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Review Article

Detection of carbapenemases-producing *Acinetobacter baumannii*: the phenotypic approaches

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ABSTRACT

Antimicrobial resistance among *Acinetobacter* species represents one of the most critical challenges within healthcare settings. *Acinetobacter baumannii* primarily causes hospital-acquired infections affecting patients with prolonged hospitalization and with long-term administration of antimicrobials. *A. baumannii* can develop antimicrobial resistance, especially to carbapenems, the last-line of antimicrobial agents to treat serious infections. Several resistance mechanisms have been reported including antimicrobial-degrading enzymes, efflux pumps, target modification and porin deficiency which likely work synergistically. Among those, the most prevalent mechanism is enzymatic degradation by carbapenemases, β-lactamase enzymes that are capable to hydrolyse carbapenems. Currently, spreading of carbapenem-resistant *A. baumannii* has been increasing worldwide. A high prevalence of *A. baumannii* with multidrug-resistance including carbapenems leads to conventional failure of common antibiotic therapy treatments. Therefore, accurate and rapid detection techniques of carbapenemases-producing *A. baumannii* are extensively required to screen and prevent the outbreak of this microorganism. Both phenotypic and genotypic methods have been developed aiming to detect carbapenemases enzymes. This review summarizes the concepts and practical approaches for phenotypic detection of this enzyme which are applicable to practise in developing countries.

1. INTRODUCTION

Acinetobacter baumannii, an aerobic Gram-negative coccobacillus, usually inhabits in natural environments such as soil and water (Figure 1). *A. baumannii* is one of the most important opportunistic nosocomial infections causing pneumonia, wound infection, and septicaemia[1]. This bacterium is notable for extensive antimicrobial resistance and capability to acquire antimicrobial resistance genes. *A. baumannii* can develop resistance to most of current antimicrobial agents used, including carbapenems. There are several resistance mechanisms of *A. baumannii* such as efflux pump, outer

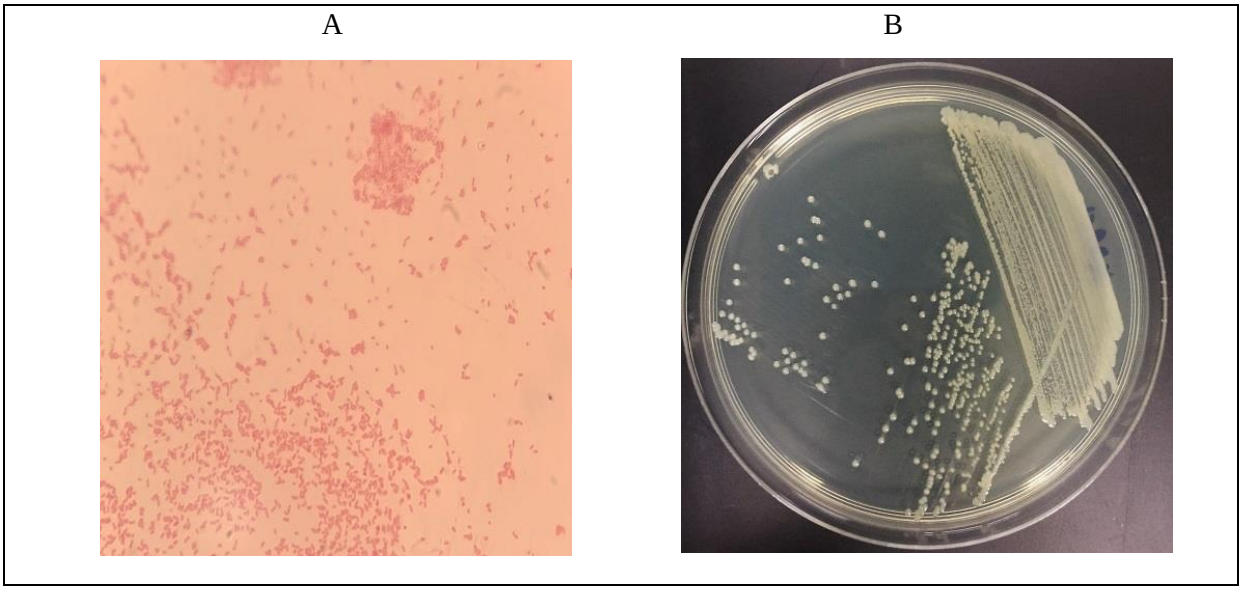

Figure 1. *Acinetobacter baumannii* (A) Gram staining (B) Culture on nutrient agar

membrane alteration, and the most important one, production of carbapenemases enzyme. Carbapenemases is a group of enzymes that is able to hydrolyse not only carbapenems but also all β-lactam antibiotics[2]. Therefore, methods for enzyme detection is highly necessary in order to prevent resistant bacterial spreading and to treat the infection at the right time. At present, detections of carbapenemases-producing bacteria are focused on phenotypic and genotypic methods. The phenotypic detection is by using non-molecular technique to detect the activity of carbapenemases. The advantages of these methods are easy to perform, convenient, and inexpensive. Another method, the genotypic detection, relies on molecular techniques to detect the gene encoding enzymes. The genotypic detections are more accurate for the detection of carbapenemase gene[3]. However, this method can detect only known genes and required the expertise and expensive instrument[3].

