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PHENOTYPIC ASSAYS FOR DETECTION OF EXTENDED SPECTRUM B-LACTAMASES AND CARBAPENEMASES: A LABORATORY GUIDE FOR MICROBIOLOGISTS

Dickson Aruhomukama[1*]

[1]Department of Medical Microbiology, College of Health Sciences, Makerere University, P.O Box 7072, Kampala, Uganda.

***Correspondence:** Dickson Aruhomukama; dickson.aruhomukama@chs.mak.ac.ug

Abstract

This review aimed at defining and classifying extended spectrum β-lactamases, ESBLs and carbapenemases, summarizing the different phenotypic methods used to detect production of these enzymes in clinically significant gram negative bacteria and also describing the methods that discourse challenges mostly encountered during detection of these enzymes in microbiology laboratories with the purpose of formulating recommendations on best practice to screen for these enzymes. We conclude that the modified double disk synergy, MDDS is not only suitable for the confirmation of ESBL production after screening isolates with the cephalosporin/clavulanate combination disc diffusion or broth micro-dilution methods but also distinguishes ESBL production and over-expression of AmpC-derepressed mutants and as well serves as an indicator for AmpC screening. Furthermore, we suggest cefotaxime, ceftazidime and cefpodoxime (for testing using a single drug) as indicator antibiotics of choice for ESBL detection. The MDDS and cefoxitin/AmpC inhibitor combination disc method, using cloxacillin and phenylboronic acid can be used as screening tests for AmpC production and either the AmpC disc test, the disc approximation test or the modified three dimensional extract test as confirmatory tests for AmpC production. We also suggest that confirmation of carbapenemase production be done with the modified hodge test, using *Klebsiella pneumoniae* ATCC 700603 as the indicator organism or the modified carbapenem inactivation method. However, to differentiate between the different classes of carbapenemases, boronic acid and EDTA based methods (double-disk synergy tests and

combined-disk tests) using imipenem, meropenem and ertapenem, in combination with 3-aminophenylboronic acid and ethylene diamine tetra acetic acid be used.

Keywords: Extended spectrum β-lactamases, AmpC Carbapenemases, Carbapenemases, Classification, Phenotypic Detection, Clinically significant bacteria

1. Introduction

Infections involving gram negative Extended Spectrum β-lactamase and Carbapenemase producing bacteria in Health-Care settings continue to increase [1-6]. The treatment of these infections remains a serious public health concern, as even the most likely non-fatal infections become fatal when they involve particularly these antibiotic resistant bacteria [1, 2, 5-8]. Extended Spectrum β-lactamase, ESBL and Carbapenemase enzymes hydrolyze and hence inactivate β-lactam antibiotics resulting in β-lactam resistance in bacteria that are producers of these enzymes [6, 9-13]. Increased β-lactam resistance in bacteria has been attributed to their continuous exposure to β-lactam antibiotics, a factor that has contributed to increased uninterrupted production and mutation of β-lactamases in bacteria [6, 14]. Despite the Clinical Laboratory Standards Institute, CLSI recommendations in regards detection of ESBL and Carbapenemase production in clinically significant bacteria, the occurrence of ESBL and Carbapenemase producing bacteria remains extremely difficult to resolve, this is attributed to various reasons notably; i) difficulty in detecting ESBL and Carbapenemase production in these bacteria; ii) inconsistences in the reporting of results in regards ESBL and Carbapenemase production and iii) co-existence of β-lactamase enzymes in the bacteria [6, 7, 15, 16]. These therefore set precedence for more accurate and practical phenotypic approaches to the detection of ESBLs and Carbapenemases in bacteria, as these are beneficial in providing substantial information in regards ESBLs and Carbapenemases in these bacteria. Additionally, these phenotypic approaches are also vital in reducing the need to apply the more expensive molecular techniques for detection of production of these enzymes in bacteria. Hence, this review defines and classifies ESBLs and Carbapenemases and summarizes the different phenotypic methods used in the

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detection of ESBLs and Carbapenemases in bacteria. Noteworthy, also described herein are methods that discourse challenges in detection of ESBLs and Carbapenemases.

