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Review Article

A Review of Phenotypic Carbapenemase Detection Methods

Fenotipik Karbapenemaz Tespit Yöntemlerinin İncelenmesi

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Abstract: Carbapenems are broad-spectrum antibiotics used to treat multidrug-resistant infections caused by Gram-negative, Gram-positive, and anaerobic bacteria. However, the overuse of these antibiotics has led to the rise of carbapenem-resistant bacteria (CRB), which present significant treatment challenges. Carbapenem resistance is primarily mediated by the production of carbapenemase enzymes, which hydrolyze carbapenems and are often encoded by plasmids, facilitating horizontal gene transfer. Rapid detection of carbapenemase-producing organisms is crucial for effective antimicrobial stewardship and preventing the spread of resistance. Phenotypic methods for detecting carbapenemases include disk diffusion, minimum inhibitory concentration (MIC) methods, gradient diffusion, automated systems, and chromogenic media. More advanced techniques, such as the Carba NP test and immunochromatographic assays, provide rapid identification of resistance profiles, while spectrophotometric analysis detects enzymatic hydrolysis. The choice of method depends on sensitivity, specificity, and clinical context. Early and accurate detection allows for timely adjustments to treatment, optimizing patient outcomes and limiting the misuse of broad-spectrum antibiotics.

Keywords: Fenotipik tespit, Karbapenemaz, Antibiyotik direnci

Öz: Karbapenemler, Gram-negatif, Gram-pozitif ve anaerobik bakteriler tarafından kaynaklanan çoklu ilaç dirençli enfeksiyonların tedavisinde kullanılan geniş spektrumlu antibiyotiklerdir. Ancak, bu antibiyotiklerin aşırı kullanımı, karbapenem dirençli bakterilerin (CRB) artışına yol açmış ve bu durum tedavi açısından önemli zorluklar yaratmıştır. Karbapenem direnci, öncelikle karbapenemleri hidrolize eden ve genellikle plazmidler tarafından kodlanan karbapenemaz enzimlerinin üretimiyle aracılık edilmektedir, bu da genetik materyalin yatay transferini kolaylaştırır. Karbapenemaz üreten organizmaların hızlı bir şekilde tespiti, etkili antimikrobiyal yönetim ve direnç yayılmasının önlenmesi için çok önemlidir. Karbapenemazları tespit etmek için fenotipik yöntemler arasında disk difüzyon, minimum inhibitör konsantrasyon (MIC) yöntemleri, gradyan difüzyon, otomatik sistemler ve kromojenik ortamlar bulunmaktadır. Carba NP testi ve immünokromatografik analizler gibi daha ileri teknikler, direnç profillerinin hızlı bir şekilde tanımlanmasını sağlarken, spektrofotometrik analizler enzimatik hidrolizi tespit eder. Yöntem seçimi, duyarlılık, özgüllük ve klinik bağlama bağlıdır. Erken ve doğru tespit, tedavinin zamanında ayarlanmasına olanak tanır, hasta sonuçlarını optimize eder ve geniş spektrumlu antibiyotikleri yanlış kullanımı sınırlayarak hastaların tedavi süreçlerini iyileştirir.

Anahtar Kelimeler: Phenotypic detection, Carbapenemase, Antimicrobial resistance

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INTRODUCTION

Carbapenems, which are derivatives of thienamycin naturally produced by Streptomyces cattleya, exhibit broad-spectrum antimicrobial activity and are classified within the β-lactam class of antibiotics (1). They are used as last resort in the treatment of multidrug-resistant Gram-negative, Gram-positive, and anaerobic bacterial infections. However, the widespread use of broad-spectrum antibiotics in recent years has led to the rapid emergence and spread of carbapenem-resistant bacteria (2,3). Over the past two decades, advancements in intensive care practices have led to prolonged hospitalizations, thereby increasing the incidence of healthcare-associated infections (HCAIs) caused by carbapenem-resistant bacteria (CRB). Such infections significantly limit available therapeutic options. According to the Centers for Disease Control and Prevention (CDC), CRB are defined as microorganisms that exhibit resistance to at least one agent within the carbapenem class and/or produce carbapenemase enzymes capable of hydrolyzing carbapenems, with potential for horizontal transfer to other bacterial species (4–6).