2. *Acinetobacter baumannii*

A. baumannii, is occasionally found as commensals on skin and mucus membrane. This bacteria is becoming one of the most critical opportunistic pathogens, causing hospital-acquired pneumonia, meningitis, and septicaemia, especially in developing countries[4,5]. At present, antibiotic-resistant *A. baumannii* become a serious concern in hospital environments worldwide. Several resistance mechanisms of *A. baumannii* have been reported, including changes in outer membrane proteins, efflux pump, and the drug target modification. The most common resistance mechanism is production of drug inactivated enzymes, especially carbapenemases. Carbapenemases production has an impact on the use of carbapenems, resulting in high failure rates of treatment[6]. This enzyme belongs to 3 classes of Ambler classification: Class A, B, and D β-lactamases[7]. Class A carbapenemases are serine β-lactamases mostly found in *Klebsiella pneumoniae*, hence, its name *K. pneumoniae* carbapenamases (KPC). However, they are also found in other Enterobacteriaceae and Gram-negative non-glucose fermenter such as *Pseudomonas aeruginosa*[8]. Until now, there is only one report on KPC-producing *A. baumannii* in Puerto Rico[9]. Other class A carbapenemases have been reported such as Guiana extended spectrum (GES), *Serratia marcescens* enzyme (SME), and Imipenem-hydrolysing β-lactamases (IMI). Among these enzymes, KPC is the most clinical important outbreak in many countries[6]. The activity of these enzymes are inhibited by β-lactamases inhibitors such as boronic acid, calvulanic acid, and tazobactam[10]. Secondly, Class B carbapenemases or metallo-β-lactamases, this group requires divalent cations, such as Zn^{2+}, for their activity. This group of enzyme usually found in Enterobacteriaceae and *P. aeruginosa*[11]. New Delhi metallo-β-lactamases (NDM), Verona integrin-encoded metallo-β-lactamases (VIM), and Imipenem-resistant Pseudomonas (IMP), are the most identified MBLs worldwide[12]. Other classes of MBLs have been reported such as German imipenmase (GIM) and Seoul

