Review of phenotypic assays for detection of extended-spectrum β-lactamases and carbapenemases: a microbiology laboratory bench guide

Dickson Aruhomukama

Department of Medical Microbiology, College of Health Sciences, Makerere University, Kampala, Uganda.

Abstract
Background: Infections caused by gram-negative antibiotic-resistant bacteria continue to increase. Despite recommendations by the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) with regards to detection of antibiotic degrading enzymes secreted by these bacteria, the true prevalence of extended-spectrum β-lactamase (ESBL) and carbapenemase producers remains a difficult task to resolve. Describing of previously designed phenotypic detection assays for ESBLs and carbapenemases in a single document avails a summary that allows for multiple testing which increases the sensitivity and specificity of detection.

Methods and aims: This review, therefore, defined and classified ESBLs and carbapenemases, and also briefly described how the several previously designed phenotypic detection assays for the same should be performed.

Conclusion: Extended-spectrum β-lactamase and carbapenemase detection assays, once performed correctly, can precisely discriminate between bacteria producing these enzymes and those with other mechanisms of resistance to β-lactam antibiotics.

Keywords: Extended-spectrum β-lactamases; carbapenemases; phenotypic detection.

DOI: https://dx.doi.org/10.4314/ahs.v20i3.11

Cite as: Aruhomukama D. Review of phenotypic assays for detection of extended-spectrum β-lactamases and carbapenemases: a microbiology laboratory bench guide. Afri Health Sci. 2020;20(3): 1090-1108. https://dx.doi.org/10.4314/ahs.v20i3.11

Introduction
Infections involving extended-spectrum β-lactamase and carbapenemase producing gram-negative bacteria continue to increase in health-care settings\(^1,2\). These infections remain difficult to treat and are a serious public health concern\(^1,2\). This is because, even the most likely non-fatal infections become fatal when they involve these antibiotic resistant bacteria\(^2\). Extended-spectrum β-lactamase and carbapenemase enzymes hydrolyze and hence inactivate β-lactam antibiotics resulting in β-lactam resistance in bacteria that are producers of these enzymes\(^5\). Increased β-lactam resistance in bacteria is due to their continuous exposure to β-lactam antibiotics, this has contributed to increased unimpeded production and mutation of β-lactamases in bacteria\(^1,4\). The true prevalence of ESBL and carbapenemase producing bacteria remains a difficult task to resolve despite the recommendations provided by CLSI and EUCAST for detection of these enzymes\(^1,5-8\). This is due to among others, incompetence of most clinical microbiology laboratory staff to correctly perform the phenotypic detection assays and report production of these enzymes\(^1,5-7\). This suggests that improvements in competence of clinical microbiology laboratory staff to correctly perform the assays and report production of these enzymes are needed\(^5,6,7\). The correct performance of these assays is beneficial in distinguishing between bacteria producing β-lactamases and those with other mechanisms of resistance to β-lactam antibiotics. Additionally, correct performance of these assays is vital in reducing the need to apply the more expensive molecular techniques used in detecting production of these enzymes. Hence, this review defines and classifies ESBLs and carbapenemases, and summarizes the several previously designed phenotypic assays used to detect production of these enzymes in gram-negative antibiotic resistant bacteria.

Corresponding author:
Dickson Aruhomukama,
Department of Medical Microbiology,
College of Health Sciences,
Makerere University, Kampala, Uganda.
Tel: +256706511287
Email: dickson.aruhomukama@chss.mak.ac.ug

Extended-spectrum β-lactamases: Definition and classification

© 2020 Aruhomukama D. Licensee African Health Sciences. This is an Open Access article distributed under the terms of the Creative commons Attribution License (https://creativecommons.org/licenses/BY/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Extended-spectrum β-lactamas es are enzymes that break down and thus inactivate β-lactam antibiotics\textsuperscript{1,3,9,10}. Additionally, ESBLs are characterized by their susceptibility to inhibition by β-lactam inhibitors particularly; clavulanic acid (CA), tazobactam and sulbactam\textsuperscript{1,3,9}. β-lactamas es are classified according to two schemes, these are; i) the Bush-Jacoby-Medeiros functional classification and ii) the Ambler molecular classification\textsuperscript{1,3,9}. The basis of the Bush-Jacoby-Medeiros functional classification is the functional properties of the β-lactama ses enzymes principally, their inhibitor and substrate profiles\textsuperscript{3,9}. The Ambler molecular classification however is based on the protein homology of the β-lactama s enzymes\textsuperscript{3,9}. The Ambler molecular classification further classifies β-lactama s enzymes into four classes namely; class A, C and D also known as the Serine β-lactama s and class B β-lactamas es also known as the zinc or metallo-β-lactamas es\textsuperscript{3,9}.

The CTX-M type forms the most common genetic variant of ESBLs\textsuperscript{1,9,11–14}. This family of β-lactamas es specially hydrolyze cefotaxime (CTX) over ceftazidime (CAZ) and are found exclusively in the functional group 2\textsuperscript{1,3,9,11–14}. Also, unique to these β-lactamas es is their susceptibility to inhibition by the β-lactam inhibitor tazobactam as compared to inhibition by the other β-lactam inhibitor, CA and sulbactam\textsuperscript{1,12,15}. The extended-spectrum of activity of the CTX-M β-lactama ses is due to the serine residue present at position 237\textsuperscript{1,11}. The CTX-M β-lactamas es are divided into five groups namely; the CTX-M group 1, 2, 8, 9 and 25 basing on their amino acid sequences\textsuperscript{1,3,9,16}. Unlike the other β-lactama se 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\textsuperscript{1}. The TEM type β-lactama s form another β-lactama s family; β-lactamas es belonging to the TEM type, TEM-1 a variant of TEM hydrolyze penicillins and first-generation cephalosporins\textsuperscript{1}. The TEM-3 β-lactamas es, which are another variant of TEM β-lactamas es hydrolyze extended-spectrum cephalosporins\textsuperscript{1,17,18}. In addition to these families is the SHV type β-lactamas es, SHV-1 β-lactamas es, a variant of the SHV hydrolyze broad spectrum penicillins, these include ampicillin, tigecycline and piperacillin\textsuperscript{18,19}; the OXA type β-lactamas es, these β-lactamas es exhibit the ability to hydrolyze oxacillin, are predominantly present in Pseudomonas aeruginosa and Acinetobacter baumannii however also occur in other gram-negative bacteria\textsuperscript{19,20,21}; the PER type β-lactamas es, unique about these β-lacta-

mases is their efficient hydrolysis of penicillins, cephalosporins and their susceptibility to CA inhibition\textsuperscript{1,3,9}; the GES type β-lactamas es, these β-lactama s hydrolyze penicillins and extended-spectrum cephalosporins, but not cephamycins or carbapenems, these are also inhibited by β-lactam inhibitors\textsuperscript{1,3,9}; other described β-lactama s families include among others the VEB-1, BES1, CME-1 and SFO-1, enzymes belonging to these families have mostly been found to exhibit β-lactama s activity\textsuperscript{1,22,23}.