Carbapenemase enzymes are a subset of β-lactamases capable of hydrolyzing penicillins, cephalosporins, and, to varying extents, carbapenems and monobactams. Resistance to carbapenems is typically multifactorial, involving mechanisms such as the production of carbapenemase enzymes, overexpression of efflux pumps, and/or the loss of carbapenem-specific outer membrane porins–such as OprD–often in conjunction with the overproduction of AmpC β-lactamases. Among these, the most clinically significant mechanism is the enzymatic hydrolysis of carbapenems by carbapenemases. The genes encoding these enzymes are frequently plasmid-borne, facilitating horizontal gene transfer within and between bacterial species and thereby accelerating the spread of resistance (7). Carbapenem-resistant bacteria (CRB), which are rapidly disseminating worldwide, represent a major threat to global public health. This is due not only to their resistance to nearly all β-lactam antibiotics but also to their capacity for harboring additional resistance mechanisms against other antimicrobial classes such as aminoglycosides and fluoroquinolones. Infections due to these microorganisms are associated with high morbidity and mortality (7,8). The increasing prevalence of CRB has led to serious clinical complications and elevated rates of treatment failure. Due to the prolonged turnaround time of conventional susceptibility testing, empiric treatment often involves the use of broad-spectrum antibiotics prior to the identification of resistance phenotypes in carbapenemase-producing isolates. Each year, an estimated 700,000 deaths worldwide are attributed to antimicrobial-resistant bacteria, and this figure is projected to rise to approximately 10 million deaths annually by 2050, resulting in substantial economic and healthcare burdens (2,4,7). Therefore, the development and implementation of rapid, simple, and reliable phenotypic methods for the laboratory detection of carbapenemases are essential for reducing unnecessary use of broad-spectrum agents, particularly the critically important carbapenem antibiotics. To address this need, the application of diagnostic assays that detect CRB in clinical specimens and identify the underlying carbapenemase enzymes is of paramount importance for optimizing antimicrobial stewardship. Accurate and timely identification of resistance profiles in clinical isolates is also crucial for preventing the spread of carbapenemase-producing organisms and guiding appropriate empirical therapy. This review aims to provide an overview of the various phenotypic methods currently employed for the detection of carbapenemase enzymes (9,10).

Phenotypic Carbapenemase Detection Methods

1. Disk Diffusion Method

The disk diffusion method, commonly performed using the Kirby-Bauer technique with a carbapenem-class antimicrobial disk, is primarily utilized for screening purposes. Among carbapenems, meropenem is considered the most reliable agent due to its optimal balance of sensitivity and specificity in detecting carbapenemase-producing organisms. Although ertapenem exhibits higher sensitivity, its specificity is comparatively lower. While ertapenem can be used as a screening agent, it should be kept in mind that resistance may also occur in isolates producing extended-spectrum beta-lactamase (ESBL) or AmpC beta-lactamase. In this method, a bacterial suspension standardized to a 0.5 McFarland turbidity is inoculated onto Mueller-Hinton agar. Subsequently, disks containing meropenem or other carbapenem antibiotics are placed onto the agar surface. After 16-24 hours of incubation, the diameter of the inhibition zones is measured and interpreted according to guidelines provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical and Laboratory Standards Institute (CLSI). Despite its ease of use, the disk diffusion method may yield inaccurate results, particularly in borderline-resistant isolates. Therefore, confirmation with more precise phenotypic methods—such as gradient diffusion (e.g., Etest®), broth microdilution, or automated antimicrobial susceptibility testing systems—is recommended to ensure diagnostic accuracy (11,12).

2. Minimum Inhibitory Concentration Method

The lowest concentration of an antimicrobial agent that inhibits visible growth of a microorganism is defined as the Minimum Inhibitory Concentration (MIC). MIC determination is considered a sensitive and reliable method for detecting carbapenem-resistant isolates and plays a crucial role in guiding effective antimicrobial therapy (13).