2. Extended Spectrum β-lactamases: Definition and Classification

Extended Spectrum β-lactamases, ESBLs are enzymes that break down and thus inactivate antibiotics known as β-lactams [6, 12, 13, 17]. Additionally, ESBLs are characterized by their susceptibility to inhibition by β-lactam inhibitors particularly; clavulanic acid, tazobactam and sulbactam [6, 12, 13]. β-lactamases are classified according to two schemes, these are; i) the Bush-Jacoby-Medeiros functional classification and ii) the Ambler molecular classification [6, 12, 13]. The basis of the Bush-Jacoby-Medeiros functional classification is the functional properties of the β-lactamase enzymes principally, their inhibitor and substrate profiles [12, 13]. The Ambler molecular classification however is based on the protein homology of the β-lactamase enzymes [12, 13]. The Ambler molecular classification further classifies β-lactamase enzymes into four classes namely; class A, C and D also known as the serine β-lactamases and class B β-lactamases also known as the zinc or Metallo β-lactamases [12, 13].

The CTX-M type forms the most common genetic variant of ESBLs, this family of β-lactamases specially hydrolyze cefotaxime over ceftazidime and are found exclusively in the functional group 2 [6, 12, 18-21]. Also, unique to these β-lactamases is their susceptibility to inhibition by the β-lactam inhibitor tazobactam as compared to inhibition by the other β-lactam inhibitors, clavulanic acid and sulbactam [6, 20, 22]. The Extended Spectrum of activity of the CTX-M β-lactamases is attributed to the serine residue present at position 237 [6, 21]. The CTX-M β-lactamases can be divided into five groups namely; the CTX-M group 1, 2, 8, 9 and 25 basing on their amino acid sequences [6, 12, 23]. unlike the other β-lactamase enzymes, the CTX-M enzymes are acquired and disseminated in bacteria via mobile genetic elements particularly conjugative plasmids and transposons in horizontal gene transfer processes [6].

The TEM type β-lactamases form another β-lactamase family; β-lactamases belonging to the TEM type, TEM-1 a variant of TEM hydrolyze penicillins and first generation cephalosporins [6]. The TEM-3 β-lactamases, which are another variant of TEM β-lactamases hydrolyze Extended Spectrum Cephalosporins [6, 24, 25]. In addition to these

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families is the SHV type β-lactamases, SHV-1 β-lactamases, a variant of the SHV hydrolyze broad spectrum penicillins, these include ampicillin, tigecycline and piperacillin [25, 26]; the OXA type β-lactamases, these β-lactamases exhibit the ability to hydrolyze oxacillin, are predominantly present in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* however also occur in other gram negative bacteria [6, 12, 27, 28]; the PER type β-lactamases, unique about these β-lactamases is their efficient hydrolysis of penicillins, cephalosporins and their susceptibility to clavulanic acid inhibition [6, 12, 13]; the GES type β-lactamases, these β-lactamases hydrolyze penicillins and Extended Spectrum Cephalosporins, but not cephamycins or carbapenems, these are also inhibited by β-lactamase inhibitors [6, 12, 13]; other described β-lactamase families include among others the VEB-1, BES-1, CME-1 and SFO-1, enzymes belonging to these families have mostly been found to exhibit β-lactamase activity [6, 29, 30].

3. Carbapenemases: Definition and Classification

Carbapenemases are enzymes that break down and thus inactivate antibiotics known as carbapenems, these enzymes represent the most versatile family of β-lactamases and are uniquely characterized by their broad spectrum of activity as compared to other β-lactam hydrolyzing enzymes [12, 13, 31-35]. Carbapenemases are classified into two major groups basing on the nature of their active sites, these groups are the serine carbapenemases belonging to the class A penicillinases and class D oxacillinases and the Metallo β-lactamases belonging to the class B carbapenemases which contain one or more zinc atoms at their active sites a characteristic future that allows them to hydrolyze the bicyclic β-lactam ring [12, 13, 31-35].

Class A Carbapenemases, also known as the class A serine carbapenemases, when present in bacteria confer a characteristic reduced susceptibility to imipenem [31, 35]. Class A carbapenemases include; IMI/NMC, KPC, GES and SME enzymes [31, 35]. These enzymes require an active serine at position 70 for their hydrolytic activity in the Ambler numbering system for class A β-lactamases, hydrolyze carbapenems, cephalosporins, penicillins and aztreonam, are inhibited by clavulanic acid and tazobactam [31, 35, 36]. IMI, NMC and SME are chromosomally encoded enzymes whereas GES and KPC enzymes are plasmid encoded [31, 35].

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Class B carbapenemases, also known as class B Metallo β-lactamases hydrolytic activity is dependent on the interaction of the β-lactams with zinc ions in the active sites of these enzymes, these interactions result in the distinctive trait of their inhibition by EDTA which is a chelator of zinc and other divalent ions [31, 33, 35, 37, 38]. These enzymes ably hydrolyze carbapenems, are resistant to commercially available β-lactamase inhibitors, are susceptible to inhibition by metal ion chelators and have a relatively broad substrate profile that includes cephalosporins and penicillins [31, 33, 35, 37, 38]. Also, characteristic to the class B carbapenemases is their inability to hydrolyze aztreonam [31, 33, 35, 37, 38]. These enzymes include; GIM, IMP, VIM, NDM and SIM [31, 35, 38]. Additionally, these enzymes mostly occur in integron structures and are mostly disseminated via mobile genetic elements particularly conjugative plasmids and transposons in horizontal gene transfer [31, 35, 38, 39].

