific resistance mechanism (64). More recently, the role of carbapenem inhibitors was explored. The addition of PBA resulted in the reappearance of MS peaks corresponding to ertapenem for class A carbapenemases, whereas MS peaks associated with the degradation products of ertapenem remain in the case of MBL producers. Conversely, when adding the chelator DPA, MS peaks representing the hydrolyzed form of ertapenem remain in settings with class A carbapenemases, but peaks associated with ertapenem reappear in the case of MBL producers (64).

The plasmid-associated peak approach functions quite differently. Investigators at the National Institutes of Health retrospectively evaluated protein profiles from the pKpQIL plasmid carrying a bla_{KPC-3} gene involved in a *K. pneumoniae* outbreak at the NIH clinical center that led to 18 affected patients and 6 deaths (65). An approximately 11,109-Da MS peak corresponding to a gene product of the bla_{KPC-3}-carrying pKpQIL plasmid was identified and was common to all *K. pneumoniae* isolates and absent from a diverse set of controls (65). Remarkably, plasmid identification using this MALDI-TOF MS method was accomplished in as little as 10 min from isolated colonies. Further work by the same investigators including 140 characterized *Enterobacteriaceae* isolates showed both sensitivity and specificity over 95% (66). The investigators propose that signature MS peaks may be useful in tracking other plasmids conferring carbapenem resistance.

MALDI-TOF-MS for carbapenemase detection can be a cost-effective approach, if the instrument is already present in a microbiology laboratory, for genus and species identification. Importantly, the use of MALDI-TOF MS for carbapenemase detection by the hydrolysis method requires MALDI-TOF instrument settings different from those traditionally used for FDA-approved microbial identification and requires in-house validation. Currently, the MALDI-TOF MS hydrolysis approach for carbapenemase detection requires reagent preparation and advanced knowledge of the MALDI-TOF MS instrument settings. It can be viewed as being relatively complex to perform and interpret. Although the plasmid-associated peak approach is promising, it has only

been evaluated for a single plasmid-associated carbapenemase gene, and more work in developing a more comprehensive database of commonly circulating plasmids and associated carbapenemase genes is needed before it becomes a feasible approach for clinical microbiology laboratories.

CONCLUSION

In summary, the spread of CPO remains a significant clinical and public health concern. Reliable detection of carbapenemase production is an essential first step in combating this problem. In an era of international travel and medical tourism, the association between specific resistance mechanisms and a given region will become less important, making both routine surveillance and further evaluation of carbapenem-resistant Gram-negative clinical isolates imperative. While there is no single phenotypic test that meets all specifications of the "perfect" test, there are a number of options that are user-friendly, accurate, and feasible for implementation in clinical microbiology laboratories of all sizes.

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