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PHENOTYPIC METHODS FOR DETECTION OF CARBAPENEMASE PRODUCTION AMONG CARBAPENEM RESISTANT ENTEROBACTERIALES: FROM CONVENTIONAL TO INNOVATIVE APPROACHES A COMPREHENSIVE OVERVIEW

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ABSTRACT:

The global health community is facing a significant challenge with the rapid spread of multidrug-resistant Gram-negative organisms. These organisms have developed various mechanisms to evade the effects of commonly prescribed antibiotics, making it increasingly difficult to treat infections caused by them. Of particular concern is the rise in carbapenemase-producing organisms (CPO), which are capable of spreading their carbapenemase genes through mobile genetic elements. This poses a serious threat as there are limited treatment options for infections caused by CPO, and the mortality rates associated with these infections are high. Identifying whether an organism is carbapenemase-producing and determining the class of carbapenemase(s) it produces is crucial for treatment decisions. Certain antibiotics have better activity against specific carbapenemases, making accurate detection important. Additionally, CPO can spread more easily between patients compared to non-CP-carbapenem-resistant organisms, necessitating stricter infection control measures. Currently, there are several phenotypic assays used in clinical practice to detect CPO. These include growth-based assays, hydrolysis methods, and lateral flow immunoassays. While no single test meets all the ideal specifications, there are user-friendly, affordable, accurate, and feasible tests available for implementation in clinical microbiology laboratories of all sizes. In conclusion, addressing the challenge of multidrug-resistant Gram-negative organisms, particularly carbapenemase-producing organisms, requires a comprehensive understanding of their mechanisms, accurate detection methods, and effective infection control measures.

Keywords: Mcim; Ecim; Carba NP; Scim; Phenotypic Test; Carbapenemase; CRE; Carbapenem Resistant; Cpos; Enterobacterales; Gnbs.

1. INTRODUCTION

Infections caused by carbapenemase-producing organisms (CPO) have been linked to alarming mortality rates. These carbapenemase genes possess a stable and transferable form of resistance, allowing them to spread through clonal expansion or by transferring genes to susceptible bacteria. The ability of carbapenemases to transcend geographical boundaries makes the prevention of CPO a significant concern for public health, necessitating international cooperation to contain its spread. It is crucial to differentiate CPO from carbapenem-resistant Gram-negative organisms that do not produce carbapenemases, as CPO can disseminate more easily between patients and require more rigorous infection control measures. The U.S. Centers for Disease Control and Prevention (CDC) advise clinical laboratories to actively screen isolates for carbapenemase production, following the CDC surveillance definition for carbapenem-resistant Enterobacteriales [1,2]. Carbapenemases are enzymes that have the ability to break down carbapenems, monobactams, cephalosporins, and penicillins. In the treatment of infections caused by gram-negative bacteria that are resistant to multiple drugs, carbapenems are the preferred choice, resulting in a rise in the occurrence of CRE, CRPA, and CRAB in recent years. Detecting carbapenem-resistant strains accurately and promptly is crucial for effective patient management and to halt the spread of resistance in the community [3]. The majority of clinical microbiology laboratories do not currently analyze the underlying mechanism of carbapenem resistance when making therapeutic decisions. However, it is crucial to determine if an organism is producing carbapenemase and, if so, the specific type of carbapenemase, as this information affects the choice of treatment. While antimicrobial susceptibility testing (AST) results are often sufficient for selecting the appropriate antibiotic therapy, the identification of carbapenemase mechanisms becomes important when access to susceptibility testing for newer antibiotics is limited. This is especially true considering that certain antibiotics, such as ceftazidime-avibactam or meropenem-vaborbactam, are effective

against certain carbapenemases (e.g., Klebsiella pneumoniae carbapenemases [KPCs]) but not others (e.g., metallo-β-lactamases [MBLs], like New Delhi metallo-β-lactamases [NDMs]). Unfortunately, there is no specific combination of AST results that can reliably differentiate between carbapenemase producers and non-carbapenemase producers.

Carbapenems

Carbapenems are a group of highly effective antibiotics commonly used to treat severe bacterial infections. These antibiotics are typically reserved for cases of known or suspected multidrug-resistant (MDR) bacterial infections and severe infections caused by extended spectrum β-lactamase (ESBL) producing bacteria [14]. The global use of carbapenems such as imipenem, meropenem, doripenem, ertapenem, and biapenem has increased due to the growing resistance to cephalosporin antimicrobials within the Enterobacterales group. Recent emerging mechanisms of resistance accumulate through the spread of carbapenem-destroying β lactamases leaving narrow therapeutic options [15].