Class D carbapenemases, also known as class D Metallo β-lactamases or the OXA β-lactamases are uniquely characterized by their association with plasmids, hence are mostly plasmid encoded, although may also be chromosomally encoded [31, 35, 40-42]. Additionally, these enzymes hydrolyze oxacillin and cloxacillin and are described as penicillinases, are poorly inhibited by clavulanic acid and EDTA and have large amounts of variability in their amino acid sequences [12, 31, 35, 42, 43].

4. Detection of Extended Spectrum β-lactamase Production

Different phenotypic methods, disk diffusion-based methods and broth micro-dilution methods have been described previously to ably detect the production of ESBLs in clinically significant bacteria [7]. However, currently some of these methods have become increasingly unreliable, this is attributed to; i) the co-existence of different classes of β-lactamases in bacteria [44-46], ii) difficulty in recognition of ESBL production due to the over-expression of AmpC β-lactamases [44-46], iii) potential masking of ESBL production by AmpC producing bacteria which serve as reservoirs of ESBLs [44-46], and also iv) the general inability of most microbiology laboratories to ably perform testing for the detection of ESBL production in bacteria [47-50], all of which affect testing and consequently result in inappropriate antimicrobial therapy [44, 51]. In the testing for ESBL production, other

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factors including inoculum density [52-54] and the distance between the discs [7, 53-56] being tested have been documented to influence the outcomes of the testing.

Testing for ESBL production in bacteria is a two stage process that involves; screening and confirmation of production of ESBLs in potential producing bacterial isolates [57, 58]. Screening and confirmation of ESBL production follows testing bacteria against array of antibiotics namely; cefpodoxime, ceftazidime, ceftriaxone, cefotaxime and aztreonam and identifying specific zone diameters indicative of potential ESBL production [57, 58]. Testing with greater than one of the antibiotics is recommended to improve the sensitivity of ESBL detection [57, 58]. It is however recommended to use cefotaxime, due to its consistent susceptibility to CTX-M and ceftazidime, due its consistent susceptibility to TEM and SHV [57, 59-61]. Cefpodoxime however should be used when testing is to be performed using a single drug [57, 59-61]. Reduced susceptibility to any of the agents as specified by the Clinical Laboratory Standards Institute, CLSI is a qualification of ESBL testing and necessitates phenotypic confirmatory testing to confirm diagnosis [58]. Phenotypic confirmation of ESBL production can be achieved using array of methods, including the use of the cephalosporin/clavulanate combination disc diffusion or broth micro-dilution method [57, 58], the double disk synergy (DDS) method [6, 56, 57, 62-64] and a modification of this method, the modified double disc synergy (MDDS) method, which confirms ESBL production and also differentiates ESBL production and over-expression of AmpC-derepressed mutants [6, 56, 57, 62-64].

In the cephalosporin/clavulanate combination disc diffusion and broth micro-dilution methods; testing is performed following the CLSI guidelines for susceptibility testing using both cefotaxime (30µg / 0.25-64 µg/ml) and ceftazidime (30µg / 0.25-128 µg/ml) alone or in combination with clavulanic acid (10 µg or 0.25/4-64/4 µg/ml / 0.25/4-128/4 µg/ml) [58]. Reporting of results is done after 16-20 hours of incubation in ambient air at 35^0C ± 2^0C as follows; in the disc diffusion method, an increase by ≥ 5mm in the zone of inhibition from either antimicrobial agent tested in combination with the clavulanate versus the zone diameter of the agent when tested alone is indicative of ESBL production [58]. In the broth micro-dilution, a ≥ 3 two fold concentration decrease in the Minimum Inhibitory

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Concentration, MIC for either antimicrobial agent tested in combination with clavulanate versus the MIC of the agent when tested alone is indicative of ESBL production [58].