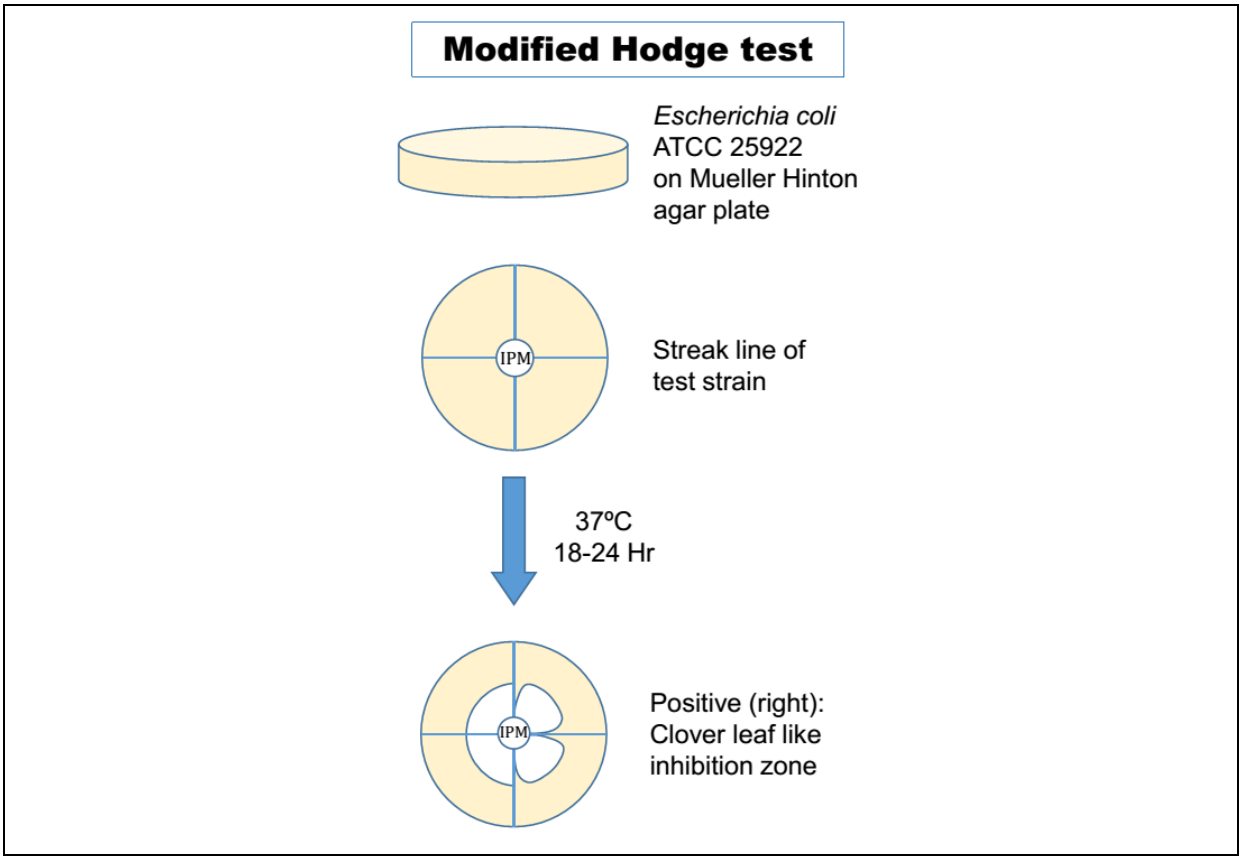

Figure 2. Schematic diagram illustrating the modified Hodge test

imipenemase (SIM). NDM-genes are usually found in *K. pneumoniae* and *E. coli* isolates but also found in *A. baumannii* and *P. aeruginosa* [6]. In addition IMP, VIM, and SIM-producing *A. baumannii* also have been reported[12]. Since the activity of MBLs depends on Zn^{2+}, it can be inhibited by chelating agents such as EDTA. Lastly, class D carbapenemases are also serine type β-lactamases but not inhibited by clavulanic acid or tazobactam[13]. This group includes oxacillinase (OXA) enzymes that hydrolyse oxacillin and the third generation cephalosporins but possess weak activity against carbapenems[6]. The OXA-type carbapenemase was first reported in *A. baumannii* in 1993[14]. There are several subtypes of these enzymes such as OXA-48 like enzymes which usually produced by Enterobacterales [15]. The other subtypes including OXA-23-like, OXA-40-like, OXA-58-like, and OXA-143-like enzymes, which are frequently produced by *A. baumannii*[16]. In particular, the main mechanisms of carbapenem resistance in *A. baumannii* are productions of MBLs and OXA carbapenemases[4].

3. DETECTION OF CARBAPENEMASES-PRODUCING *A. baumannii*

The appropriate methods for detection of carbapenemase-producing *A. baumannii* are seriously concerned because the limitation of treatment options. At present, the methods used to detect carbapenemases can be classified into 2 groups: phenotypic and genotypic detections. The scope of this review is focusing only the phenotypic method. Several phenotypic methods have been developed to detect the production of carbapenemases such as modified Hodge test, combined disc test, carbapenemase inactivation method, and CarbaNP. These methods are mainly detected either the growth of bacteria in the presence of antibiotics or the by-products of carbapenem hydrolysis.

3.1. Modified Hodge test

Modified Hodge test (MHT) is one of the most recognized assay for carbapenemases detection and has been recommended by the clinical and laboratory standards institute (CLSI)