**Carbapenemases: Definition and classification**

Carbapenemases are enzymes that break down and thus inactivate carbapenem antibiotics; these enzymes represent the most versatile family of β-lactamas es and are uniquely characterized by their broad spectrum of activity as compared to other β-lactam hydrolyzing enzymes\textsuperscript{3,9,24–28}. 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 penicillinas es and class D oxacillinases, and the metallo β-lactamas es belonging to the class B carbapenemas es which contain one or more zinc atoms at their active sites a characteristic future that allows them to hydrolyze the bicyclic β-lactam ring\textsuperscript{3,9,24–28}. Class A carbapenemases, also known as the class A serine carbapenemas es, when present in bacteria confer a characteristic reduced susceptibility to imipenem\textsuperscript{24,28}. Class A carbapenemases include; IMI/NMC, KPC, GES and SME enzymes\textsuperscript{24,28}. These enzymes need an active serine at position 70 for their hydrolytic activity in the Ambler numbering system for class A β-lactamas es, hydrolyze carbapenems, cephalosporins, penicillins and aztreonam, are inhibited by CA and tazobactam\textsuperscript{24,28,29}. IMI, NMC and SME: are chromosomally encoded enzymes whereas GES and KPC enzymes are plasmid encoded\textsuperscript{24,28}.

Class B carbapenemas es, also known as class B metallo β-lactamas es 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\textsuperscript{24,26,28,30,31}. These enzymes ably hydrolyze carbapenems, are resistant to commercially available β-lactam inhibitors, are susceptible to inhibition by metal ion chelators and have a relatively broad substrate profile that includes cephalosporins and penicillins\textsuperscript{24,26,28,30,31}. Also, characteristic to the class B carbapenemas es is their inability to hydrolyze
These enzymes include; GIM, IMP, VIM, NDM and SIM. 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. 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. These enzymes hydrolyze oxacillin and cloxacillin, are penicillinases, are poorly inhibited by CA and EDTA, and have large amounts of variability in their amino acid sequences.

### AmpC β-lactamases: Definition and classification

AmpC β-lactamases are chromosomally encoded cephalosporinases that mediate resistance to most penicillins, cefoxitin, cefazolin, cephalothin, and β-lactamase inhibitor-β-lactam combinations. These enzymes belong to class C in the Ambler molecular classification of β-lactamases, while they are assigned to group 1 in the Bush Jacoby-Medeiros functional classification scheme. They mostly occur in the Enterobacteriaceae, however, they have also been reported to occur in a few non-Enterobacteriaceae. In most of these bacteria, AmpC β-lactamases are inducible and are expressed at high levels by mutation. The over expression of these enzymes is reported to confer resistance to extended-spectrum cephalosporins that include: CTX, CAZ and ceftriaxone. Evidence of induction of resistance in bacterial isolates belonging to Enterobacter spp. initially susceptible to these extended-spectrum cephalosporins upon therapy is available. The prevalence of resistance mediated by plasmid-mediated AmpC β-lactamases has been shown to be low in most parts of the world as compared to the ESBLs, these have also been documented to be harder to detect as compared to ESBLs, and to mostly exhibit a broad-spectrum antibiotic activity. Current detection assays of these enzymes have been reported to lack specificity and/or sensitivity, they have also been reported to be inconvenient, subjective, and to require reagents mostly unavailable to many clinical microbiology laboratories. In addition, recent times have witnessed identification of bacteria previously lacking and/or poorly expressing chromosomally-mediated AmpC genes as AmpC β-lactamases producers. This has been possible through the acquisition of these genes via conjugative plasmids in horizontal gene transfer, a phenomenon typically seen to occur in Enterobacteria coli, Klebsiella pneumoniae, and Proteus mirabilis. Both chromosomally encoded and plasmid-mediated AmpC β-lactamases continue to evolve, through evolution, these attain potentials to hydrolyze extended-spectrum cephalosporins more efficiently. Just like the AmpC β-lactamases, phenotypic detection methods for the same continue to evolve, and are mostly not yet optimized for the clinical microbiology laboratory.

### Phenotypic detection of extended-spectrum β-lactamase production

Disk diffusion and broth micro-dilution methods have been described to ably detect production of ESBLs in gram-negative bacteria. However, currently some of these methods have become increasingly unreliable, this is due to: i) the co-existence of different classes of β-lactamases in gram-negative bacteria; ii) difficulties recognizing ESBL production due to the over-expression of AmpC β-lactamases; iii) potential masking of ESBL production by AmpC producing bacteria which serve as reservoirs of ESBLs, and iv) the general inability of most clinical microbiology laboratory staff to ably perform testing for detection of ESBL production. These affect testing and so result in inappropriate antimicrobial therapy. In the testing for ESBL production, other factors including inoculum density and the distance between the disks being tested influence the outcomes of the testing.

Testing for ESBL production in bacteria is a two stage process that involves screening and confirmation that follows testing bacteria against array of antibiotics namely: cefpodoxime, CAZ, CRO, CTX and aztreonam and identifying specific zone diameters indicative of potential ESBL production. Testing with greater than one of the antibiotics is recommended to improve sensitivity of ESBL detection. It is however recommended to use CTX, due to its consistent susceptibility to CTX-M and CAZ, due its consistent susceptibility to TEM and SHV. Cefpodoxime should be used when testing using a single drug. Reduced susceptibility to any of the agents as specified by CLSI is a qualification of ESBL testing and necessitates phenotypic confirmatory testing to confirm diagnosis. Phenotypic confirmation of ESBL production is achieved using array of methods, including; the use of the cephalosporin/clavulanate combination disk diffusion or broth micro-dilution method, use of DDS method, and MDDS method which is a modification of the DDS method that confirms ESBL production.
and differentiates ESBL production and overexpression of AmpC-derepressed mutants.\textsuperscript{51,53,57,58}

