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# Combined Disc Test and Modified Hodge Test for Detection of Carbapenemase-Producing Gram-Negative Bacilli

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## ABSTRACT

**Introduction:** Identification of carbapenem resistance and its mechanism in clinical isolates is important for patient management, epidemiological studies and control of their spread. The objective of this study was to evaluate phenotypic methods; the Modified Hodge Test (MHT) and Combined Disc Test (CDT) in the detection and typing of carbapenemase-producing isolates.

**Methods:** A total of 396 clinical isolates (117 *Pseudomonas* spp, 126 *Acinetobacter* spp, 86 *E. coli* and 67 *Klebsiella pneumoniae*) were screened for carbapenem resistance by modified Kirby-Bauer Disc diffusion method using Imipenem. Carbapenem-resistant isolates were further tested for carbapenemase production by MHT and CDT.

**Results:** Seventy-four isolates were carbapenem-resistant isolates which included 24 *Pseudomonas* spp, 27 *Acinetobacter* spp, 14 *Klebsiella pneumoniae* and 9 *E. coli*. MHT detected 59(79.72%) while CDT detected 62(83.43%) as carbapenemase producer (p-value >0.05). CDT further distinguished carbapenemases as; 20 molecular class A, 33 molecular class B, and 9 co-producing class A and B while 12 were negative in CDT for any carbapenemase. As high as 85.71% of *Klebsiella pneumoniae* isolates, 85.15% *Acinetobacter* spp, 77.77% *E. coli* and 70.83% *Pseudomonas* spp were found to be carbapenemase producers by MHT. On the other hand, carbapenemase detection by CDT was noticed among 95.83% *Pseudomonas* spp, 92.85% *Klebsiella pneumoniae*, 88.88% *E. coli*, and only 66.66% *Acinetobacter* spp.

**Conclusion:** MHT and CDT are acceptable phenotypic methods for the detection of carbapenemase production and differentiation. These methods are easier, cheaper and can be performed along with routine AST.

***Keywords:*** *Betalactamase; Carbapenem; Carbapenemase*

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## INTRODUCTION

Antimicrobial resistance has been a global problem, contributing to increased treatment costs, hospital stay, morbidity and mortality.[1] Production of antibiotic hydrolyzing enzymes is one of the important mechanisms that makes microorganisms resistant to antibiotics. In recent years, bacteria producing antibiotic hydrolyzing enzymes like ESBLs (extended spectrum β-lactamases), carbapenemases, etc. are increasing in numbers and also spreading globally. Many nonfermenting gram-negative bacteria (e.g. *Pseudomonas* spp., *Acinetobacter* spp.) as well as members of *Enterobacteriaceae* are becoming resistant to commercially available carbapenems.[2] Carbapenems are the group of beta-lactam antibiotics, regarded as last resort antibiotics and have superior activity against gram positive as well as gram negative bacteria.[3] The resistance mechanisms include production of enzyme beta lactamase (e.g. carbapenemase), efflux pump, mutations altering expression and function of PBPs and porin channels and sometimes combination of different mechanism.[4,5]

Carbapenemases are family of beta lactamase enzymes capable of hydrolysing almost all types of beta lactam antibiotics and are not inhibited by commercially available beta lactamase inhibitors.[6] Carbapenemase encoding genes are located in chromosome as well as plasmids and latter one possesses greater risk of spread in homogenous as well as heterogenous bacterial population.[7]

It is important to know the prevalence of carbapenem resistance among the gram-negative clinical isolates for the empirical patient management and epidemiological studies. Unfortunately, the pattern, prevalence of antibiotic resistance keeps changing. This emphasises the need of regular surveillance for updated recent reports. The methods of detection are either PCR (Polymerase chain reaction) or phenotypic methods like Modified Hodge Test (MHT), Combined Disc Test (CDT) etc. PCR is expensive though is very specific and sensitive while phenotypic methods are cost effective, easier to perform and have acceptable sensitivity and specificity. Clinical laboratories are not using these phenotypic tests as data are less on their sensitivity and specificity.

Thus, this study was aimed to assess the potential of the phenotypic tests e.g. CDT and MHT in detection of carbapenemase production and typing.

## METHODS

This was a prospective study conducted in Manipal Teaching Hospital, Pokhara. A total of 396 non-repeating clinical isolates of *Pseudomonas, Acinetobacter spp, Escherichia coli and Klebsiella pneumoniae* were collected. These isolates were obtained from various clinical samples like urine, blood, sputum, throat swab, pus, body fluids from different wards which were sent for routine bacteriological culture and antibiotic sensitivity. The isolates were identified by standard microbiological phenotypic methods.[8] All these isolates were screened for imipenem(10mcg) and meropenem(10mcg) resistance by Kirby-Bauer Disk diffusion method following CLSI guidelines.[9]

The isolates that were resistant to carbapenem were tested for carbapenemase production phenotypically by Modified Hodge test (MHT) and combined disk test (CDT). Differentiation of carbapenemase type was done by CDT by using specific carbapenemase inhibitors.