Carbapenem Mode of Activity and Structure-Function

Carbapenems, a type of antimicrobial agent within the β-lactam family, are closely related to penicillins in terms of their chemical structure. The mechanism of action of carbapenems begins with their ability to penetrate the bacterial cell wall and attach to specific enzymes called penicillin-binding proteins (PBPs) [16, 17]. The main inhibitory PBPs are 1a, 1b, 2, and 3, which deactivate an autolytic enzyme inhibitor in the cell wall, resulting in the bacteria's death [18, 19]. Previous studies have shown that the inhibition of PBPs 2 and 3 typically causes Gram-negative bacilli to become spherical cells and filamentous organisms [20].

According to current knowledge, the inhibition of transpeptidase is considered the key mechanism by which carbapenems target bacterial cell wall synthesis. Transpeptidation involves the formation of a co-valent bond by PBPs, which are composed of carboxypeptidase and transpeptidase enzymes. This inhibitory action effectively prevents their peptide cross-linking activities during the biosynthesis of peptidoglycan. The resulting lethal effects are believed to induce cell death through autolytic action within the bacterial cell [21]. According to Papp-Wallace K.M et al., [22] the precise mode of action of carbapenems remains uncertain. The rigid structure of the glycan backbone is given significant importance. When PBPs are repressed, the vitality of the cell wall is impacted, leading to the weakening of the glycan backbone through autolysis. Eventually, the cell succumbs to osmotic pressure, resulting in its destruction in Gram-negative bacteria [23].

Carbapenem use and side effects

The role of carbapenemases in resistance has been examined in relation to the effective use of carbapenems in combination therapy to improve patient outcomes. Bacteria produce enzymes called β-lactamases that degrade β-lactam antimicrobials. However, they struggle to break down carbapenem agents combined with β-lactamase inhibitors for in vivo use. The first compound used was imipenem with cilastatin, which inhibits renal metabolism and extends its half-life. Imipenem, meropenem, and doripenem have half-lives of about 1 hour, while ertapenem has a half-life of around 4 hours, making it suitable for once-daily dosing. Imipenem is known for causing dose-dependent gastrointestinal side effects compared to other carbapenems. Ertapenem has the lowest activity against Pseudomonas species and other non-fermentative Gram-negative bacteria. Doripenem, imipenem, and meropenem are commonly used as antipseudomonal agents in clinical practice. Ertapenem, imipenem, and meropenem are poorly absorbed orally and must be administered parenterally for effectiveness. Doripenem, a recently approved carbapenem, is valuable for treating serious infections [25].

Carbapenems possess a fused beta lactam ring that is impervious to most beta lactamases. They exhibit strong efficacy against streptococci, enterococci, staphylococci, listeria, enterobacteriaceae, as well as numerous pseudomonas, bacteroides, and acinetobacter species. Nevertheless, most methicillin-resistant staphylococci are resistant to carbapenems. The safety

profile of carbapenems is akin to that of other beta lactam antibiotics like cephalosporins and penicillins. Common adverse effects include injection site reactions, diarrhea, nausea, vomiting, skin rash, and pruritus. Carbapenems may lead to transient, mild-to-moderate, and asymptomatic increases in serum aminotransferase levels that typically resolve upon discontinuation of therapy. Rare instances of cholestatic liver injury have been reported during or shortly after carbapenem treatment, particularly in patients with multiple medical issues and other causes of liver disease (e.g., parenteral nutrition, sepsis). Carbapenems are primarily excreted unchanged in the urine with minimal hepatic metabolism, which likely accounts for the rarity of clinically significant hepatotoxicity with jaundice associated with these antibiotics [24].

Carbapenem resistance

The emergence of Carbapenem-Resistant Enterobacteriaceae (CRE) infections poses a major challenge in healthcare environments and is a growing worry worldwide [26,27]. Carbapenem medications are powerful antimicrobials that are usually administered intravenously in hospitals with low risk of allergic reactions [28]. Each carbapenem medication has its own specific function, necessitating careful selection for treating severe infections in clinical practice [29]. The importance of using carbapenems against Gram-negative pathogens cannot be emphasized enough. Organisms that are resistant to at least one of the carbapenem antibiotics, such as imipenem, meropenem, ertapenem, or doripenem, are categorized as carbapenem-resistant organisms. The most prevalent carbapenem-resistant organisms are Enterobacterales (CRE), *Acinetobacter baumannii* (CRAB), and *Pseudomonas aeruginosa* (CRPA) [3].