**Double disk synergy (DDS) and modified double disk synergy (MDDS)**

Both the DDS and MDDS are used in the detection of ESBL production in gram-negative bacteria\textsuperscript{48,51,57–59}. In the DDS, testing is performed by inoculating Mueller-Hinton agar with a lawn culture of organisms for the standard diffusion method and placing onto the agar plate a commercially available disc of CTX (30μg) and/or ceftriaxone (30μg) and/or CAZ (30μg) and/or aztreonam (30μg) and a disc of amoxicillin-clavulanic acid (20/10μg) at a distance of 30mm center to center as shown in figure 1.\textsuperscript{5,51,56,57,60} However, narrower distances (< 30mm) between the discs is reported to increase the sensitivity of the test.\textsuperscript{5,51,56,57,60} Interpretation of the results is done after incubation at 350°C ± 20°C for 18-24 hrs 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 CA containing disc, often resulting in a characteristic shape-zone referred to as “champagne-cork” or key hole as seen in figure 1 is indicative of ESBL production.\textsuperscript{5,51,56,57,60}

In the MDDS, the following antibiotic discs are used: amoxicillin-clavulanic acid (20/10 μg) or piperacillin-tazobactam (100/10μg) along three third-generation cephalosporins: CAZ (30μg), ceftriaxone (30 μg), and cefpodoxime (30 μg) as well as a fourth-generation cephalosporin, ceftazidime (30 μg).\textsuperscript{5,57} This test is performed by inoculating a lawn culture of organisms on Mueller-Hinton agar for the standard diffusion method and placing onto the agar plate a commercially available amoxicillin-clavulanic acid disc, preferentially in the center of the testing area.\textsuperscript{5,51,56,60} The discs including the third and fourth-generation cephalosporins are then placed 15mm and 20mm respectively, center to center to that of the amoxicillin-clavulanic acid disc on the agar plate as shown in figure 2 and 3.\textsuperscript{5,51,57,60} Interpretation of the results is done after incubation at 350°C ± 20°C for 18-24 hrs in ambient air as follows: any distortion or increase in the zone towards the disc of amoxicillin-clavulanic acid is indicative of ESBL production as seen in figure 2 and 3.\textsuperscript{5,51,57,60} In addition, strains are

---

*Fig. 1. DDS. Photograph showing extension and broadening of inhibitory zones of the third generation cephalosporins towards the amoxicillin-clavulanic acid disc. Reprinted from the European Journal of Clinical Microbiology and Infectious Diseases (vol. 8, Issue 6, pp 527–529), by Legrand, P., et al., 1989. Copyright 1989 by the European Journal of Clinical Microbiology and Infectious Diseases.*
divided into three groups basing on the interpretation with MDDS and their susceptibilities to CTX and CAZ, these groups include: Group 1 (Wild type) that includes ESBL non-producers sensitive to CTX and CAZ, Group 2 (derepressed mutants) that includes ESBL non-producers resistant to CTX and CAZ, and Group 3 (ESBL producers) includes ESBL producers sensitive, intermediate or resistant to CTX and CAZ. Worth mentioning, reporting of results is 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.5,57,58

This test can also be used to presumptively identify AmpC β-lactamase producing gram-negative bacteria5,36,49,56. This is because, cefepime, the fourth-generation cephalosporin included in the testing is less rapidly inactivated by AmpC β-lactamases than by ESBLs5,36,49,56. Cefepime improves the detection of synergy with the amoxicillin-clavulanic acid in circumstances when simultaneous stable hyper-production of the AmpC β-lactamases occurs5,36,49,56.

**Three-dimensional tests (direct and indirect)**

The three-dimensional tests are also used in detection of ESBL production in gram-negative bacteria5,51,59,61. In the direct-three dimensional test, a 0.5 McFarland of the test organisms is inoculated onto the surface of a Mueller-Hinton agar plate, this is then dispensed with inoculums between 109-1010 cells/ml in a circular slit cut in the agar so that the slit is filled5,51,59,61. Following this, antibiotic discs are placed on the agar plate, 3mm outside the strain-containing slit as shown in figures 4, 5 and 6.5,51,59,61. By inspecting the margin of the inhibition zone in the vicinity of intersection with the strain containing slit, enzymatic inactivation of each antibiotic is then detected5,51,59,61. Interpretation of the test as positive is dependent on inactivation of the antibiotic as it diffuses through the slit resulting in a distortion or discontinuity in the expected circular inhibition zone, or production of discrete colonies in the vicinity of the inoculated slit5,51,59,61. The indirect three-dimensional test is a modification of the direct three dimensional test; in this test, the defining difference from the direct three dimensional test is that the surface of agar plate is inoculated with a fully susceptible indicator strain, *E. coli* ATCC 259225,51,59,61.
Bacterial producers of AmpC β-lactamases are susceptible to fourth-generation cephalosporins (cefepime and cefpirome)\textsuperscript{36,41}, and are poorly inhibited by CA\textsuperscript{36,41}. As a result, these are resistant to β-lactam/β-lactamase-inhibitor combinations and give a positive and negative test during ESBL screening and confirmation respectively\textsuperscript{62}. These features form the basis of screening and confirmation of AmpC β-lactamase production in gram-negative bacteria\textsuperscript{36,62–64}. Presumptive identification of AmpC β-lactamase producing Enterobacteriaceae can be achieved following the criteria in figure 7.