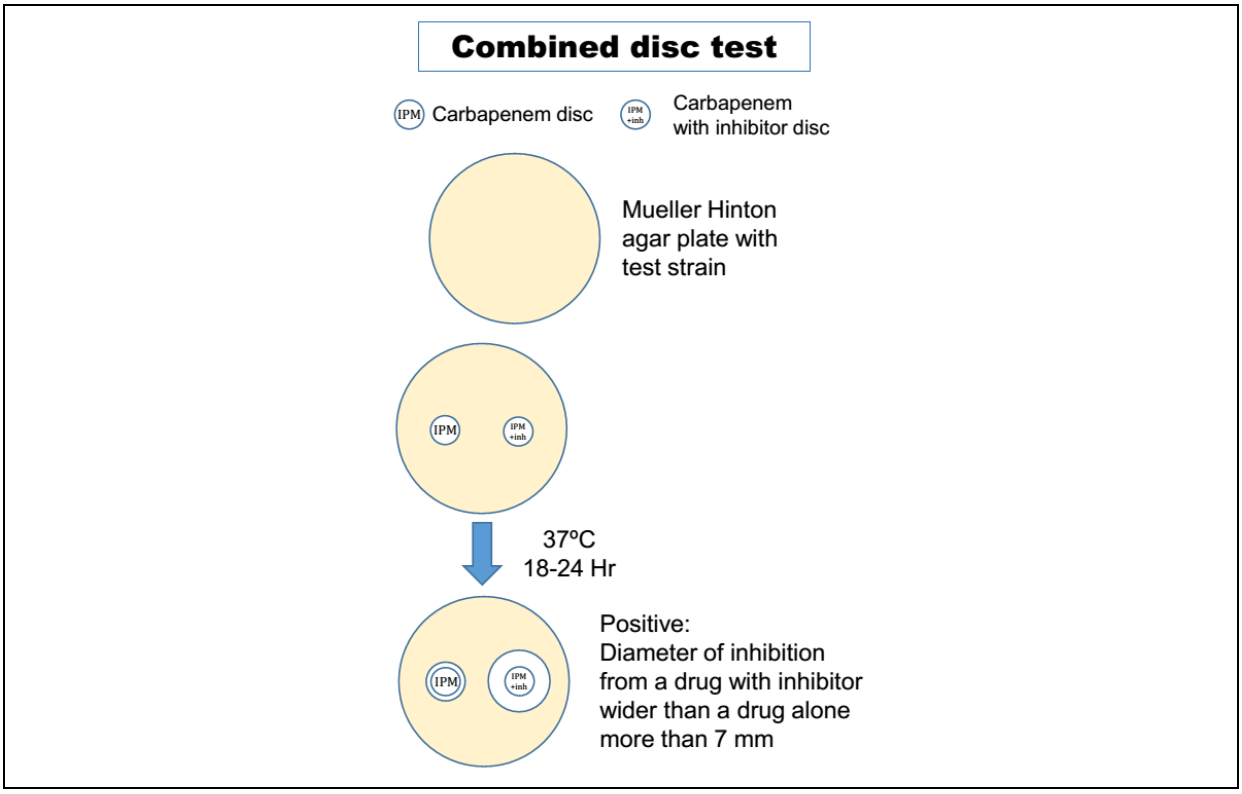

Figure 3. Schematic diagram illustrating the combined disc test

since 2009[17]. The MHT is a phenotypic screening test designed to measure the growth of carbapenem-susceptible *Escherichia coli* ATCC 25922 in the presence of a carbapenemases-producing strain. Briefly, an *E. coli* suspension culture of 0.5 McFarland turbidity is cultivated thoroughly on a Muller-Hinton agar plate. Then, the test strain is streaked as a straight line from the center of the plate, where carbapenem disc is placed (imipenem, ertapenem, or meropenem). If the tested strain produces carpabenemases enzyme, the *E. coli* standard strain can grow around the streak line due to the degradation of carbapenem, as displayed the cloverleaf-like pattern (Figure 2). This method is cheap and easy to perform; however, 24-hour incubation period is still required and false positive results are often observed[18]. In addition, this method is not sensitive for all carbapenemases[6]. It shows high sensitivity and specificity on the detection of class A and class D carbapenemases but not class B carbapenemase[19]. Sachdeva and colleagues compared three phenotypic methods to detect carbapenemases-producing *P. aeruginosa*. The MHT showed positive results only 62.5% while the double synergy and combined disc give 82.3% and 98.6%, respectively[20]. Kim et al., used MHT to detect 33 metallo-β-lactamase producers and found that MHT could detect only 60.6% of MBL producers and 68.0% of NDM producers[21]. The authors also reported that culture media and types of carbapenem drugs used had a significant effect on the sensitivity of MHT detection of MBL subtypes. However, this method was not included in the recent performance standards for antimicrobial susceptibility testing by CLSI[22].

3.2. Combined disc test

The principle of this method is to compare the diameters of inhibition zones between a carbapenem disc and a carbapenem with an inhibitor disc on the culture plate. Several compounds have been used as inhibitors for this method. For example, boronic acid and its derivatives including phenylboronic acid (PB) and 3-aminophenylboronic acid (APBA) have been used as class A carbapenemase inhibitor. Chelating agents such as ethylenediaminetetra-acetate (EDTA) are often used to inhibit class B carbapenemases. For this technique, a carbapenem disc and a carbapenem with inhibitor disc are placed on the lawn of tested bacteria cultured on the surface of Muller-Hinton agar plate. After incubation at 37°C for 18-24 hours, the diameters of each inhibition zones are measured. The positive result is obtained when the diameter of inhibition from a