In the DDS, testing is performed following the CLSI guidelines for susceptibility testing using cefotaxime (30µg) and/or ceftriaxone (30µg) and/or ceftazidime (30µg) and/or aztreonam (30µg) and a disc of amoxicillin-clavulanic acid (20/10µg) at a distance of 30mm center to center; However, narrower distances between the discs have been associated with increased sensitivity of the test and have been previously described [7, 56, 65-67]. Reporting of the results is done after incubation in ambient air at 35^0C ± 2 ^{0}C for 16-20 hours as follows; a decreased susceptibility to the antibiotic disc used combined with a clear-cut enhancement of the inhibition zone of same antibiotic disc in front of the clavulanic acid containing disc, often resulting in a characteristic shape-zone referred to as “champagne-cork” or key hole is indicative of ESBL production whereas absence of the same is indicative of non ESBL production [7, 56, 65-67].

In the MDDS, the following antibiotic discs are used; amoxicillin-clavulanic acid (20/10 µg) or piperacillin tazobactam (100/10µg) along two third generation cephalosporins, ceftazidime (30µg) and cefotaxime (30µg); a fourth generation cephalosporin, cefepime (30µg) used as a replacement for ceftriaxone (30µg) and aztreonam (30µg) [7, 63-65]. This test is performed following the CLSI guidelines for susceptibility testing as follows; cefotaxime (30µg) is placed 20 mm from the amoxicillin-clavulanic acid (20/10 µg), aztreonam (30µg) disc is placed at 25 mm, ceftazidime (30µg) at 30 mm and cefepime (30µg) at 30 mm, incorporation of this modification into a gram negative template involves placing a piperacillin tazobactam (100/10µg) disc 25 mm from cefepime (30µg) [7, 63-65]. Reporting of results is done after 16-20 hours of incubation in ambient air at 35^0C ± 2^0C as follows; Strains are divided into three groups basing on interpretation with MDDS and their susceptibilities to cefotaxime and ceftazidime, Group 1 (Wild type) includes ESBL non producers sensitive to cefotaxime and ceftazidime; Group 2 (derepressed mutants) includes ESBL non producers resistant to cefotaxime and ceftazidime; Group 3 (ESBL producers) includes ESBL producers sensitive, intermediate or resistant to cefotaxime and ceftazidime [7, 63-65]. Worth mentioning, reporting of results is done

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following CLSI guidelines as follows; isolates with a positive confirmatory test are reported as resistant to all penicillins, cephalosporins and aztreonam except for cephamycins and cefoxitin irrespective of the MIC of the particular cephalosporin [7, 63-65].

5. Detection of AmpC β-lactamase production

AmpC β-lactamases are enzymes that belong to class C in the Ambler structural classification or group 1 of the Bush's functional classification [13, 46, 68, 69]. AmpC β-lactamases may be chromosomally encoded, chromosomal AmpCs [69-71] or plasmid mediated, plasmid-mediated AmpCs [68, 69, 71, 72]. Both types of AmpC β-lactamases have been studied and documented to occur in clinically significant bacteria [45, 68, 71, 73]. These enzymes confer resistance to β-lactam antibiotics notably penicillins, third generation cephalosporins, cephamycins (cefoxitin and cefotetan) and monobactams notably aztreonam [45, 69, 71, 74]. Bacterial producers of these enzymes are however susceptible to fourth generation cephalosporins (cefepime and cefpirome) [46, 69] , are also poorly inhibited by clavulanic acid [46, 69], hence are resistant to β-lactam/β-lactamase-inhibitor combinations and give a positive and negative test during ESBL screening and confirmation respectively [68]. These features form the basis of screening and confirmation of AmpC β-lactamase production in bacteria [68, 69, 75, 76].

Screening for the production of AmpC β-lactamases in bacteria is done using cefoxitin (30µg) and cefoxitin (30µg) supplemented with an AmpC inhibitor either 200µg of cloxacillin or 400µg of phenylboronic acid or using cefpodoxime (10µg) and cefpodoxime (10 µg) supplemented with an AmpC inhibitor, either 200µg of Cloxacillin or 400µg of Phenylboronic acid [76-80]. Reporting of results is done after 16-20 hours of incubation in ambient air at 35^0C ± 2^0C as follows; an increase in the zone of inhibition by ≥ 4mm in the cefoxitin or cefpodoxime discs supplemented with either of the AmpC inhibitors as compared to the unsupplemented disc of either cefoxitin or cefpodoxime is indicative of AmpC production [76-79, 81].

Another similar method with minor modifications that can be used to screen for the production of AmpC β-lactamases in bacteria [65] is performed by making a lawn culture of *E.coli* ATCC 25922 adjusted to a 0.5 McFarland standard on Mueller Hinton agar, MHA

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following the CLSI guidelines for standard disc diffusion testing [58, 65]. Following this, a cefoxitin (30µg) disc is placed on the surface of the inoculated MHA plate [65]. Then a sterile plain 6mm paper disc initially inoculated with several colonies of the test organism is placed besides the cefoxitin disc almost making contact with it [65]. Reporting of results is done after 16-20 hours of incubation in ambient air at $35^0C \pm 2^0C$ as follows; indentation or a flattening of the inhibition zone is indicative of enzyme inactivation of the cefoxitin hence AmpC production whereas the absence of distortion is indicative of non-significant enzyme inactivation of the cefoxitin hence non AmpC production [65].