2.1. Broth Microdilution or Agar Dilution Method

The most commonly employed methods for determining the MIC of antimicrobial agents are the broth microdilution and agar dilution techniques. In these methods, antimicrobial agents are serially diluted in broth or solid medium in accordance with the recommendations of EUCAST or CLSI guidelines, followed by the addition of a standardized inoculum of the test organism (11–13). For instance, when determining the MIC value of meropenem using a U-shaped microtiter plate in the broth microdilution method, 100 µl of Mueller-Hinton broth is first dispensed into each of the 12 wells designated for testing. A previously prepared meropenem solution at a concentration of 256 µg/ml is used to create a two-fold serial dilution starting from the first well to the eleventh. The twelfth well serves as the growth control and does not contain any antimicrobial agent. At this stage, the final concentration of meropenem in the first well is 128 µg/ml. The bacterial suspension is prepared by adjusting the isolate to a turbidity equivalent to a 0.5 McFarland standard. Subsequently, 100 µl of the bacterial suspension (approximately 10^5 CFU/mL, as recommended by EUCAST or CLSI) is added to each well. Following this step, due to the dilution effect, the final meropenem concentration in the first well is adjusted to 64 µg/ml. After incubation at 35–37°C for 18–24 hours, the MIC is defined as the lowest concentration of the antimicrobial agent that completely inhibits visible bacterial growth. Interpretation of MIC values for carbapenem-class antimicrobials should be performed according to the latest breakpoints established by EUCAST or CLSI. With few exceptions, EUCAST guidelines recommend broth microdilution as the reference method for MIC determination. Regardless of the method, Mueller-Hinton broth or agar is the standard medium utilized for quantitative MIC analysis (11,12).

2.2. Gradient Diffusion Method

The gradient diffusion method is a quantitative in vitro technique used to determine antimicrobial susceptibility, in accordance with the standards established by EUCAST and CLSI. In this method, commercially available test strips impregnated with a gradient of antimicrobial concentrations are utilized. After the bacterial suspension is adjusted to 0.5 McFarland turbidity and inoculated evenly onto Mueller-Hinton agar, a test strip is placed onto the surface of the agar plate. Following 18–24 hours of incubation at 35–37°C, the MIC value is read at the point where the elliptical inhibition zone intersects with the scale on the strip. Commercially manufactured strips are available for various carbapenem agents individually, as well as in combination with specific inhibitors, allowing for both susceptibility testing and preliminary enzyme characterization. For example, strips containing meropenem and boronic acid are used for the detection of Klebsiella pneumoniae carbapenemases (KPC), while those incorporating imipenem/imipenem-EDTA or meropenem/meropenem-EDTA combinations are used for identifying metallo-β-lactamase (MBL) production (10,13,14).

2.3. Automated Systems

Automated systems such as BD Phoenix, MicroScan WalkAway, Sensititre Autoreader, VITEK, and VITEK 2 are widely used in clinical microbiology laboratories for the determination of MIC values of carbapenem-class antimicrobials. These platforms offer standardized, high-throughput, and reproducible results, allowing for rapid and reliable antimicrobial susceptibility testing, including detection of resistance to carbapenems.

3. Chromogenic Media

Chromogenic media are utilized primarily for screening purposes to detect carbapenemase-producing organisms. These culture media contain selective agents that inhibit the growth of Gram-positive bacteria and non-carbapenemase-producing Gram-negative bacteria. Additionally, they include chromogenic substrates that are hydrolyzed by carbapenemase enzymes. When hydrolyzed, these substrates release chromophores that induce a visible color change in the medium, facilitating the presumptive identification of carbapenemase-producing colonies based on colony color. Several chromogenic agar formulations are available for this purpose, including ChromID ESBL, ChromID CARBA, ChromID OXA-48 (bioMérieux, France); CHROMagar KPC (CHROMagar, France); HardyChrom CRE (Hardy Diagnostics, USA) and SpectraCRE, Brilliance CRE (Thermo Fisher Scientific, USA) (15). These media serve as effective tools for the initial detection of carbapenemase-producing Enterobacterales, Acinetobacter spp., and Pseudomonas spp.