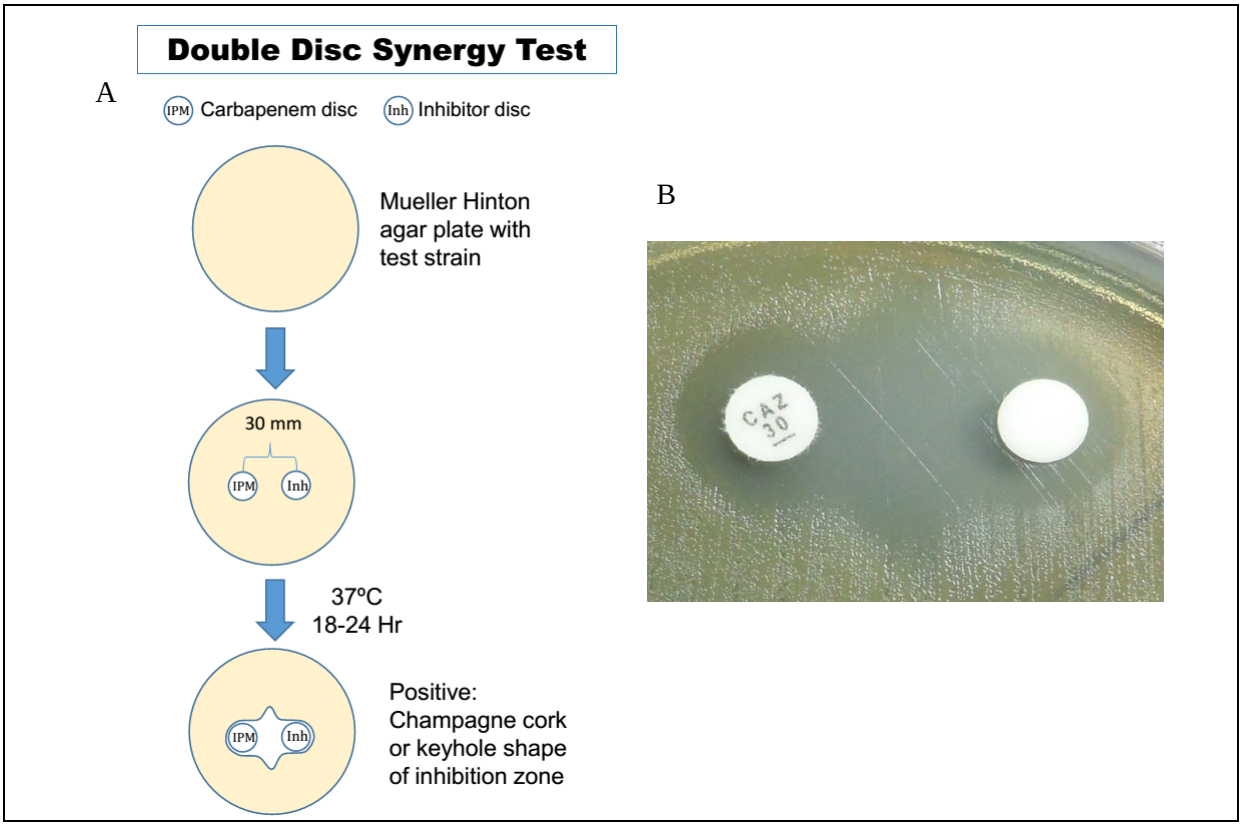

Figure 4. The double disc synergy test: (A) Schematic diagram illustrating the double disc synergy test; (B) Positive result showing the champagne cork shape of inhibition zone.

drug with inhibitor wider than a drug alone more than 7 mm. (Figure 3). However, the criteria used for interpretation may be varied depending on the types of inhibitors and enzymes. For examples, Yong and colleagues used imipenem and EDTA at a concentration of 750 µg to detect metallo-β-lactamases production in *Pseudomonas* spp. and *A. baumannii*. The positive results were obtained when the diameter of inhibition zone showed more than 7 mm. in difference. This method is highly specific to *Pseudomonas* spp. However, 4.3% and 9.1% false negative was reported for metallo-β-lactamases-positive and metallo-β-lactamases-negative *A. baumannii* strains, respectively[23]. Moreover, another factor that influences the inhibitory zone is the concentration of inhibitors. Aktas and Kayzcan used EDTA at the concentration of 0.5 M and 0.1 M with imipenem to detect metallo-β-lactamases production in *A. baumannii*. The results are interpreted as positive when a zone of inhibition increases ≥7 and ≥4 mm. in the presence of 0.5 and 0.1 M EDTA, respectively. The authors reported that 100% metallo-β-lactamases positive was obtained from 0.5 M EDTA. In contrast, metallo-β-lactamases positive was 0% when the criteria of 0.1 M EDTA was applied. The increase in inhibition zone could be possibly by the susceptibility of the microorganism to 0.5 M EDTA, rather than the presence of metallo-β-lactamases[24].

In agreement with Askoy and colleagues, this group used the same criteria to examine metallo-β-lactamases production in 52 strains of *A. baumannii*. The 98% positive results were obtained from 0.5 M EDTA, while only 21% positive results were detected from the same set of samples when using 0.1 M EDTA. When confirmed with PCR, no metallo-β-lactamase-producing strains were detected. It could be possible that high concentrations of EDTA alter bacterial membrane transport[25]. Therefore, the antimicrobial activity of chelating agent should be examined before testing to prevent false positive results. Interestingly, class D carbapenemases are not inhibited by clavulanic acid, tazobactam, boronic acid, and EDTA. There was no specific inhibitor for this enzyme so far. Sodium chloride at a high concentration (100 mM) was applied as an OXA inhibitor by Poirel et al. but this method has not been used in laboratory to detect carbapenemase yet[26].