Intrinsic Resistance of Gram-Negative Bacilli

Resistance to carbapenems can occur due to intrinsic resistance mechanisms, acquired resistance mechanisms, or a combination of both. It is crucial to acknowledge that a significant number of bacteria, including both commensal and pathogenic strains, naturally possess resistance to specific classes of antimicrobial agents. This inherent insensitivity, referred to as intrinsic resistance, presents challenges and complexities in the selection of appropriate drugs for treatment. Furthermore, it can also contribute to the development of acquired resistance. For instance, Gram-negative organisms have the capability to reduce the uptake of β-lactam drugs by selectively modifying their cell membrane porin channels. This reduction in outer membrane permeability hinders the effective delivery of β-lactams to their intended targets [25].

Acquired Resistance of Gram-Negative Bacilli

Bacteria have developed a range of resistance methods, including enzymatic inactivation, target site mutation, and efflux pumps. Among these, inactivating enzymes appeared early on after the discovery and clinical use of β-lactam antibiotics. These enzymes, which break down β-lactams, have evolved from penicillinases to cephalosporinases, ESBLs, and more recently, MBLs and other carbapenemases. The emergence of MBLs has had a significant impact on the effectiveness of carbapenems, which are often considered a last resort for treating multi-resistant Gram-negative bacteria [31]. Plasmid-mediated carbapenemases are commonly found in Enterobacteriaceae, and there are multiple pathways through which they can be spread among bacterial isolates. Furthermore, recent studies have identified other important mechanisms that confer resistance to carbapenems. One such mechanism involves the combination of plasmid AmpCs and ESBL enzymes, which can make Gram-negative bacteria resistant to hydrolysis-resistant carbapenems [32].

Overproduction of efflux pumps

Efflux pumps are essential for transporting substances across the cell membrane. These proteins can be found in both prokaryotic and eukaryotic organisms. They help maintain the

proper potential and pH gradient, assist in intercellular signaling, play a role in microbial virulence, and remove harmful metabolites and toxins from the cell. Overall, efflux pumps are crucial for maintaining cell homeostasis [34-36]. MDR efflux pumps have the ability to remove different antimicrobials that do not share a common structure. P. aeruginosa and Acinetobacter species, both Gram-negative bacteria, are known for their resistance to β-lactam antibiotics due to efflux mechanisms. Overexpression of efflux pumps targeting carbapenems may lead to resistance against this class of antibiotics [30].

Carbapenemase production

Carbapenemases are enzymes known as β-lactamases that possess a wide range of hydrolytic capabilities. These enzymes have the ability to break down penicillins, cephalosporins, monobactams, and carbapenems. Bacteria that produce these β-lactamases can cause severe infections, as the carbapenemase activity renders many β-lactam antibiotics ineffective. Carbapenemases are classified into three molecular classes: A, B, and D β-lactamases. Class A and D enzymes utilize a hydrolytic mechanism based on serine, whereas class B enzymes are metallo-β-lactamases that contain zinc in their active site. The class A carbapenemase group includes various families such as *SME, IMI, NMC, GES*, and *KPC*. Among these, the *KPC* carbapenemases are the most prevalent and are predominantly found on plasmids in *Klebsiella pneumoniae*. The class D carbapenemases consist of OXA-type β-lactamases, which are frequently detected in *Acinetobacter baumannii*. The metallo-β-lactamases belong to families such as *IMP, VIM, SPM, GIM,* and *SIM,* and have primarily been detected in *Pseudomonas aeruginosa*. However, there is an increasing number of reports worldwide of this group of β-lactamases in the Enterobacteriales [25].

Dissemination of carbapenamse was initially species specific until the 1990s but the identification of different types of metallo-β-lactamases in *pseudomonas aeruginosa* [37], *bla-KPC* in *Klebsella pneumonia* [38], *bla-SME,* in *Serratia marcescens* [39] exhibit different pattern of the spread of carbapenamases. Carbapenamses are hydrolytic in nature due to the presence of metal atom in their active sites, they allow the bacteria to survive under adverse conditions in the presence of antibiotics. Among the ESCAPE pathogens *Pseudomonas aeruginosa* strains have shown a wide range of carbapenamase producing genes [40-43]. Those genes are generally derived from plasmids, get transmitted easily from one bacterium to another and enhance their virulence [44].