Among these, the SuperCarba medium is a specialized chromogenic agar designed specifically for the detection of carbapenemase production. It demonstrates high sensitivity, particularly for identifying OXA-48-like enzymes, and is currently the only known chromogenic medium capable of detecting OXA-244, a less prevalent variant of the OXA-48 family (15,16). Brilliance CRE, ChromID CARBA, and CHROMagar KPC can also be used in combination with

other selective media containing carbapenem antibiotics to enhance screening accuracy.

4. Combination Disk Test

The combination disk test is used to identify the specific types of carbapenemase enzymes contributing to resistance against carbapenem-class antimicrobials. This method offers several advantages, including low cost, ease of implementation in routine laboratory settings, commercial availability, and the potential to provide insight into the bacterial genotype. The test is performed using four meropenem (10 µg) disks. One disk is supplemented with 10 µl of 40 mg/ml phenylboronic acid (PBA) to inhibit Klebsiella pneumoniae carbapenemase (KPC); another contains 10 µl of 0.1 M EDTA to inhibit metallo-β-lactamase (MBL); a third disk includes both PBA and EDTA to assess the simultaneous inhibition of KPC and MBL; and the fourth disk contains meropenem alone, serving as a control. Cloxacillin may also be incorporated to distinguish carbapenem resistance due to overproduction of AmpC enzymes and porin loss from true carbapenemase production. Bacterial suspensions adjusted to 0.5 McFarland turbidity are inoculated onto Mueller-Hinton agar (MHA), and after a 10-minute absorption period, the disks are placed onto the same medium. The plates are then incubated overnight at 37°C. Following incubation, the inhibition zones around the meropenem disk and those containing PBA, EDTA, or both are compared to determine the presence and type of carbapenemase enzyme (10,13,17).

5. Modified Hodge Test

The Modified Hodge Test (MHT) is designed to evaluate alterations in the inhibition zone of a susceptible indicator strain (E. coli ATCC 25922) caused by carbapenemase-producing isolates that inactivate the carbapenem-class antimicrobial used in the assay. The E. coli ATCC 25922 reference strain is prepared to a 0.5 McFarland turbidity in broth or physiological saline and subsequently diluted at a 1:10 ratio before being inoculated onto Mueller-Hinton agar (MHA). A 10 µg meropenem or 10 µg ertapenem disk is placed at the center of the plate. The test isolate is then applied using a swab or applicator stick in a straight line approximately 20–25 mm in length, starting from the edge of the carbapenem disk toward the periphery of the plate. After incubation at 35 ± 2 °C for 16–24 hours, the presence of a cloverleaf-like indentation in the inhibition zone of E. coli ATCC 25922—along the inoculation streak of the test organism—is interpreted as a positive MHT result. Each test plate must include both positive and negative controls. Klebsiella pneumoniae ATCC BAA-1705 serves as the positive control, while K. pneumoniae ATCC BAA-1706 is used as the negative control. Although the MHT is inexpensive and simple to perform, it frequently yields false-positive results, particularly in extended-spectrum β-lactamase (ESBL)-producing isolates with porin loss or alterations. The test shows higher reliability in detecting KPC and OXA-48 producers but exhibits low sensitivity for metallo-β-lactamase (MBL) producers. To enhance the detection of MBLs, supplementation of the MHA medium with zinc (Zn) is recommended, while the addition of cloxacillin can help reduce false positives associated with resistance mechanisms such as AmpC production and porin deficiency. However, due to variability in sensitivity and specificity and challenges in result interpretation, both EUCAST and CLSI no longer recommend the use of MHT (11,12,18).