Fig. 4. The circular three-dimensional inoculation (indicated by the arrow) intersected the inhibition zone margins to produce major distortions (i.e., positive three-dimensional test results) that indicated enzymatic inactivation of piperacillin (A), cefamandole (D), cefoperazone (E), CTX (F), and ceftriaxone (G). Distortions did not occur (i.e., negative three-dimensional test results) in tests with aztreonam (B), imipenem (C), CAZ (H), or cefoxitin (I). The outer circle is the plastic rim on the bottom of the petri dish. Fig. 5. The three-dimensional inoculation (arrow) resulted in minor distortions that indicated antibiotic inactivation (i.e., positive three-dimensional test results) at the intersection with the zone margins of cefamandole (D), cefoperazone (E), CTX (F), and ceftriaxone (G). Distortions did not occur in tests with aztreonam (B), imipenem (C), or cefoxitin (I). The inhibition zones were too small to interpret in the direct three-dimensional test in tests with piperacillin (A) and CAZ (H). Fig. 6. Major zone distortions that indicated antibiotic inactivation (i.e., positive three dimensional test results) occurred in tests with piperacillin (A), cefuroxime (B), cefamandole (D), cefoperazone (E), CTX (F), ceftriaxone (G), and cefoxitin (I) but not in tests with aztreonam (C) or CAZ (H). Note the growth of small colonies in the cefoperazone (E) and CTX (F) zones in the vicinity of the three-dimensional inoculation site. The small colonies in this part of the zone also indicated enzymatic drug inactivation (i.e., positive three-dimensional test results). Reprinted from the Journal of Antimicrobial Agents and Chemotherapy (vol. 36, (9), pp.1877-1882), by Thomson, K.S. and Sanders, C.C., 1992. Copyright 1992 by the Journal of Antimicrobial Agents and Chemotherapy.
Furthermore, AmpC β-lactamase detection can also be achieved using the three dimensional extract test. In this 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. Following this, the culture is grown at 35°C ± 2°C for 4 hrs. The bacterial cells are then centrifuged and the crude enzyme prepared by freezing-thawing the bacterial cell pellets five times. The surface of the Mueller-Hinton agar plate to be used is then inoculated with E. coli ATCC 25922 or E. coli ATCC 11775 following the standard diffusion method. A commercially available cefoxitin (30μg) disc is then aseptically placed on the inoculated agar plate. A slit beginning 5mm from the edge of the disc is then cut in the agar in an outward direction using a sterile scalpel blade. Then, 25-30 mls of enzyme preparation is dispensed into the slit using a pipette, beginning near the disc and moving outwards with care being taken not to over fill the slits as shown in figure 8. The inoculated media is then incubated at 35°C ± 2°C for 18-24 hrs. Interpretation of the results is as follows: enhanced growth of the surface organism at the point where the slit intersect the zone of inhibition is reported as a positive three-dimensional extract test as seen in figure 8. Testing involving swarming organisms is done on MacConkey agar.
In another method, commercially available discs of cefoxitin (30μg) and cefoxitin (30μg) supplemented with 200μg of cloxacillin, an AmpC β-lactamase inhibitor or cefpodoxime (10μg) and cefpodoxime (10 μg) supplemented with an AmpC inhibitor, either 200μg of cloxacillin or 400μg of phenylboronic acid (PBA) are used. Testing using either of the options involves, inoculating a Mueller-Hinton agar plate with the test organism for the standard diffusion testing and placing a cefoxitin disc un-supplemented with an AmpC inhibitor and another cefoxitin disc supplemented with the AmpC inhibitor onto the inoculated agar plate. This is then followed by incubation at 35°C ± 2°C for 18-24 hrs. Interpretation of the results is as follows: the inhibition zone diameters in the un-supplemented cefoxitin disc and the supplemented cefoxitin disc are measured, an increase in the inhibition zone diameter in the cefoxitin disc supplemented with an AmpC inhibitor by ≥ 4mm as compared to the un-supplemented disc is indicative of AmpC production.

Another method of AmpC β-lactamase detection involving the use cefoxitin (30μg) has been described. In this test, a lawn culture of E. coli ATCC 25922 adjusted to a 0.5 McFarland standard is inoculated on Mueller-Hinton agar for standard disc diffusion testing. Following this, a commercially available cefoxitin (30μg) disc is placed on the surface of the inoculated agar plate. 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 as shown in figure 9. This is followed by incubation at 35°C ± 2°C for 18-24 hrs. Interpretation is done after examination of the plates as follows: indentation or a flattening of the inhibition zone as seen in figure 9 is indicative of enzyme inactivation of the cefoxitin and is reported as a positive result whereas the absence of distortion is indicative of non-significant enzyme inactivation of the cefoxitin hence is reported as a negative result.
Detection of inducible AmpC β-lactamase production
Inducible AmpC β-lactamase activity in bacteria is detected using imipenem (10μg), cefoxitin (30μg) and amoxicillin-clavulanic acid (20/10μg) discs: in this testing, these are used as inducing substrates. In the same experiments: CAZ (30μg), CTX (30μg) and PBA (400μg) are used as reporter substrates. Other combinations of the inducing and reporter substrates that can be used have previously been described. The discs both supplemented and the un-supplemented are placed aseptically at a distance of 20mm apart, after a 0.5 McFarland suspension of the test organism is inoculated on a Mueller-Hinton agar plate following the standard diffusion testing. Following this, interpretation is done after the agar plate has been incubated in ambient air at 35°C ± 2°C for 18-24 hrs based on any obvious blunting or flattening of the zone of inhibition between the disc serving as the inducing substrate and the disc serving as the reporter substrate. This observation confirms inducible AmpC β-lactamase production in the bacteria. In the testing micro colonies present in the clear zones of inhibition are mostly ignored and measurements of inhibition zone diameters are done to the edge of the obvious inhibition.

Phenotypic detection of carbapenemase production
Phenotypic detection of carbapenemase production in gram-negative bacteria is achieved by performing either: i) MHT or ii) mCIM, a modification of the carbapenem inactivation method (CIM). These methods have not only been documented to be simple and cost effective but have also been documented to have high sensitivity. Despite the high sensitivity of MHT, it has a high frequency of false-positive results especially in carbapenem resistant Enterobacteriaceae that are producers of ESBLs and AmpC β-lactamases and low sensitivity in regards detection of NDM-1 producing bacteria. In addition to this, the use of E. coli ATCC 25922 has also been implicated with low sensitivity, specificity and repeatability of MHT. However, replacing E. coli ATCC 25922 with Klebsiella pneumoniae has been shown to provide high sensitivity, specificity and repeatability of the test. 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 difficulty in interpretation of results is reported. Despite these, MHT remains a phenotypic reference method for confirmation of carbapenemase production.

Modified hodge test (MHT)
The modified hodge test (MHT) is used in detection of carbapenemase production in gram-negative bacteria. In MHT, a 0.5 McFarland dilution of E. coli ATCC 25922, an indicator strain in 5 ml of sterile saline or nutrient broth is prepared. The modified hodge test shown by indentation of the zone of inhibition around cephoxitin disc. Reprinted from the Journal of Clinical and Diagnostic Research (vol. 7, 2 (2013): 229–233), by Kaur, Jaspal et al., 2013. Copyright 2013 by the Journal of Clinical and Diagnostic Research.
ATCC 70060331,63,73–75,83. Following this, a 1:10 dilution of the indicator strains is each streaked as a lawn onto a Mueller-Hinton agar plate using a sterile swab31,63,73–75,83. Then, a commercially available meropenem (10μg) or ertapenem (10μg) or imipenem (1μg) disc is placed at the center of the test area on the Mueller-Hinton agar plates31,63,73–75,83,84. 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 as shown in figure 10 31,63,73–75,83. *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 test31,63,73–75,83. Incubation of the test plates is done at 35°C ± 2°C for 18-24 hrs in ambient air31,63,73–75,83. Interpretation of the tests is done after incubation as follows: the test organism(s) is positive for MHT when a clover leaf-like indentation of the *E. coli* ATCC 25922 or *Klebsiella pneumoniae* ATCC 700603 growing along the test organism growth streak within the disc diffusion zone is observed as seen in figure 1031,63,73–75,83. MHT negative organism(s) show no growth of the *E. coli* 25922 or *Klebsiella pneumoniae* ATCC 700603 along the test organism growth streak within the disk diffusion as seen in figure 1031,63,73–75,83.