The spread of carbapenamses was noticed first among *Klebsiella* species and was identified as KPC (Klebsiella producing carbapenemases) in North Carolina [45], which gradually began to spread throughout the US. Over the years, KPC producers have spread rapidly among different gram negative bacteria [46]. In India the occurrence of KPC was first reported in 2011 from a middle aged patient admitted to a tertiary care hospital in Chennai with the gene name KPC-2 [47]. The treatment of infections caused by KPC-producing organisms is severely limited, posing a high risk of complications and requiring an extended recovery period for patients [48]. The KPCs are now endemic in Israel, Italy and found in different Asian and European countries leading to healthcare associated infections and treatment challenges [49, 50]. KPC producing organisms have also been reported in Australia and New Zealand due to travel across countries which are endemic to KPC [51].

Mechanism of Action of Carbapenemases

Carbapenemases are a type of β lactamases which contain Zn2+ ions in their active sites and hydrolyse penicillins, cephalosporins, monobactams, and carbapenems [52]. They have broad spectrum antimicrobial activity and bind to PBPs, reducing their permeability and causing mutation. They dissociate the –CO–NH structure of the β-lactam ring by mimicking the alanyl-alanine residue of the peptidoglycan layer [52]. Based on their function, the carbapenemases are classified into different classes.

The functions and characteristics of various carbapenemases: These enzymes are classified into different groups, such as Non-Metallo-Carbapenemases, Class A, C, and D, Metallo β lactamases, which consist of Class B [53]. Their activity can be suppressed by chelating agents like EDTA shown in Figure 1.

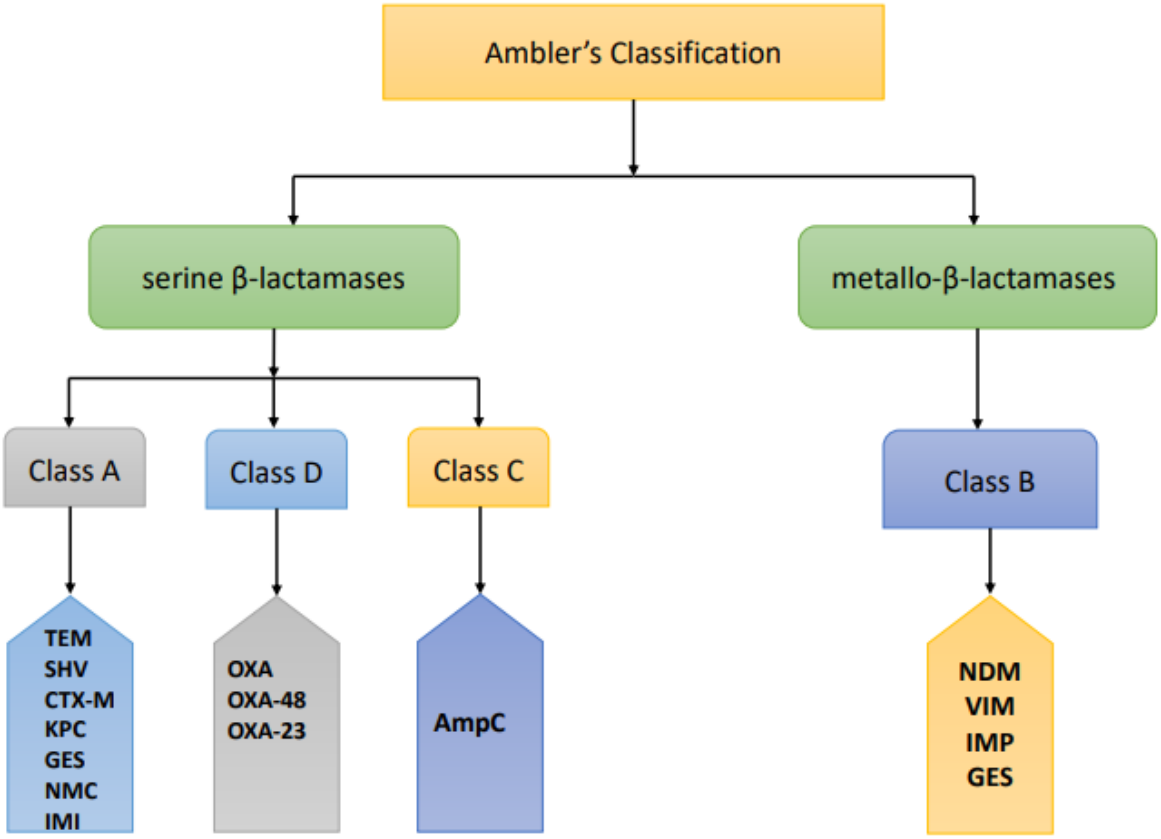

Figure 1: Illustrates the Ambler Molecular Classification of carbapenemases, featuring KPC

(Klebsiella pneumoniae carbapenemase), NMC (non metallo carbapenemase), SME (Serratia marcescens enzyme), AmpC (Ampicillin chromosomal cephalosporinases), OXA (Oxacillin carbapenemase/oxacillinase), NDM (New Delhi metallo-β-lactamase), VIM (Verona integron-encoded metallo-β-lactamase), and IMP (Imipenemase metallo-β-lactamase).