6. Carbapenemase Nordmann–Poirel (Carba NP) Test

The Carba NP test is a phenotypic method employed for the detection of carbapenemase production in Enterobacterales and Pseudomonas species. The principle of the test lies in detecting pH changes in the reaction medium, which result from the in vitro hydrolysis of imipenem by bacterial lysates. Phenol red is used as a pH indicator, shifting from red to yellow or orange in response to acidification. Studies evaluating the Carba NP test have reported 100% sensitivity and specificity for Enterobacterales, as well as 100% specificity and 94.4% sensitivity for carbapenemase-producing Pseudomonas species. To detect carbapenemase production in Acinetobacter species, a modified version of the assay—referred to as the CarbAcineto NP test—has been developed. Furthermore, the recently introduced Rapidec Carba NP test has demonstrated high sensitivity and specificity for identifying carbapenemase production in clinically significant Gram-negative bacteria (19–21).

7. Blue-Carba Test

The Blue-Carba test is a biochemical test derived from the Carba NP test, designed to rapidly detect carbapenemase production. This modified test employs 0.04% bromothymol blue at a final pH of 7.0 as a pH indicator, along with $ZnSO_4$ (0.1 mmol/L) and imipenem (3 mg/mL) in the reaction mixture. A pure bacterial colony is inoculated into a 96-well microtiter plate containing both test and negative control solutions. Following incubation at 37°C for two hours, the presence of carbapenemase is indicated by imipenem hydrolysis, which leads to a pH shift and a corresponding color change in bromothymol blue—from blue to green/yellow or green to yellow. In the absence of carbapenemase activity, the color remains blue or green. Blue-Carba is a simpler and more cost-effective alternative to the Carba NP test and has demonstrated 100% sensitivity and specificity in studies. It enables the rapid detection of carbapenemase in Gram-negative microorganisms directly

from culture. While the test exhibits excellent sensitivity for class A and B carbapenemases, false results may occur in the detection of OXA-48-type enzymes (21,22).

8. NitroCarba NP Test / NitroSpeed-Carba NP Test

The NitroCarba NP test, also known as the NitroSpeed-Carba NP test, is a biochemical assay developed for the rapid detection of carbapenemase production. It is effective in identifying a broad range of carbapenemase classes, including KPC, OXA-48-like, NDM, VIM, and IMP enzymes. Following the addition of nitrocefin, the test yields results within 10 to 15 minutes, contributing to a total turnaround time of approximately 30 minutes. Due to its high sensitivity and specificity, the NitroSpeed-Carba NP test represents a reliable and efficient tool for the rapid identification of carbapenemase-producing bacterial isolates (22,23).

9. Modified Carbapenem Inactivation Method (mCIM) and EDTA Modified Carbapenem Inactivation Method (eCIM) Test

The Carbapenem Inactivation Method (CIM) is based on the principle that carbapenemase-producing isolates inactivate a carbapenem disk during co-incubation. Standardization of bacterial concentration, incubation time, disk content, and inhibition zone diameter is essential for consistent results. The method is simple, practical, cost-effective, and yields results within a short time. To perform the test, 1 µL of an overnight bacterial suspension is added to 2 mL of Tryptic Soy Broth (TSB). For the EDTA-modified version (eCIM), 20 µL of 0.5 M EDTA is added to a separate tube containing 2 mL of TSB to achieve a final concentration of 5 mM, followed by the addition of 1 µL of the same bacterial suspension. A 10 µg meropenem disk is placed into each tube and incubated at 37 °C for 4 hours. After incubation, the disks are removed and placed on Mueller-Hinton agar plates freshly inoculated with a 0.5 McFarland suspension of the carbapenem-susceptible E. coli ATCC 25922 strain. Plates are incubated at 37 °C for 18 to 24 hours, after which the results of the mCIM and eCIM tests are evaluated. For carbapenemase-producing isolates, an inhibition zone of 6–15 mm is interpreted as mCIM positive, while a zone ≥19 mm indicates a negative result. An inhibition zone of 16–18 mm with pinpoint colonies is considered intermediate. eCIM results are only interpreted if mCIM is positive. An increase in the inhibition zone diameter by ≥5 mm in eCIM compared to mCIM is indicative of the presence of metallo-β-lactamase (Ambler class B) carbapenemases. An increase of <4 mm is considered negative for metallo-carbapenemase production (20,21,24).