**Metallo β-lactamase test (MBL)**

Detection of metallo β-lactamase production in gram-negative bacteria is achieved by using two methods, namely: the imipenem-ethylene diamine tetra acetic acid, EDTA method and another double disc synergy method that involves use of a CAZ (30μg) disc and a CAZ (30μg)-2-mercaptopropionic acid disc85,86.

In the imipenem-EDTA method, testing is by inoculating the test organism(s) onto Mueller-Hinton agar for the standard diffusion method85,86. This is then followed by adding to the agar plates two commercially available imipenem (10μg) discs, one of the discs un-supplemented with EDTA and another supplemented with EDTA at a distance of 15 mm center to center as shown in figure 11; however narrower distances are associated with high test sensitivity and have been described85,86. The supplementation of the imipenem disc is done by adding 10 μl of 0.5M EDTA to obtain the desired concentration of 1000 μg74,84,85. A concentration of 750 μg can also be used in the testing63,74. The 0.5M EDTA stock solution is prepared by dissolving 186.1g of disodium EDTA. 2H2O in 1000 ml of distilled wa-
ter and adjusting the solution PH to 8.0 using NaOH85. After preparation of the mixture, it is sterilized by autoclaving85. Incubation of the Mueller-Hinton test agar plates is done at 35°C ± 2°C for 18-24 hrs in ambient air85. 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 production74,84,85. Noteworthy, the supplemented discs prepared maybe kept at 4°C or at -20°C in airtight vials without desiccant and under these conditions, these remain stable for 12 and 16 weeks respectively85.

In the CAZ disc method, a CAZ disc and another supplemented with 2-mercaptopropionic acid are used as shown in figures 12 and 1385. In this method, the procedure and interpretation is done as documented in the previous method, the imipenem-EDTA85.
Detection of KPC, differentiation of KPC and MBL, and KPC and MBL co-production

The detection and differentiation of *Klebsiella pneumoniae* carbapenemase production and metallo β-lactamase production is achieved using a phenotypic algorithm that involves the use of three combined-disc tests\(^7,8,7,8,8,8\). These tests consist of; i) meropenem alone and meropenem supplemented with PBA, ii) meropenem alone and meropenem supplemented with ethylene diamine tetra acetic acid (EDTA), and iii) both tests\(^7,8,7,8,8,8\). Additionally, these tests are used to detect co-production of both carbapenemases in bacteria\(^7,8,9\). In these tests, the concentrations of the PBA and EDTA used are 400 μg of PBA and 292 μg of EDTA\(^7,8,8,8\). The stock solution of PBA is prepared by dissolving PBA in DMSO at a concentration of 20 mg/ml\(^7,8,8,8\). 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\(^7,8,8,8\). Similarly, the stock solution of EDTA is prepared by dissolving anhydrous EDTA in distilled water at a concentration of 0.1M\(^7,8,8,8\). 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. Following disc preparation, the discs are dried and used within 60 minutes. Testing is performed by inoculating Mueller-Hinton agar for the standard diffusion method 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 as shown in figure 13. Incubation of the agar plates is done at 37°C for 18-24 hrs. 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. 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. Bacterial isolates are considered positive for KPC and MBL co-production only when, the inhibition zone diameters 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.

**Detection of ESBLs in KPC producing bacteria using boronic acid based tests**

Boronic acid based tests are used to detect ESBL production in KPC positive isolates. In this testing, combined disc tests are used, namely: i) CTX with or without CA supplemented with PBA and ii) CAZ with or without CA supplemented with PBA. The stock solution of PBA is prepared by dissolving PBA in DMSO 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. Incubation of the agar plates is done at 37°C for 18-24 hrs. These tests are interpreted after measuring the inhibition zone diameters around the CTX or CAZ disc with or without CA not supplemented with BA and the CTX or CAZ disc with or without CA supplemented with 400 μg of PBA as shown in figure 14. 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. 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.
The modified carbapenem inactivation method (mCIM)
The modified carbapenem inactivation method is a phenotypic method used in detection of carbapenemase production\textsuperscript{77,78}. In this method, a sterile inoculating loop is used to add 1μl of the test organism to a tube containing 2 mls of tryptic soy broth, following this, the bacterial suspension is then mixed by vortexing for 15 seconds\textsuperscript{77,78}. This is then followed by aseptically adding a commercially available meropenem disk (10μg) to the bacterial suspension\textsuperscript{77,78}. Incubation of the bacterial suspension containing the meropenem disk is then done at 35°C ± 2°C in ambient air for 4 hrs ± 15 minutes\textsuperscript{77,78}. Before completion of the incubation time, a suspension of the mCIM indicator organism, a carbapenem susceptible \textit{Escherichia coli} ATCC 25922 with a turbidity equivalent to a 0.5 McFarland is prepared and the surface of a Mueller-Hinton agar plate is inoculated following the standard disk diffusion method and placing onto the agar plate the meropenem disc previously aseptically removed from the tryptic soy broth suspension after dragging it on the walls of the tube to drain off the excess fluid\textsuperscript{80,81}. Incubation of the plate is then done at 35°C ± 2°C in ambient air for 18-24 hrs\textsuperscript{77,78}. 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 as shown in figures 15 and 16\textsuperscript{77,78}.

**Fig. 14.** Representative results of the CLSI ESBL confirmatory test (A and C) and its modification using antibiotic disks containing BA (B and D) for ESBL PCR-positive (A and B) and ESBL PCR-negative (C and D) KPC-possessing isolates. Reprinted from the Journal of Clinical Microbiology (vol. 47.11 (2009): 3420-3426), by Tsakris, Athanassios, et al., 2009. Copyright 2009 by the Journal of Clinical Microbiology.
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.

Conclusion
The review and description of previously designed phenotypic detection assays for ESBLs and carbapenemases in a single document avails a summary that allows for multiple testing which increases the sensitivity and specificity of detection. Also, extended-spectrum β-lactamase and carbapenemase detection assays when performed correctly can precisely discriminate between bacteria producing these enzymes and those with other mechanisms of resistance to β-lactam antibiotics.

Declarations
Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Conflicting interests
None declared.

Author contributions
DA, drafted, edited and reviewed both the original and final manuscripts.