Class A Carbapenamases

Class A carbapenemases can be divided into six distinct groups based on phylogenetic analysis. Enzymes from the *GES*, *KPC*, *SME*, and *IMI/NMC-A* families fall into four of these groups, while SHV-38 and SFC-1 each have their own separate group. The genes encoding these carbapenemases can be located on plasmids or within the host organism's chromosome. The bla(GES) genes are usually found within gene cassettes on class I integrons, while bla(KPC) genes and a single bla(IMI-2) gene are flanked by transposable elements on plasmids. Class A carbapenemases can hydrolyze various antibiotics, including penicillins, cephalosporins, monobactam, imipenem, and meropenem. These enzymes are further classified into four phenotypically distinct groups: 2br, 2be, 2e, and 2f, according to the Bush-Jacoby-Medeiros classification system. Clavulanate and tazobactam, like other class A beta-lactamases, can inhibit class A carbapenemases [52].

Class B carbapenemases

A recent comprehensive examination of metallo-lactamases has provided a valuable summary and update on epidemiology. This particular group of lactamases is characterized by its ability to degrade carbapenems and its resistance to commonly used lactamase inhibitors, while still being susceptible to inhibition by metal ion chelators. These enzymes have a broad range of

substrates, including cephalosporins and penicillins, but they are unable to break down aztreonam. The hydrolysis mechanism relies on the interaction between lactams and zinc ions in the enzyme's active site, making them uniquely susceptible to inhibition by EDTA, a chelator of Zn2+ and other divalent cations [53].

The initial metallo-lactamases that were revealed and investigated were chromosomal enzymes found in environmental and opportunistic pathogenic bacteria like *Bacillus cereus,* Aeromonas spp., and *Stenotrophomonas maltophilia*. These specific enzymes were frequently detected in bacteria that also produced at least one serine lactamase, and both lactamases were inducible following exposure to lactams. Fortunately, with the exception of *S. maltophilia,* these bacteria are not commonly associated with severe nosocomial infections as they are generally opportunistic pathogens, and the chromosomal metallo-lactamase genes are not easily transferable. Early classification based on functional analyses of purified proteins indicated that these lactamases were distinct from other groups of enzymes that utilized a serine-based hydrolytic mechanism. Noteworthy differences included the requirement of Zn2+ for efficient lactam hydrolysis and the resistance to inhibition by clavulanic acid and tazobactam. A defining characteristic of their substrate range was their ability to hydrolyze carbapenems [53].

Class C carbapenemases

The Ambler class C β-lactamases, also referred to as AmpC cephalosporinases, provide resistance against various antibiotics, including penicillin, oxyiminocephalosporins, and cephamycins. One intriguing variant, ACC-1, is a plasmid-encoded class C β-lactamase that has been discovered in clinical strains of *K. pneumoniae, P. mirabilis, Salmonella enterica,* and *E. coli*. Bacteria that produce ACC-1 are susceptible to cefoxitin but resistant to oxyiminocephalosporins due to alterations in the structure of the enzyme. Clavulanic acid is not an effective inhibitor of AmpC cephalosporinases, but they can be inhibited by boronic acid and cloxacillin. These enzymes can be encoded by the chromosome of various Enterobacteriaceae, such as E. cloacae and S. marcescens, as well as a few non-enterobacterial organisms like *Pseudomonas aeruginosa*. In certain cases, they can be induced by antibiotics or expressed at high levels due to mutation. However, AmpC enzymes can also be acquired through transmissible plasmids, resulting in their presence in bacteria that lack or poorly express a chromosomal blaAmpC gene, such as *Escherichia coli, Klebsiella pneumoniae,* and *Proteus mirabilis* [54].