Phenotypic confirmation of AmpC production is done by performing either the following tests; i) the AmpC disc test [45, 68], ii) the disc approximation test [64, 78, 80, 81] or iii) the modified three dimensional extract test [64, 80, 82-84].

In the AmpC disc test, a plate of MHA is inoculated with a lawn culture of *E.coli* ATCC 25922 [45, 68]. Following this, sterile 6mm paper discs infused with 20µl of 1:1 Tris-EDTA : saline solution or not infused with Tris-EDTA are moistened with 20µl of sterile saline and inoculated with several colonies of the test organisms are aseptically placed onto the MHA next to the cefoxitin disc (almost touching) [45, 68]. Reporting of results is done after 16-20 hours of incubation in ambient air at $35^0C \pm 2^0C$ as follows; flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disc is indicative of AmpC production whereas absence of flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disc is indicative of AmpC non production [45, 68].

The disc approximation method is performed using *E.coli* ATCC 25922 and inducing substrates; imipenem (10µg), cefoxitin (30µg) and amoxicillin clavulanic acid (20/10µg) discs [64, 68, 78, 80, 81] as follows, a 0.5 McFarland bacterial suspension of *E.coli* ATCC 25922 is inoculated onto a plate of MHA. Then onto the MHA plate is placed a ceftazidime (30µg) or cefotaxime (30µg) or phenyl-boronic acid (400µg) (in the center) and at a distance of 20mm from this disc, discs of imipenem (10µg), amoxicillin/clavulanic acid (20/10µg). Reporting of results is done after 16-20 hours of incubation in ambient air at $35^0C \pm 2^0C$ as follows; any obvious blunting or flattening of the zone of inhibition between the ceftazidime or cefotaxime or phenyl-boronic acid disc and the inducing substrates

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(imipenem , cefoxitin and amoxicillin/clavulanic acid) is indicative of AmpC production [64, 68, 78, 80, 81].

In the three dimensional extract test, 50mls of a bacterial suspension adjusted to a 0.5 McFarland turbidity standard prepared from an overnight culture on blood agar is inoculated into 12mls of tryptic soy broth [77, 80, 82, 83, 85]. Following this, the culture is grown at 35^0C-37^0C for 4 hrs [77, 80, 82, 83, 85]. The bacterial cells are centrifuged and the crude enzyme prepared by freezing-thawing the bacterial cell pellets five times [77, 80, 82, 83, 85]. The surface of the MHA plate to be used is then inoculated with *E.coli* ATCC 25922 or *E.coli* ATCC 11775 following the CLSI guidelines for the standard diffusion method [77, 80, 82, 83, 85]. A cefoxitin (30µg) disc is then aseptically placed on the inoculated MHA plate [77, 80, 82, 83, 85]. A linear slit (3mm) beginning 3mm from the edge of the disc is then cut in the agar in an outward direction using a sterile scalpel blade and a small circular well is made at the other end of the slit near the disc [80, 82, 83]. Then, 30-40 µl of enzyme preparation is dispensed into the small circular well using a pipette at a 10 µl increments, beginning near the disc and moving outwards with care being taken not to over fill the slits [77, 80, 82, 83, 85]. The inoculated media is then incubated at 35^0C-37^0C for 18-24hrs after allowing the liquid to dry in an upright position [77, 80, 82, 83, 85]. Interpretation of the results is done following incubation as follows; three results may be reported, these are isolates showing clear distortion in the zone of inhibition of cefoxitin are reported as AmpC producers, isolates with no clear distortion as AmpC non producers and isolates with minimal distortion as intermediate strains [77, 82, 83, 85].