10. Immunochromatographic Method

This method is based on an immunological reaction and utilizes antibody-based immunochromatographic techniques to detect carbapenemase enzymes. Monoclonal antibodies developed against epidemiologically significant carbapenemases enable the specific detection of these enzymes. When the antigen present in the bacterial sample binds to the antibody immobilized on a chromatographic strip, an immune complex is formed, resulting in a color change that visually indicates the presence of the targeted antigen or antibody. This approach allows for the identification of carbapenemases such as IMP, KPC, VIM, NDM, and OXA-48-like enzymes. For testing, a single colony from a Mueller-Hinton agar plate is suspended in 150 µL of extraction buffer. Then, 100 µL of the resulting extract is applied to the sample well of the immunochromatographic test cassette. Test results are interpreted within 15 minutes based on the appearance of visible bands along the migration lines, indicating a positive result. In routine clinical microbiology practice, commercially available immunochromatographic assays designed for the rapid detection of NDM, IMP, OXA-48-like, KPC, and other major carbapenemases have been shown to be practical, easy to use, highly sensitive, and highly specific (18,19).

11. Spectrophotometric Analysis

This method employs spectrophotometry to detect and differentiate carbapenemase enzymes. Imipenem is used as the substrate, and hydrolytic activity is measured via UV spectrophotometric analysis at a wavelength of 297 nm. The primary parameter evaluated is the difference in the absorbance slope per minute between the hydrolysis reaction involving the bacterial culture extract and a control reaction containing only imipenem. This approach enables the detection and classification of various carbapenemase enzymes in Gram-negative bacteria based on their enzymatic activity. When compared to conventional PCR, the spectrophotometric method demonstrates 100% sensitivity and 98.5% specificity (20,25).

12. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be employed for the rapid detection of carbapenemase production. Two principal approaches are commonly used, both capable of identifying carbapenemase activity within approximately 60 minutes. In the hydrolysis-based approach, bacterial protein extracts are incubated with a carbapenem substrate, and the resulting degradation products are detected using MALDI-TOF MS. In the plasmid-associated peak approach, specific protein peaks associated with plasmids encoding known

carbapenemase genes are identified. Despite the advantages of speed and versatility, the detection of OXA-48-type enzymes remains challenging with MALDI-TOF MS, often leading to false-positive or false-negative results (26).

CONCLUSION

Public health is increasingly threatened by the rapid global spread of multidrug-resistant carbapenemase-producing bacteria (CRB). These pathogens are commonly isolated from patients with severe, life-threatening infections, particularly those who have been hospitalized for extended periods (27,28). Delays of even one hour in diagnosing CRB infections and initiating appropriate antibiotic treatment are associated with an approximately 8% increase in mortality and a rise in hospital stays from 5.1 days to 8.5 days (29). Recently, rapidly advancing molecular techniques have enabled the swift and accurate detection of carbapenemase enzymes. Methods such as polymerase chain reaction (PCR), whole genome sequencing, and microarrays are some of the molecular approaches employed. PCR-based techniques allow for the rapid identification of various carbapenemase genes across different classes, while whole genome sequencing provides comprehensive insights into the mechanisms of carbapenem resistance, bacterial genome characteristics, and the identification, classification, and analysis of carbapenem-resistant isolates, including plasmids. Additionally, microarray technology can be used to detect carbapenemase genes.

In this context, it is necessary to be more careful in order to prevent the spread and selection of CRB. It is possible that CRB may be spread to the community from hospital settings. As a result, detection of CRB by phenotypic or molecular methods would be beneficial to take infection control measures to prevent the spread of these bacteria to the community and hospital settings as well as epidemiological surveillance.

Declarations

Narrative review article based entirely on previously published data and do not involve any human or animal subjects, experiments, or patient-identifiable information. Therefore, ethical approval was not required for this type of article.

Authors' Contributions

YD: conceptualization, methodology, writing - review & editing, supervision, project administration.

ST: conceptualization, methodology, investigation, data curation, writing - review & editing. project administration.

AA: methodology, investigation, data curation, writing - original draft, visualization.

All authors have read and approved the final version of the manuscript. Each author meets the ICMJE authorship criteria and accepts responsibility for the integrity and accuracy of the work.

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