Class D Carbapenemases (Oxacillinases)

Class D β-lactamases, also known as oxacillinases, were initially rare and transmitted via plasmids. They exhibit a preference for degrading isoxazolylpenicillins such as oxacillin, methicillin, and cloxacillin more rapidly than traditional penicillin. However, they are less effective against first-generation cephalosporins. The name "OXA" is derived from their preferred substrate, oxacillin. These enzymes possess a conserved serine-based structure in their active site, while the rest of the molecule varies in amino acid sequences. They are not susceptible to β-lactamase inhibitors or metal chelators. The emergence of these enzymes coincided with the introduction of flucloxacillin and methicillin for the treatment of staphylococcal infections. Early OXA β-lactamases like *OXA-1, OXA-2,* and *OXA-3* were identified in Gram-negative bacteria and primarily functioned as penicillinases with superior oxacillin hydrolysis compared to penicillin. Subsequently, *OXA-11*, the first extended-spectrum OXA variant, was detected in *P. aeruginosa* and exhibited enhanced ceftazidime hydrolysis. Other extended-spectrum *OXA* enzymes such as *OXA-13, OXA-14, OXA-15, OXA-16, OXA-17, OXA-19, OXA-28,* and *OXA-45* were also found in *P. aeruginosa* but did not spread widely. Presently, OXA enzymes with carbapenem-hydrolyzing activity include groups like *OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-58-like, OXA-143-like,* and *OXA-235* [54].

Screening test

When conducting tests in laboratories, it is crucial to be alert to the possibility of carbapenemase production when faced with a carbapenem-intermediate or -resistant result. Additionally, any reduction in carbapenem susceptibility within the susceptible range among Enterobacteriaceae and Acinetobacter spp. isolates should be viewed with suspicion. The optimal carbapenem for screening purposes has yet to be determined. Multiple screening approaches involve the Disc diffusion method, E-test for MIC determination, and automated antimicrobial susceptibility systems including Vitek-2 (Biomerieux, France) [58].

Confirmatory phenotypic methods for the detection of carbapenemase production

While there are multiple tests available for identifying carbapenemase producers, most clinical microbiology laboratories are increasingly adopting six established phenotypic methods. These methods have been evaluated in various multicenter studies and include the MHT, disc-inhibitors synergy test, mCIM, Carba NP test [4] spectrophotometry, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Modified Hodge test (MHT)

The lawn culture of Escherichia coli ATCC 25922 was prepared by diluting it at a 1:10 ratio, following the guidelines of CLSI. The culture was then streaked on a Mueller Hinton agar plate, with a meropenem susceptibility disk placed at the center of the test area. To ensure accuracy, four strains were tested on a single plate, and the plate was incubated overnight at a temperature of 35°C ± 2°C in ambient air for a duration of 16-24 hours [5].

The interpretation of the results took place following an incubation period of 16-24 hours. A positive outcome from the Modified Hodge test displayed a distinctive clover leaf-shaped depression on the Escherichia coli 25922 strain, which was growing alongside the disk diffusion zone. This indicated the presence of carbapenemase production. Conversely, a negative test showed no growth of Escherichia coli ATCC 25922 along the disk diffusion zone [6].

Multiple studies have reported inaccurate identification of AmpC and CTX-M hyper-producers [7] (Clinical and Laboratory Standards Institute (CLSI), 2012) and limited success in detecting NDM producers [8] through the MHT.

CDDT

The test strains were adjusted to meet the McFarland 0.5 standard and were then inoculated onto MHA. On the MHA, an oxoid imipenem disk of 10 μg and a HiMedia imipenem plus 750-μg ethylenediamine tetraacetic acid were placed. After an overnight incubation, the presence of a zone diameter difference of ≥7mm between the imipenem disk and imipenem plus ethylenediamine tetraacetic acid was interpreted as a positive result for metallo-β-lactamase (MBL) [9].

Ellipse test (E test)

A HiMedia Ezy MIC E test MBL strip was utilized. A direct colony suspension of 0.5 McF standard was produced and cultivated on MHA. The Ezy MIC strip was removed with an applicator and placed on an agar plate. The plates were incubated at 37°C for 16-18 hours in ambient air. The concentration at the intersection of the ellipse and the strip's scale was recorded. Results were interpreted according to the kit insert [9].

Modified Carbapenem Inactivation Method (mCIM) in conjugation with EDTA-carbapenem inactivation method

The mCIM and eCIM methods were used to detect CPE according to CLSI-2023 guideline. For each strain, two tubes containing 2 mL of Trypticase soy broth (TSB) were utilized simultaneously. One tube was supplemented with 20 μL of 0.5 M EDTA (Sigma), while the other tube remained EDTA-free. A fresh colony of the tested strain was transferred to each tube using a 1 μL inoculating loop. A 10-mg meropenem disk (HiMedia) was then incubated with the suspension of the tested strain for 2 to 4 hours at 35 ℃. Subsequently, meropenem

disks from both tubes were placed on Mueller Hinton agar plates and inoculated with the E. coli ATCC 25922 indicator strain. The mCIM yielded a positive result when the diameter of the inhibition zone ranged from 6 to 15 mm, or from 16 to 18 mm with small colonies within the inhibitory zone shown in Table 1. The interpretation of eCIM results should only be done if the mCIM result indicates the presence of carbapenemase. If the zone diameter for eCIM is 5 mm larger than that for mCIM, it suggests the likelihood of producing metallo-carbapenemase shown in Table 2 [4].