6. Phenotypic Detection of Carbapenemase Production

Phenotypic detection of carbapenemase production in bacteria is achieved by performing either; i) the modified Hodge test (MHT) [38, 84, 86-91] or ii) the modified carbapenem inactivation method (mCIM), a modification of the carbapenem inactivation method (CIM) [92-94]. These methods have not only been documented to be simple and cost effective but have also been documented to have high sensitivity [38, 84, 86-90]. Despite the high sensitivity of MHT, it has been linked to a high frequency of false-positive results

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especially in carbapenem resistant Enterobacteriaceae that are producers of ESBLs and AmpC β-lactamases and low sensitivity in regards the detection of NDM-1 producing bacteria [95, 96]. In addition to this, the use of *E.coli* ATCC 25922 has also been implicated with low sensitivity, specificity and repeatability of MHT [91, 96]. However, replacing *E.coli* ATCC 25922 with *Klebsiella pneumoniae* ATCC 700603 has been shown to provide high sensitivity, specificity and repeatability of the test [91]. MHT has also been associated with inability to discriminate between the different classes of carbapenemases (i.e. *Klebsiella pneumoniae* carbapenemase, Metallo β-lactamase and Oxacillinases) and also difficulty in interpretation of results has been reported [97]. Despite these, MHT remains a phenotypic reference method for confirmation of carbapenemase production [97].

In MHT, a 0.5 McFarland dilution of *Klebsiella pneumoniae* ATCC 700603, an indicator strain in 5 ml of sterile saline or nutrient broth is prepared [38, 86-90]. Following this, a 1:10 dilution of the indicator strain is streaked as a lawn onto an MHA plate using a sterile swab [38, 86-90]. Then, a meropenem (10µg) or ertapenem (10µg) or imipenem (10µg) disc is placed at the center of the test area on the MHA plate [38, 86-90, 98]. The test organism(s), positive control and negative control are then streaked in straight lines from the edge of the disc to the edge of the plate [38, 86-90]. *Klebsiella pneumoniae* ATCC BAA-1705 and *Klebsiella pneumoniae* ATCC-1706 are used as positive and negative controls respectively, these are ran with each batch of the test [38, 86-90]. Reporting of results is done after 16-20 hours of incubation in ambient air at $35^0C \pm 2^0C$ as follows; the test organism(s) is positive for MHT when a clover leaf-like indentation of the *Klebsiella pneumoniae* ATCC 700603 growing along the test organism growth streak within the disc diffusion zone is observed [38, 86-90]. MHT negative organism(s) show no growth of the *Klebsiella pneumoniae* ATCC 700603 along the test organism growth streak within the disk diffusion [38, 86-90].

In the mCIM method, a sterile inoculating loop is used to add 1µl of the test organism to a tube containing 2 mls of Tryptic soy broth [92, 93]. Then the bacterial suspension is mixed by vortexing for 15 seconds [92, 93]. This is then followed by aseptically adding a commercially available meropenem disk (10µg) to the bacterial suspension [92, 93].

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Incubation of the bacterial suspension containing the Meropenem disk is then done at 35^0C-37^0C in ambient air for 4hrs ± 15 minutes [92, 93]. Before the completion of the incubation time, a suspension of the mCIM indicator organism, a carbapenem susceptible *Klebsiella pneumoniae* ATCC 25922 with a turbidity equivalent to a 0.5 McFarland is prepared and the surface of an MHA plate is inoculated following the standard disk diffusion method as recommended by the CLSI and placing onto the agar plate the meropenem disc removed aseptically from the Tryptic soy Broth suspension after dragging it on the walls of the tube to drain off the excess fluid [92, 93]. Incubation of the plate is then done at 35^0C ± 2^0C in ambient air for 18-24hrs [92, 93]. The test is interpreted after measuring the inhibition zone diameter of the meropenem disk as follows; inhibition zone diameters of 6-10 mm are reported as positive for carbapenemase production, 11-19 mm as intermediate results and ≥ 20 mm as negative for carbapenemase production [92, 93]. Another interpretation criteria has been previously described, in this criteria, uninhibited growth of the indicator strain is indicative of carbapenemase production whereas inhibition zone diameters of ≥ 20 mm are indicative of non-carbapenemase production [92, 93, 99].

To differentiate between the different classes of carbapenemases, novel boronic acid and EDTA based methods (double-disk synergy tests and combined-disk tests) using imipenem, meropenem and ertapenem, in combination with 3-aminophenylboronic acid and ethylene diamine tetra acetic acid have been documented as confirmatory tests [100-102].

Detection of Metallo β-lactamase production in bacteria can be achieved by using two methods, namely; the imipenem-ethylene diamine tetra acetic acid method and another double disc synergy method that involves the use of a ceftazidime (30µg) disc and a ceftazidime (30µg)-2-Mercaptopropionic acid disc [101, 102].