Acknowledgements
Not applicable.
References
1. Shaikh S, Fatima J, Shahil S, Rizvi SMD, Kamal MA. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi J Biol Sci*. 2015;22(1):90–101.
2. Sidra Shan, Saira Sajid KA. Detection of blaIMP Gene in Metallo-β-Lactamase Producing Isolates of Imipenem Resistant Pseudomonas aeruginosa; an Alarming Threat. *J Microbiol Res*. 2015;5(6):175–80.
3. Ambler RP. The structure of β-lactamases. *Philos Trans R Soc London B, Biol Sci*. 1980;289(1036):321–31.
4. Ruppé É, Woerther P-L, Barbier F. Mechanisms of antimicrobial resistance in Gram-negative bacilli. *Ann Intensive Care*. 2015;5(1):21.
5. Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. *Clin Microbiol Infect*. 2008;14:90–103 PubMed.
6. Steward CD, Wallace D, Hubert SK, Lawton R, Fridkin SK, Gaynes RP, et al. Ability of laboratories to detect emerging antimicrobial resistance in nosocomial pathogens: a survey of project ICARE laboratories. *Diag Microbiol Infect Dis*. 2000;38(1):59–67.
7. Tsakris A, Poulou A, Pournaras S, Voulgaris E, Vrioni G, Themeli-Digalaki K, et al. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. *J Antimicrob Chemother [Internet]*. 2010 Aug 1;65(8):1664–71. Available from: http://dx.doi.org/10.1093/jac/dkq210
8. Testing EC on AS. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. Version; 2019.
9. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother*. 1995;39(6):1211.
10. Pfäffler MA, Segreti J. Overview of the epidemiological profileand laboratory detection of extended-spectrum β-Lactamases. *Clin Infect Dis*. 2006;42(Supplement_4):S153–63.
11. Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ. CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes. *Int J Antimicrob Agents*. 2000;14(2):137–42.
12. Bradford PA, Yang Y, Sahm D, Gropo I, Gardovska D, Storch G. CTX-M-5, a Novel Cefotaxime-Hydrolyzing β-Lactamase from an Outbreak of Salmonella typhimurium in Latvia. *Antimicrob Agents Chemother*. 1998;42(8):1980–4.
13. Paterson DL, Ko W-C, Von Gottberg A, Caselas JM, Mulizimoglu L, Klugman KP, et al. Outcome of cephalosporin treatment for serious infections due to apparently susceptible organisms producing extended-spectrum β-lactamases: implications for the clinical microbiology laboratory. *J Clin Microbiol*. 2001;39(6):2206 PubMed –12.
14. Walsh C. Opinion—anti-infectives: where will new antibiotics come from? *Nat Rev Microbiol*. 2003;1(1):65.
15. Ma L, Ishii Y, Ishiguro M, Matsuzawa H, Yamaguchi K. Cloning and sequencing of the gene encoding Toho-2, a class A β-lactamase preferentially inhibited by tazobactam. *Antimicrob Agents Chemother*. 1998;42(5):1181–6.
16. Bonnet R. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother*. 2004;48(1):1–14.
17. Sirot D, Sirot J, Labia R, Morand A, Courvalin P, Darfeuille-Michaud A, et al. Transferable resistance to third-generation cephalosporins in clinical isolates of Klebsiella pneumoniae: identification of CTX-1, a novel β-lactamase. *J Antimicrob Chemother*. 1987;20(3):323–34.
18. Sougakoff W, Goussard S, Courvalin P. The TEM-3 β-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino acid substitutions. *FEMS Microbiol Lett*. 1988;56(3):343–8.
19. Livermore DM. beta-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev*. 1995;8(4):557–84.
20. Weldhagen GF, Poirel L, Nordmann P. Ambler class A extended-spectrum β-lactamases in Pseudomonas aeruginosa: novel developments and clinical impact. *Antimicrob Agents Chemother*. 2003;47(8):2385–92.
21. Neuhauser MM, Weinstein RA, Rydman R, Danziger LH, Karam G, Quinn JP. Antibiotic resistance among gram-negative bacilli in US intensive care units: implications for fluoroquinolone use. JAMA. 2003;289(7):885–8.
22. Naas T, Poirel L, Nordmann P. Ambler class A extended-spectrum β-lactamases in Pseudomonas aeruginosa; implications for fluoroquinolone use. *J Antimicrob Chemother*. 2004;54(1):21.
23. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev*. 2001;14(4):933 PubMed –51.
24. Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. *Clin Microbiol Rev*. 2007;20(3):440–58.
25. Antunes NT, Lamoureux TL, Toth M, Stewart NK, Frase H, Vakulenko SB. Class D ß-lactamases: Are they all carbapenemases? *Antimicrob Agents Chemother*. 2014;58(4).
26. Walsh TR, Toleman MA, Poirel L, Nordmann P.
Metallo-β-lactamases: the Quiet before the Storm? Clin Microbiol Rev [Internet]. 2005 Apr 1;18(2):306–25. Available from: http://cmr.asm.org/content/18/2/306.abstract

27. Rasmussen BA, Bush K. Carbapenem-hydrolyzing beta-lactamases. Antimicrob Agents Chemother. 1997;41(2):223.

28. Diene SM, Rolain J-M. Carbapenemase genes and genetic platforms in Gram-negative bacilli: Enterobacteriaceae, Pseudomonas and Acinetobacter species. Clin Microbiol Infect. 2014;20(9):831–8.

29. Ambler RP, Coulson AF, Frere J-M, Ghuysen J-M, Joris B, Forsman M, et al. A standard numbering scheme for the class A beta-lactamases. Biochem J. 1991;276(Pt 1):269.

30. Walsh TR. The emergence and implications of metallo-β-lactamases in Gram-negative bacteria. Clin Microbiol Infect. 2005;11:2–9 PubMed .

31. Miriagou V, Cornaglia G, Edelstein M, Galani I, Giske CG, Gniadkowski M, et al. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin Microbiol Infect. 2010;16(2):112–22.

32. Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, et al. Cloning and characterization of bla VIM, a new integron-borne metallo-β-lactamate gene from a Pseudomonas aeruginosa clinical isolate. Antimicrob Agents Chemother. 1999;43(7):1584–90.

33. Matthew M. Plasmid-mediated β-lactamases of gram-negative bacteria: properties and distribution. J Antimicrob Agents Chemother. 1979;5(4):349–58.

34. Simpson IN, Harper PB, O’Callaghan CH. Principal beta-lactamases responsible for resistance to beta-lactam antibiotics in urinary tract infections. Antimicrob Agents Chemother. 1980;17(6):929–36.

35. Bush K. Recent developments in β-lactamase research and their implications for the future. Clin Infect Dis. 1988;10(4):681–90.

36. Jacoby GA. AmpC β-lactamases. Clin Microbiol Rev. 2009;22(1):161–82.

37. Black JA, Moland ES, Thomson KS. AmpC disk test for detection of plasmid-mediated AmpC β-lactamases in Enterobacteriaceae lacking chromosomal AmpC β-lactamases. J Clin Microbiol. 2005;43(7):3110–3.