Table 1. Modified Carbapenem Inactivation Method (mCIM) interpretation

mCIM result	eCIM result	Interpretation
Negative	Not set up	Carbapenemase not detected
Positive	Not set up	Carbapenemase detected
Indeterminate	Not set up	Testing inconclusive for the presence of carbapenemase.

Table 2. MCIM and eCIM combination test interpretation

mCIM result	eCIM result	Interpretation
Negative	Do not interpret	Carbapenemase not detected
Positive	Negative	Serine Carbapenemase detected
Positive	Positive	Metallo-β-lactamase detected
Indeterminate	Do not interpret	Testing inconclusive for the presence of carbapenemase.

RAPIDEC Carba NP test

Carba NP test is a method used to detect carbapenemase through a phenotypic approach. In their study, Patrice Nordmann, Laurent Poirel, and Laurent Dorlet explained that this test relies on the hydrolysis of the β-lactam ring of carbapenem. The underlying principle of this test is that the in vitro hydrolysis of a carbapenem causes a pH change (decreased pH), resulting in a colour change of the medium from red to yellow or light orange, as indicated by phenol red.
After a 30-minute incubation period, the test strip should be placed on the support card, which is black and white in colour. The incubation lid should be removed to conduct the initial reading. The observation of a color change from red to yellow, light orange, orange, or dark orange in well e indicates a positive result, as indicated in Table 3 [10].

Table 3. Interpretation of Results strip reading of RAPIDEC Carba NP

Control well(d)	Test well(e)	Interpretation
Red	Red	Negative
Orange	Orange	Negative
Red	Yellow, light orange, orange, dark orange	Positive
Orange	Yellow	Positive
Any colour other than red or orange	Not applicable	Un interpretable
Orange	Red	Un interpretable

Carba NP test

A solution was created by combining phenol red (0.05%) and ZnSO4.7H2O (0.1 mmol/L) with Clinical Laboratory Reagent Water; the pH was set to 7.8 ± 0.1, and the solution was stored at 4°C in amber-colored bottles for a maximum of 15 days. The B solution was freshly made by adding 12 mg/ml of imipenem-cilastatin injectable form (doubling the amount to adjust for the cilastatin component; equivalent to 6 mg/ml of imipenem standard grade powder) to the A solution and kept at 4°C until needed. Two calibrated loops, each containing 10 µl of bacterial colony cultured on 18 to 24 h SBA, were mixed with 200 µl of a bacterial lysis buffer that was prepared in-house (consisting of Tris-HCL 20 mmol/L and 0.1% Triton X-100) and then vortexed for 5 seconds. Subsequently, 100 µl of bacterial lysate was divided into two microcentrifuge tubes labeled "a" and "b." Reagents A and B were added to tubes a and b, respectively, followed by an incubation at 37°C, with readings taken at 10 min, 30 min, and 120 min by three different observers. A positive test result was confirmed if tube "a" turned red and tube "b" turned orange/yellow, while in a negative test, both tubes remained red. Quality control was assured through the use of Klebsiella pneumoniae ATCC BAA 1705 (positive control), K. pneumoniae ATCC BAA 1706 (negative control), and plain A and B reagents with lysis buffer (reagent control) [10, 11].

Simplified Carbapenem Inactivation Method (sCIM)

The sCIM is an upgraded version of the mCIM that integrates improved experimental procedures. In contrast to the mCIM, where the antibiotic disk is incubated in the organism culture media for 4 hours, the sCIM involves directly applying the organism to be tested onto an antibiotic disk. To perform the sCIM for Enterobacteriaceae, a 0.5 McFarland standard suspension of E. coli ATCC 25922, prepared using the direct colony suspension method, is introduced onto the MHA plate following the routine disk diffusion procedure. For A. baumannii and P. aeruginosa, a 0.5 McFarland standard suspension of E. coli ATCC 25922, diluted 1:10 in saline, is introduced onto the MHA plate using the direct colony suspension method. The plates are then left to dry for 3-10 minutes. Afterwards, 1-3 overnight colonies of the test organisms grown on blood agar are applied onto an imipenem disk (10 µg; HiMedia, Mumbai, India).