In the imipenem-EDTA method, testing is performed by inoculating the test organism(s) onto MHA for the standard diffusion method as recommended by the CLSI [101]. This is then followed by adding to the MHA plates two commercially available imipenem (10µg) discs, one of the discs un-supplemented with EDTA and another supplemented with

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EDTA at a distance of 15 mm center to center; However narrower distances have been associated with high test sensitivity and have been previously described [101-103]. The supplementation of the imipenem disc is done by adding 10 µl of 0.5M EDTA to obtain the desired concentration of 1000 µg [87, 89, 101]. A concentration of 750 µg can also be used in the testing [101]. The 0.5M EDTA stock solution is prepared by dissolving 186.1g of disodium EDTA. $2H_2O$ in 1000 ml of distilled water and adjusting the solution PH to 8.0 using NaOH [101]. After preparation of the mixture, it is sterilized by autoclaving [101]. Incubation of the MHA plates is done at $35^0C \pm 2^0C$ for 18-24hrs in ambient air [87, 89, 101]. These tests are interpreted after measuring the inhibition zone diameters around the imipenem disc not supplemented with EDTA and the imipenem discs supplemented with EDTA as follows; the inhibition zone diameters of the imipenem disc supplemented with EDTA are each compared to the inhibition zone diameter of the un-supplemented imipenem disc and an increase in inhibition zone diameter of ≥ 5 mm in the EDTA-supplemented disc is interpreted as positive for Metallo β-lactamase production [87, 89, 101]. Noteworthy, the Supplemented discs prepared maybe kept at 4^0C or at -20^0c in airtight vials without desiccant and under these conditions, these remain stable for 12 and 16 weeks respectively [101].

In the ceftazidime disc method, a ceftazidime disc and another supplemented with 2-Mercaptopropionic acid are used [101]. In this method, the procedure and interpretation is done as documented in the previous method, the imipenem-EDTA [101].

The detection and differentiation of *Klebsiella pneumoniae* carbapenemase production and Metallo β-lactamase production can be achieved using a phenotypic algorithm that involves the use of three combined-disc tests [16, 104-107]. These tests consist of i) Ertapenem alone and ertapenem supplemented with phenylboronic acid (PBA); ii) ertapenem alone and ertapenem supplemented with ethylene diamine tetra acetic acid (EDTA); iii) both tests [16, 104-107]. Additionally, these tests can be used to detect co-production of both carbapenemases in bacteria [16, 107]. In these tests, the concentrations of the PBA and EDTA used are 400 µg of PBA and 292 µg of EDTA [16, 106]. The stock solution of PBA is prepared by dissolving PBA in DMSO at a

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concentration of 20 mg/mL [16, 106, 108]. From this solution, 20 µl (Containing 400 µg of PBA) are then added onto commercially available meropenem discs to obtain the PBA supplemented meropenem discs [16, 106, 108]. Similarly, the stock solution of EDTA is prepared by dissolving anhydrous EDTA (Sigma-Aldrich) in distilled water at a concentration of 0.1M [16, 106, 108]. From this solution, 10 µl (containing 292 µg of EDTA) are then added onto commercially available meropenem discs to obtain the EDTA supplemented meropenem discs [16, 106, 108]. Following disc preparation, the discs are dried and used within 60 minutes [16]. Testing is performed by inoculating MHA for the standard diffusion method as recommended by the CLSI and placing onto agar plates a disc of meropenem that is not supplemented with any of the inhibitors, (PBA and EDTA) and three discs of meropenem supplemented with 400 µg of PBA, 292 µg of EDTA or both 400 µg of PBA and 292 µg of EDTA [16, 106]. Incubation of the agar plates is done at 35^0C ± 2^0C for 18-24hrs [16, 106, 107]. These tests are interpreted after measuring the inhibition zone diameters around the meropenem disc not supplemented with any inhibitor and the meropenem discs supplemented with either PBA, EDTA or both PBA and EDTA as follows; the inhibition zone diameters of the meropenem disc supplemented with either PBA, EDTA or both PBA and EDTA are each compared to the inhibition zone diameter of the un-supplemented meropenem disc [16, 106]. An increase in the inhibition zone diameter in any of the supplemented meropenem discs by ≥ 5 mm is considered positive for *Klebsiella pneumoniae* carbapenemase production [16, 106]. Bacterial isolates are considered positive for KPC and MBL co-production only when, the inhibition zone diameter around the meropenem disc supplemented with both PBA and EDTA is ≥ 5 mm compared to the inhibition zone diameter of the meropenem disc not supplemented with any inhibitor while the inhibition zone diameters of the meropenem disc supplemented with PBA and that supplemented with EDTA each are ≤ 5 mm compared to the meropenem disc not supplemented with any of the inhibitors [16, 106].