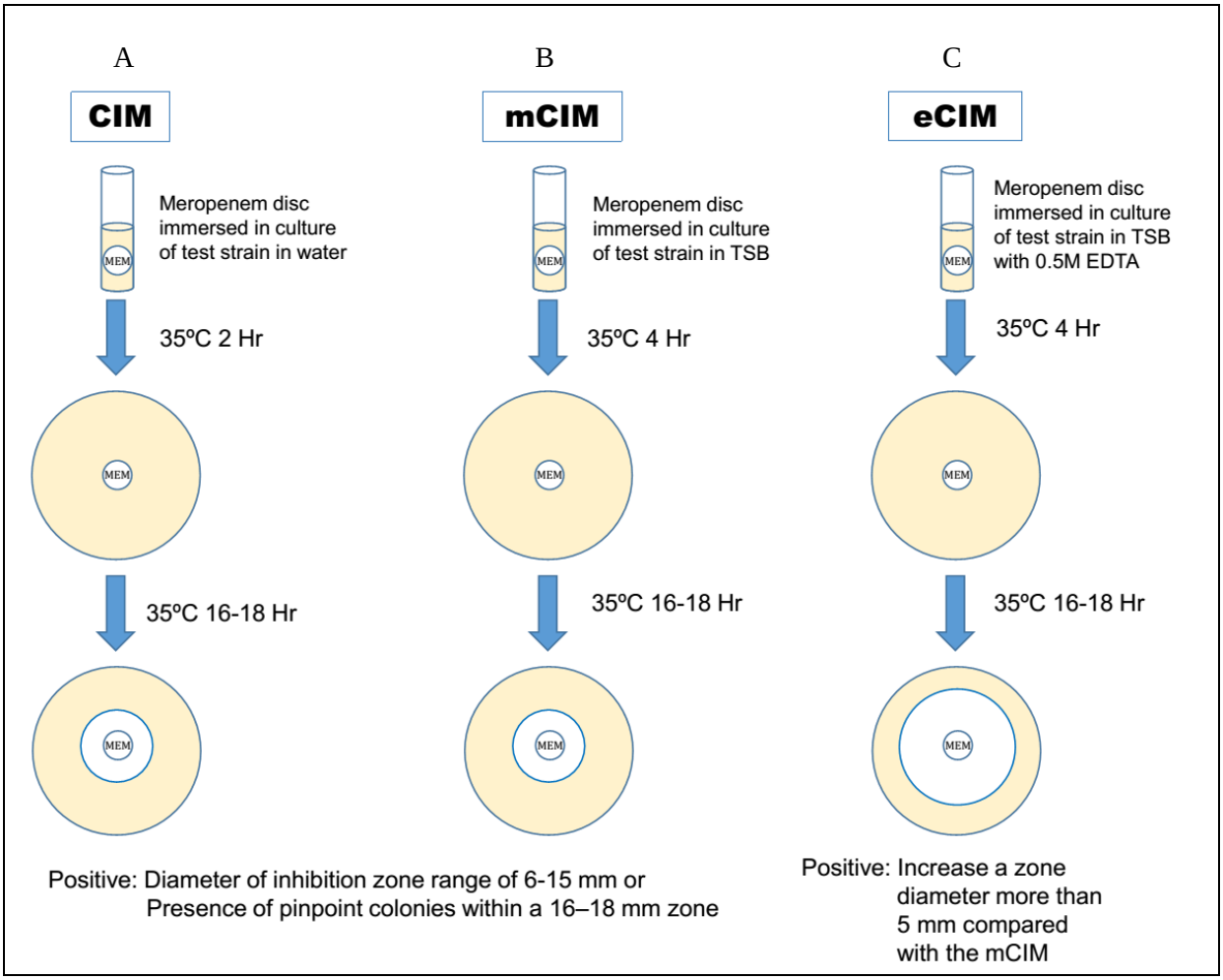

Figure 5. Schematic diagram illustrating the carbapenem inactivation method (A), the modified carbapenem inactivation method (B), and the EDTA carbapenem inactivation method (C).

3.3. Double disc synergy test

This method was developed by Jarlier and colleagues for detection of extended spectrum β-lactamase (ESBLs) producing *Enterobacterales*[27]. Double disc diffusion was performed by applying a carbapenem disc and a carbapenem with inhibitor disc on the bacterial culture plate. The distance between two discs is usually 30 mm. from centre to centre. The positive result is reported when the inhibition zone size of a carbapenem disc at the side closed to a carbapenem with inhibitor disc is increased compared to the other side. The shape of inhibition zone should look like champagne cork or keyhole (Figure 4).

Interestingly, it has been reported that the sensitivity and specificity of this method will be increased when the distance between 2 discs is reduced to 20 mm. due to the diffusion capacity. Moreover, the concentration of inhibitor also has an influence on the results of this method. Aktas and Kayzcan used EDTA at the concentrations of 0.5 M and 0.1 M to detect metallo-β-lactamase-producing-*A. baumannii* strains. The higher concentration of EDTA gave 63.6% positive results, while no positive strains were detected when used only 0.1 M EDTA. This study was confirmed by PCR method and no bla_{MBL} genes were detected[24]. Askoy also examined imipenem-resistant-*A. baumannii* using 0.5 M EDTA and 21% positive results were obtained by double disc synergy test. However, no bla_{MBL} gene was detected by by PCR method. Therefore, it is possible that EDTA may affect the susceptibility of bacteria as reported in the combined disc method[25]. The concentration of EDTA is effected the size of inhibition zone in both metallo-β-lactamases and class D carbapenemases-producing-*A. baumannii*. Because oxacillinases are converted to a less active state leading to increase of the inhibition zone, which can give the false positive results[28]. For double disc synergy test,

there is still no specific inhibitor for class D carbapenemases similar to the combined disc method. Huang et al. suggested temocillin disc or avibactam-supplemented combination disc for the detection of class D OXA-48 enzymes but this study was reported in carbapenemase-producing *Enterobacterales*[29]. Further investigation is still needed to confirm the possibility to be used for carbapenemases-producing-*A. baumannii*.