38. Philippopon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type β-lactamases. Antimicrob Agents Chemother. 2002;46(1):1–11.

39. Oberoi I, Singh N, Sharma P, Aggarwal A. ESBL, MBL and Ampc β lactamases producing superbugs—Havoc in the Intensive Care Units of Punjab India. J Clin diagnostic Res JCDR. 2013;7(1):70.

40. Singhal S, Mathur T, Khan S, Upadhyay DJ, Chugh S, Gaid R, et al. Evaluation of methods for AmpC beta-lactamase in gram negative clinical isolates from tertiary care hospitals. Indian J Med Microbiol. 2005;23(2):120.

41. Shoorashetty RM, Nagarathnamma T, Prathibha J. Comparison of the boronic acid disk potentiation test and cefepime-clavulanic acid method for the detection of ESBL among AmpC-producing Enterobacteriaceae. Indian J Med Microbiol. 2011;29(3):297.

42. Hageman JC, Fridkin SK, Mohammed JM, Steward CD, Gaynes RP, Tenover FC. Antimicrobial proficiency testing of National Nosocomial Infections Surveillance System hospital laboratories. Infect Control Hosp Epidemiol. 2003;24(5):356–61.

43. Tenover FC, Mohammed MJ, Stelling J, O’Brien T WR. Ability of laboratories to detect emerging antimicrobial resistance: proficiency testing and quality control results from the World Health Organization’s external quality assurance system for antimicrobial susceptibility testing. J Clin Microbiol. 2001;39((1)):241–50.

44. Pitout JD, Nordmann P, Laupland KB PL. Emergence of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) in the community. J Antimicrob Chemother. 2005;56((1)):52–59.

45. Stevenson KB, Samore M, Barbera J, Moore JW, Hannah E, Houck P, et al. Detection of antimicrobial resistance by small rural hospital microbiology laboratories: comparison of survey responses with current NCCCLS laboratory standards. Diagn Microbiol Infect Dis. 2003;47(1):303–11.

46. Grover N, Sahni AK, Redt SB. Therapeutic challenges of ESBLs and AmpC beta-lactamase producers in a tertiary care center. Med J Armed Forces India. 2013;69(1):4–10.

47. Queenan AM, Foleno B, Gownley C, Wira E, Bush K. Effects of inoculum and β-lactamase activity in AmpC-and extended-spectrum β-lactamase (ESBL)-producing Escherichia coli and Klebsiella pneumoniae clinical isolates tested by using NCCCLS ESBL methodology. J Clin Microbiol. 2004;42(1):269–75.

48. Willems E, Verhaegen J, Magerman K, Nys S, Cartuyvels R. Towards a phenotypic screening strategy for emerging β-lactamases in Gram-negative bacilli. Int J Antimicrob Agents. 2013;41(2):99–109.

49. Legrand P, Fournier G, Bure A, Jarlier V, Nicolas MH, Deere D, et al. Detection of extended broad-spectrum beta-lactamases in Enterobacteriaceae in four French hospitals. Eur J Clin Microbiol Infect Dis. 1989;8(6):527–9.
50. M’Zali FH, Chanawong A, Kerr KG, Birkenhead D, Hawkey PM. Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae: comparison of the MAST DD test, the double disc and the Etest ESBL. *J Antimicrob Chemother.* 2000;45(6):881–5.

51. Thomson KS, Sanders CC. Detection of extended-spectrum beta-lactamases in members of the family Enterobacteriaceae: comparison of the double-disc and three-dimensional tests. *Antimicrobial Agents Chemotherapy.* 1992;36(9):1877–82.

52. Rawat D, Nair D. Extended-spectrum β-lactamases in Gram Negative Bacteria. *J Glob Infect Dis.* 2010;2(3):263.

53. (CLSI) C and LSI. Performance Standards for Antimicrobial Susceptibility Testing. CLSI supplement M100. Clinical and Laboratory Standards, 2018. 28th ed. 2018;(28th ed.).

54. Livermore DM, Paterson DL. Pocket guide to extended-spectrum β-lactamases in resistant. Current Medicine Group; 2006.

55. Steward CD, Biddle JW, Raney PM, Anderson GJ. Characterization of clinical isolates of Klebsiella pneumoniae from 19 laboratories using the NCCLS extended-spectrum betalactamase detection methods. *J Clin Microbiol.* 2001;39:2864–72 PubMed .

56. Jarlier V, Nicolas M-H, Fournier G, Philippin A. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Clin Infect Dis.* 1988;10(4):867 PubMed –78.

57. Kaur J, Chopra S, Sheevani GM. Modified double disc synergy test to detect ESBL production in urinary isolates of Escherichia coli and Klebsiella pneumoniae. *J Clin diagnostic Res JCDR.* 2013;7(2):229.

58. Garrec H, Drieux-Rouzet L, Golmard J-L, Jarlier V, Robert J. Comparison of nine phenotypic methods for detection of extended-spectrum β-lactamase production by Enterobacteriaceae. *J Clin Microbiol.* 2011;49(3):1048 PubMed –57.

59. Pitout JDD, Laupland KB. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis.* 2008;8(3):159 PubMed –66.

60. Tzelepi E, Giakoupi P, Sofianou D, Loukova V, Kmeroglou A, Tsakris A. Detection of Extended-Spectrum β-Lactamases in Clinical Isolates of Enterobacter cloacae and Enterobacter aerogenes. *J Clin Microbiol.* 2000;38(2):542 PubMed –6.

61. Hemalatha V, Padma M, Sekar U, Vinodh TM, Arunkumar AS. Detection of Amp C beta lactamases production in Escherichia coli & Klebsiella by an inhibitor based method. *Indian J Med Res.* 2007;126(3):220 PubMed .

62. El-Hady SA, Adel LA. Occurrence and detection of AmpC β-lactamases among Enterobacteriaceae isolates from patients at Ain Shams University Hospital. *Egypt J Med Hum Genet.* 2015;16(3):239–44.

63. Brenwald NP, Jevons G, Andrews J, Ang L, Fraise AP. Disc methods for detecting AmpC β-lactamase-producing clinical isolates of Escherichia coli and Klebsiella pneumoniae. *J Antimicrob Chemother.* 2005;56(3):600–1.

64. Ingram PR, Inglis TJ, Vanzetti TR, Henderson BA, Harnett GB, Murray RJ. Comparison of methods for AmpC β-lactamase detection in Enterobacteriaceae. *J Med Microbiol.* 2011;60(6):715–21.