Boronic acid based tests can also be used to detect ESBL production in KPC positive isolates [106, 108]. In this testing, combined disc tests are used, namely; i) cefotaxime (CTX) with or without clavulanic acid (CA) supplemented with boronic acid (BA) and ii) ceftazidime (CAZ) with or without clavulanic acid supplemented with boronic acid [106,

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108]. The stock solution of BA is prepared by dissolving phenylboronic acid (PBA) in dimethyl sulfoxide and water at a concentration of 20 mg/ml. From the stock solution, 20 µl are added onto commercially available discs containing CTX (30 µg) or CAZ (30 µg) with or without CA (10 µg). Addition of this volume to either of the discs makes the final volume on the discs 400 µg [106, 108]. Following this, discs are dried and used within 60 minutes [106]. Testing is performed by inoculating MHA for the standard diffusion method as recommended by the CLSI and placing onto agar plates a disc of CAZ or CTX with or without CA that is not supplemented with BA and two discs of CAZ or CTX with or without CA supplemented with 400 µg of BA [16, 106]. These tests are interpreted after measuring the inhibition zone diameters around the CAZ or CTX disc with or without CA not supplemented with BA and the CTX or CAZ disc with or without CA supplemented with BA as follows; the inhibition zone diameters of the CTX or CAZ disc with or without CA disc supplemented with BA are each compared to the inhibition zone diameter of the un-supplemented CTX or CAZ disc with or without CA [106]. An increase in the inhibition zone diameter in any of the supplemented CAZ or CTX discs with or without CA by ≥ 5 mm is considered positive for ESBL production [106].

7. Conclusion

Different phenotypic screening and confirmation methods have overtime been developed and evaluated for the detection of extended spectrum β-lactamases and carbapenemases in clinically significant gram negative bacteria. In this review we summarize approved guidelines, the different methods described in literature for the detection of these enzymes and also methods that discourse challenges mostly encountered during detection of these enzymes in clinical microbiology laboratories. In this section, we provide recommendations on the best and/or acceptable methods that might be useful in formulating efficient approaches for optimizing the detection of extended spectrum β-lactamases and carbapenemases in clinically significant bacteria in microbiology laboratories.

It is recommended that screening for ESBL production in bacteria be done using the cephalosporin/clavulanate combination disc diffusion and broth micro-dilution methods using cefotaxime, ceftazidime and cefpodoxime (for testing using a single drug). Testing

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with more than one antibiotic is also recommended as it increases sensitivity of the testing. The modified double disc synergy test using amoxicillin-clavulanic acid (20/10 µg) or piperacillin tazobactam (100/10µg) along two third generation cephalosporins, ceftazidime (30µg) and cefotaxime (30µg); a fourth generation cephalosporin, cefepime (30µg) used as a replacement for ceftriaxone (30µg) and aztreonam (30µg) remains the preferred method for confirmation of ESBL production, this not only confirms ESBL production but also ably distinguishes ESBL production and over-expression of AmpC derepressed mutants. Additionally, the method also sets precedence for AmpC screening in bacteria.

In the detection of AmpC production, screening of isolates may be done using the MDDS and the cefoxitin/AmpC inhibitor combination disc method, using cloxacillin and phenylboronic acid, confirmation of AmpC production can then be done by using either the AmpC disc test, the disc approximation test or the modified three dimensional extract test.

It is also recommended that confirmation of carbapenemase production be done with the modified hodge test, using *Klebsiella pneumoniae* ATCC 700603 as the indicator organism or the modified carbapenem inactivation method. However, to differentiate between the different classes of carbapenemases, boronic acid and EDTA based methods (double-disk synergy tests and combined-disk tests) using imipenem, meropenem and ertapenem, in combination with 3-aminophenylboronic acid and ethylene diamine tetra acetic acid can be used.

Detection of Metallo β-lactamase production in bacteria can be achieved by using two methods, namely; the imipenem-ethylene diamine tetra acetic acid method and another double disc synergy method that involves the use of a ceftazidime (30µg) disc and a ceftazidime (30µg)-2-Mercaptopropionic acid disc.

Detection and differentiation of *Klebsiella pneumoniae* carbapenemase production and Metallo β-lactamase production can be achieved using a phenotypic algorithm that involves the use of three combined-disc tests, these tests consist of i) Ertapenem alone and ertapenem supplemented with phenylboronic acid (PBA); ii) Ertapenem alone and ertapenem supplemented with ethylene diamine tetra acetic acid (EDTA); iii) both tests.

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8. Declarations

8.1 Ethical approval and consent to participate

Not applicable

8.2 Consent for publication

Not applicable

8.3 Conflicting interests

Not applicable

8.4 Author contributions

DA, Drafted, Edited and Reviewed Original Manuscript, Drafted and Reviewed Final Manuscript

8.5 Acknowledgements

Not applicable

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