3.4. Carbapenem inactivation method

Carbapenem inactivation method (CIM) was developed by van der Zwaluw and colleagues in 2015[30]. In this method, meropenem disc is immersed in the suspension of bacteria, prepared by inoculation of a loopful culture in 400 µL of water, and further incubated at 35°C for 2 hours. After incubation, meropenem disc was taken form suspension and placed on a culture plate of *E. coli* ATCC25922, a susceptible indicator strain, and incubated at 35°C for 16-18 hours. If the bacteria produce carbapenemases, meropenem is subsequently inactivated allowing the growth of *E. coli* indicator strain (Figure 5A). Nevertheless, this method has limitation in detection capacity for low carbapenemase activities such as OXA-type, and metallo-β-lactamases that required divalent cations for its activity. To overcome these limitations, the modified carbapenem inactivation method (mCIM) has been developed. The difference between CIM and mCIM is the preparation of bacterial suspension and inactivation time. In mCIM, bacterial suspension is prepared by using a loopful inoculum in tryptic soy broth and extended inactivation time to 4 hours[31] (Figure 5B). It is clearly demonstrated that the sensitivity of mCIM is increased from 82% to 93% compared to the CIM and the specificity is still 100%[31]. This method has been included in CLSI 2017 for detection of carbapenemase producing *Enterobacterales*[32]. The positive results are reported when the diameter of inhibition zone range of 6-15 mm. or presence of pinpoint colonies within a 16–18 mm. zone[22]. When the inhibition zone diameter is more than 19 mm., it is categorized as negative results. The indeterminate results are obtained when the diameter between 16-18 mm. or $\geq$ 19 mm. and the presence of pinpoint colonies within the zone. When the presence or absence of a carbapenemases cannot be confirmed, the confirmation test is required.

It is noteworthy to point out that the mCIM can accurately determine the presence of carbapenemases but cannot distinguish the types of carbapenemases. Therefore, the mCIM is further modified by addition of EDTA (eCIM). This method is performed by adding 20 µL of 0.5M EDTA into another 2-mL tube in parallel with the mCIM[21]. When the mCIM gives a positive result, the eCIM should be interpreted. The production of metallo-β-lactamases will theoretically increase a zone diameter more than 5 mm. compared with the mCIM[22] (Figure 5C). The CLSI is recommended the eCIM as a method that can distinguish metallo-β-lactamases from other carbapenemases. However, in some cases, two carbapenemases are co-produced by only one isolate and the false negative result may occur[33]. In 2017, Uechi and colleagues modified the inactivation process by using Tris-HCL buffer pH7.6 instead of tryptic soy broth (CIMTris) to detect carbapenemase production in *Acinetobacter* spp. and *Pseudomonas* spp[34]. However, CIMTris gave a high percentage of false positive results after 4 hours inactivation[34]. Moreover, Tris-HCl could inactivate meropenem in the absence of an organism[35]. Therefore, the CIMTris method might not be suitable to detect the production of carbapenemases. However, neither eCIM nor mCIM was recommended by CLSI for detecting carbapenemases-producing-*A. baumannii*.

3.5. *Carba NP* test

This method was developed by Nordmann and Poirel (NP) in 2012 to detect carbapenemases production in *Enterobacterales*[36]. The principle of this method relies on pH change when carbapenems are hydrolysed. The colour of solution can be changed from red to orange or yellow, due to a phenol red indicator in the solution. In this test, two tubes must be performed simultaneously. The first tube is containing solution A, consisting of phenol red zinc sulphate and water at pH of 7.8 ± 0.1. The second tube, solution B is prepared by dissolving 6 mg imipenem in 1 mL solution A. Bacterial hydrolysates, prepared in Tris-HCl lysis buffer (B-PERII), are added in both tubes and further incubated at 37°C for 2 hours. The