65. Coudron PE, Moland ES, Thomson KS. Occurrence and detection of AmpC beta-lactamases among Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis isolates at a veterans medical center. *J Clin Microbiol.* 2000;38(5):1791–6.

66. Tan TY, Ng LSY, He J, Koh TH, Hsu LY. Evaluation of screening methods to detect plasmid-mediated AmpC in Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis. *Antimicrobial Agents Chemotherapy.* 2009;53(1):146–9.

67. Vercauteren E, Descheemaeker P, Ieven M, Sanders CC, Goossens H. Comparison of screening methods for detection of extended-spectrum beta-lactamases and their prevalence among blood isolates of Escherichia coli and Klebsiella spp. in a Belgian teaching hospital. *J Clin Microbiol.* 1997;35(9):2191–7.

68. M’Zali FH, Heritage J, Gascoyne-Binzi DM, Denton M, Todd NJ, Hawkey PM. Transcontinental importation into the UK of Escherichia coli expressing a plasmid-mediated AmpC-type beta-lactamase exposed during an outbreak of SHV-5 extended-spectrum beta-lactamase in a Leeds hospital. *J Antimicrob Chemother.* 1997;40(6):823–31.

69. Peter-Getzlaff S, Polsfuss S, Polledica M, Hombach M, Giger J, Böttger EC, et al. Detection of AmpC beta-lactamase in Escherichia coli, Klebsiella pneumoniae, and their prevalence among blood isolates of Escherichia coli and Klebsiella spp. in a Belgian teaching hospital. *J Clin Microbiol.* 1997;35(9):2191–7.

70. Coudron PE. Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in Klebsiella spp., Escherichia coli, and Proteus mirabilis. *J Clin Microbiol.* 2005;43(8):4163–7.

71. Polsfuss S, Bloemberg G V, Giger J, Meyer V, Böttger EC, Hombach M. Practical approach for reliable detection of AmpC beta-lactamase-producing Enterobacteriaceae. *J Clin Microbiol.* 2011;49(8):2798–803.
72. Yagi T, Wachino J, Kurokawa H, Suzuki S, Yamane K, Doi Y, et al. Practical methods using boronic acid compounds for identification of class C β-lactamase-producing Klebsiella pneumoniae and Escherichia coli. J Clin Microbiol. 2005;43(6):2551–8.

73. Amjad A, Mirza IA, Abbasi SA, Farwa U, Malik N, Zia F. Modified Hodge test: A simple and effective test for detection of carbapenemase production. Iran J Microbiol. 2011;3(4):189.

74. Kateete DP, Nakanjako R, Namugenyi J, Erume J, Joloba ML, Najjuka CF. Carbapenem resistant Pseudomonas aeruginosa and Acinetobacter baumannii at Mulago Hospital in Kampala, Uganda (2007–2009). Springerplus [Internet]. 2016 Aug 9;5(1):1308. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4978656/.

75. Okoche D, Asimwe BB, Katabazi FA, Karo L, Najjuka CF. Prevalence and Characterization of Carbapenem-Resistant Enterobacteriaceae Isolated from Mulago National Referral Hospital, Uganda. PLoS One [Internet]. 2015 Aug 18;10(8):e0135745. Available from: https://doi.org/10.1371/journal.pone.0135745

76. Asthana S, Mathur P T V. Detection of carbapenem production in Gram-negative bacteria. J Lab Physicians. 2014;6(2):69.

77. Pierce VM, Simner PJ, Lonsway DR, Roe-Carpenter DE, Johnson JK, Brasso WB, et al. Modified carbapenem inactivation method for phenotypic detection of carbapenemase production among Enterobacteriaceae. J Clin Microbiol. 2017;55(8):2321–33.

78. van der Zwaluw K, de Haan A, Pluister GN, Bootsma HJ, de Neeling AJ, Schouls LM. The carbapenem inactivation method (CIM), a simple and low-cost alternative for the Carba NP test to assess phenotypic carbapenemase activity in gram-negative rods. PLoS One. 2015;10(3):e0123690.

79. Hrabáč J, Chudáčková E, Papagiannitsis CC. Detection of carbapenemases in Enterobacteriaceae: a challenge for diagnostic microbiological laboratories. Clin Microbiol Infect. 2014;20(9):839–53.

80. Pasteran F, Mendez T, Rapoport M, Guerrero L, Corso A. Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class A carbapenemase in species of Enterobacte-riaceae by incorporating boronic acid. J Clin Microbiol. 2010;48(4):1323–32.

81. Pasteran F, Veliz O, Rapoport M, Guerrero L, Corso A. Sensitive and specific modified Hodge test for KPC and metallo-beta-lactamase detection in Pseudomonas aeruginosa by use of a novel indicator strain, Klebsiella pneumoniae ATCC 700603. J Clin Microbiol. 2011;49(12):4301–3.

82. Bialvaei AZ, Kabul HS, Asgharzadeh M, Yousef Memar M, Yousefi M. Current methods for the identification of carbapenemases. J Chemother. 2016;28(1):1–19.

83. Tenover FC, Emery SL, Spiegel CA, Bradford PA, Eells S, Endimiani A, et al. Identification of plasmid-mediated AmpC β-lactamases in Escherichia coli, Klebsiella spp., and Proteus species can potentially improve reporting of cephalosporin susceptibility testing results. J Clin Microbiol. 2009;47(2):294–9.

84. Asthana S, Mathur P T V. Detection of carbapenemase production in Gram-negative bacteria. J Lab Physicians. 2014;6(2):69–75.

85. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol. 2002;40(10):3798–801.

86. Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H, et al. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J Clin Microbiol. 2000;38(1):40–3.

87. Song W, Bae IK, Lee Y-N, Lee C-H, Lee SH, Jeong SH. Detection of extended-spectrum β-lactamases by using boronic acid as an AmpC β-lactamase inhibitor in clinical isolates of Klebsiella spp. and Escherichia coli. J Clin Microbiol. 2007;45(4):1180–4.

88. Tsakris A, Poulou A, Themeli-Digalaki K, Voulgaris E, Pittaras T, Sofianou D, et al. Use of boronic acid disk tests to detect extended-spectrum β-lactamases in clinical isolates of KPC carbapenemase-producing Enterobacteriaceae. J Clin Microbiol. 2009;47(11):3420–6.

89. Pournaras S, Poulou A, Tsakris A. Inhibitor-based screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J Clin Microbiol. 2002;40(10):3798–801.

90. Tijet N, Patel SN, Melano RG. Detection of carbapenemase activity in Enterobacteriaceae: comparison of the carbapenem inactivation method versus the Carba NP test. J Antimicrob Chemother. 2015;71(1):274–6.