Bacterial strains that possess the ability to produce carbapenemase have the capability to break down imipenem, resulting in unrestricted growth of the susceptible indicator strain. On the contrary, the presence of a 6-20 mm diameter zone of inhibition around the disk or the satellite growth of E. coli ATCC 25922 colonies around the disk with a zone diameter of ≤22 mm indicates the isolate's ability to produce carbapenemase. A negative result is indicated by a zone of inhibition ≥26 mm, while a zone of inhibition ranging from 23-25 mm is considered an indeterminate result for carbapenemase production [12, 13].

Maldi-Tof Ms

Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) is an advanced analytical technique used for rapid and precise identification and characterization of microorganisms. This technique involves evaluating the mass of ionized sample molecules using short laser pulses, following co-crystallization with a low-molecular-weight organic acid called matrix. MALDI-TOF MS has become a major advancement in recent times, especially in clinical microbiology. It allows for the detection of pathogenic bacteria and fungi, along with antimicrobial drug resistance. By examining the protein profiles of bacteria and fungi, MALDI-TOF MS provides outstanding sensitivity, high throughput, ease of use, and cost-effectiveness, despite the higher initial cost of the spectrometer [56].

MALDI-TOF MS technology is widely utilized in clinical microbiology labs for identifying microbial genus and species. Two main applications are currently in progress for quickly detecting carbapenemase production. The first method, called the "hydrolysis approach," involves recognizing carbapenem degradation products by incubating bacterial protein extracts with a carbapenem substrate. The second method, known as the "plasmid-associated peak

approach," concentrates on identifying a particular protein peak linked to a known carbapenemase-bearing plasmid [57].

MALDI-TOF MS is a highly effective and reliable technique that has been successfully integrated into clinical microbiology laboratories to either replace or supplement traditional methods of identifying bacteria and fungi. By utilizing MALDI-TOF/MS, we can significantly reduce turnaround times by an average of 1.45 days compared to conventional approaches. The automation of the manual process involved in MALDI target spotting brings additional benefits to the diagnostic workflow, minimizing human errors and allowing for complete traceability. Ready-to-use reagents, combined with specific modules to enhance the instrument's capabilities, have proven to be reliable in routine diagnostics. The selection and utilization of these reagents should be based on the laboratory's activity volumes and intended application of the MALDI-TOF MS device. Additionally, MALDI-TOF MS can detect antimicrobial drug resistance mechanisms, providing prompt identification of resistant isolates at least 24 hours earlier than traditional methods. Its ability to simultaneously identify bacteria, fungi, and antimicrobial resistance markers makes it a rapid and dependable tool in clinical microbiology laboratories [56].

2. CONCLUSION

The spread of CPO continues to be a significant concern in both clinical and public health settings. Detecting carbapenemase production reliably is crucial in addressing this issue effectively. With the rise of international travel and medical tourism, the correlation between specific resistance mechanisms and geographical regions becomes less relevant. Therefore, it is imperative to prioritize routine surveillance and further evaluation of carbapenem-resistant Gram-negative clinical isolates. Although there is no single phenotypic test that fulfills all requirements of an ideal test, there are several user-friendly, accurate, and feasible options available for implementation in clinical microbiology laboratories of any scale. The escalating health concerns associated with CPOs have been highlighted in various global studies, underscoring the urgent need for improved diagnostics, therapeutics, and infection prevention measures. Consequently, the importance of understanding the mechanisms behind carbapenem resistance has become increasingly apparent. In various studies findings have indicated that the combination of mCIM and eCIM tests outperforms the CarbaNP test. The introduction of newer classes of β-lactam and β-lactamase inhibitors, such as ceftazidime-avibactam, has shown promising therapeutic effects on serine carbapenemase producers. Additionally, concurrent treatment with ceftazidime-avibactam, aztreonam, and colistin has demonstrated high efficacy against MBL producers. Given that mCIM with eCIM can effectively differentiate between these two types of carbapenemases, it could serve as a straightforward, dependable, and cost-efficient method for carbapenemase detection, ultimately aiding in the development of more effective treatment strategies to reduce therapeutic failures. Furthermore, this approach could assist resource-limited laboratories in making informed decisions about genotypic testing for carbapenemase production.

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Authors' contributions

GV and PK was involved in the conception and design of the study. GV, AP, SG, BC was involved in the articulation of the contents, and in editing the manuscript. SA, US was involved in the editing the manuscript. GV was involved in the design of figures and editing the manuscript. SKR was involved in editing the manuscript. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

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Not applicable.

Competing interests

The author declares that he has no competing interests.

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