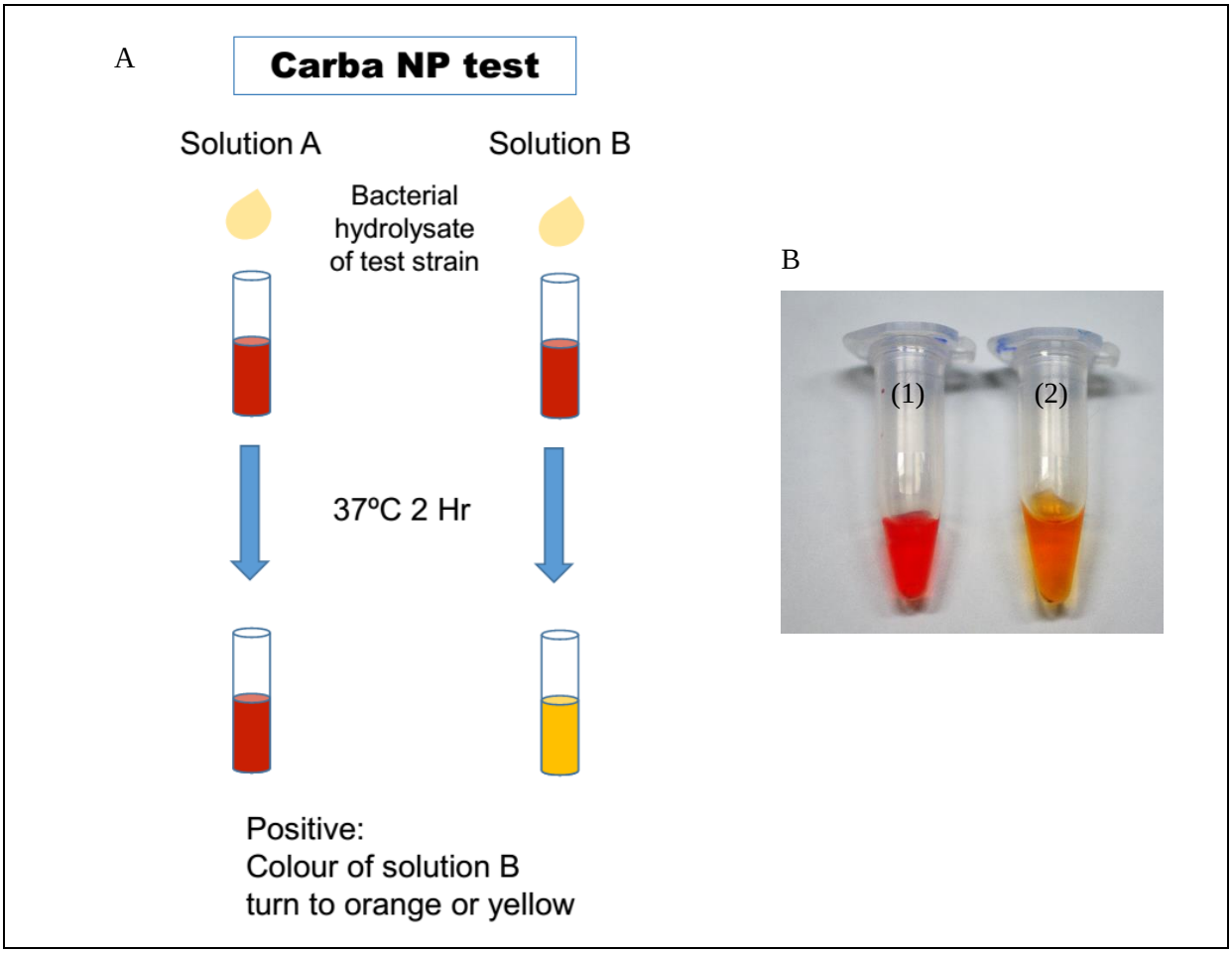

Figure 6: The carba NP test: (A) Schematic diagram illustrating the carba NP test; (B) Result showing the positive result of the test strain, colour of solution A was no change (B1) while colour of solution B turned into orange (B2)

colour of the solution should turn from red to yellow when the test strain produces carbapenemases (Figure 6). In the study of Nordmann, this method showed 100% sensitivity and specificity[36]. The Carba NP method has been recommended by the CLSI to apply for *Enterobacterales*, *P. aeruginosa*, and *Acinetobacter* spp. since 2016[37]. This method gives high sensitivity for class A and class B carbapenemases, ranged from 73-100%[33]. However, the sensitivity is lower when used for detection of OXA-type carbapenemases, found mostly in *A. baumannii*. This is because the OXA-type has weaker activity than class A and B carbapenemases and cannot completely hydrolyse imipenem. Therefore, CLSI did not recommended this method for detection of carbapenemase-producing *Acinetobacter* spp since 2018[38]. In addition, most carbapenem-resistant *A. baumannii* generally involve with other mechanisms[39]. Therefore, the modified Carba NP has been developed to overcome these limitations such as CarbaAcineto NP[40]. In CarbaAcineto NP test, 5M sodium chloride solution is used to break bacterial cells instead of B-PERII to avoid any buffer effects. The other modification is the bacterial inoculum. The amount of bacterial lysate is increased from a half of calibrated inoculum loop (10µL) to a full inoculum loop to augment the quantity of enzyme[40]. It has been reported that the specificity and sensitivity of CarbaAcineto NP are 100% and 94.7%, respectively. However, the CarbaAcineto NP is not recommended by the CLSI for OXA-type carbapenemases detection.

4. CONCLUSION

Increasing recovery of carbapenemases-producing-*A. baumannii* is a serious public health problem worldwide. The accurate and reliable detection method is essential for selection of an appropriate treatment and controlling the spread of resistant bacteria. Several phenotypic methods have been developed for rapid detection of

Table 1. Comparison of phenotypic detection methods for carbapenemase-producing *A. baumannii*

Method	Pros	Cons
MHT	Inexpensive Easy to perform	Longer incubation time False-negative with NDM producers
CIM	Higher sensitivity than carba NP Lower cost than Carba NP Easy interpretable	Longer incubation time False-positive with AmpC over producers
Combined disc method/ double disc synergy	Inexpensive Easy to perform	Longer incubation time Concentration of inhibitor effect the inhibition zone No specific inhibitor for OXA-type carbapenemases
Carba NP	Rapid (2 hours)	False-negative with mucoid isolates and OXA-type producers Reagent has short shelf-life Subjective endpoint interpretation

carbapenemase producing-microorganisms (Table 1). These methods are cheap, easy to perform, and can implement in routine laboratory. The sensitivity and specificity of phenotypic methods are depended on the methods and type of enzymes. With an exception of the Carba NP test, all methods require long incubation time for at least 16-24 hours. Therefore, the quest for specific and sensitive methods is still challenging due to the rapid spread of multi-drug resistant *A. baumannii*.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

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