**Modified Hodge Test (MHT):**

MHT was performed according to CLSI guidelines for detection of carbapenemase production in *Enterobacteriaceae*.[9] It is phenotypic method for test of carbapenemase production.

Briefly, bacterial suspension of indicator strain *E. coli* ATCC 25922 was prepared and was matched with 0.5 MacFarland standard

and diluted to 1:10 in normal saline. Lawn culture of indicator *E. coli* ATCC 25922 was made on Muller Hinton agar plate. Imipenem (10 mcg) was kept at the centre of the plate and test isolates were streaked straight away from the antibiotic disk. If the test isolate produced carbapenemase, it allowed growth of indicator *E. coli* towards a carbapenem disk. The positive result gave a characteristic clover-leaf like indentation.

## Combined Disk Test (CDT):

This is a phenotypic method to identify and classify the type of carbapenemase produced by carbapenem resistant isolates and was performed in accordance with the standsrds improvised earlier.[10] This method is based on the synergy between inhibitors like ethylenediamine tetra-acetic acid (EDTA) plus carbapenem for MBL detections, Phenyl Boronic Acid (PBA) plus carbapenem for class A carbapenemase detection and EDTA, PBA together plus carbpenem for co-production of class A and class B carbapenemase.

Imipenem discs were soaked with calculated amount of solution of carbapenemase inhibitors so that each imipenem discs contain 400 mcg of PBA, 292 mcg of EDTA and both 400 mcg of PBA plus 292 mcg of EDTA.

Lawn culture of test organism was done on Muller-hinton agar. Imipenem(10mcg) without any inhibitor and 3 discs of imipenem containing PBA, EDTA and PBA plus EDTA were placed on agar surface. The agar plates were incubated at 37$^{o}$C overnight. The diameter of inhibited zone around the imipenem disc with PBA, EDTA and PBA plus EDTA were measured and interpretation was done. Increase of zone diameter by ≥5mm with addition of PBA, EDTA, and PBA plus EDTA were regarded as production of molecular class A, class B and both class A and B carbapenemase respectively.

## RESULTS

The total number of isolates screened for carbapenem resistance was 396. The isolates composed of 117 *Pseudomonas spp*, 126 *Acinetobacter spp*, 86 *E. coli*, and 67 *Klebsiella pneumoniae.* Overall 74 isolates were found to be resistant to imipenem (10 mcg) and meropenem (10 mcg). Out of these 74 carbapenem resistant isolates, 59 (79.72%) were detected as carbepenemase producer by MHT and 62 (83.78%) by CDT (Table 1).

**Table 1: Distribution of carbapenem-resistant and carbapenemase producers**

| Isolate | Carbapenemase producer | | | |
|---|---|---|---|---|
| | MHT | | CDT | |
| | Positive N (%) | Negative N (%) | Positive N (%) | Negative N (%) |
| ***Pseudomonas* spp (n=24)** | 17 (70.83) | 7 (29.16) | 23 (95.83) | 1 (4.16) |
| ***Acinetobacter* spp (n=27)** | 23 (85.15) | 4 (14.81) | 18 (66.66) | 9 (33.33) |
| ***E. coli* (n=9)** | 7 (77.77) | 2 (22.22) | 8 (88.88) | 1 (11.11) |
| ***Klebsiella pneumoniae* (n=14)** | 12 (85.71) | 2 (14.28) | 13 (92.85) | 1 (7.14) |
| **Total (n=74)** | 59 (79.72) | 15 (20.27) | 62 (83.78) | 12 (16.21) |

**Table 2: Type of carbapenemase by CDT**

| Isolates | Class A | Class B | Both A and B | Not detected | Total |
|---|---|---|---|---|---|
| *Pseudomonas* spp | 2 | 15 | 6 | 1 | 24 |
| *Acinetobacter* spp | 8 | 10 | 0 | 9 | 27 |
| *E. coli* | 3 | 4 | 1 | 1 | 9 |
| *Klebsiella pneumoniae* | 7 | 4 | 2 | 1 | 14 |
| Total | 20 | 33 | 9 | 12 | 74 |

**Table 3: Detection of carbapenemase by CDT and MHT**

| | Detected (%) | Not detected (%) | P value |
|---|---|---|---|
| MHT | 59 (79.72) | 15 (20.27) | >0.05 |
| CDT | 62 (83.78) | 12 (16.21) | |

With the use of CDT, it was observed that, 20 (27.02%) of 74 carbapenem resistant isolates were molecular class A producers, 33 (44.59%) were molecular class B producers, 9 (12.16%) isolates were identified as co-producers of both molecular class A and B (Table 2). Carbapenemase production was not detected among 12 (16.21%) and 15 (20.27%) isolates by CDT and MHT respectively (Table 3). As high as 85.71% of *Klebsiella pneumoniae* isolates, 85.15% *Acinetobacter* spp, 77.77% *E. coli* and 70.83% *pseudomonas* spp were found to be carbapenemase producers by MHT. On the other hand, carbapenemase detection by CDT was noticed among 95.83% *Pseudomonas* spp, 92.85% *Klebsiella pneumoniae*, 88.88% *E. coli,* and only among 66.66% *Acinetobacter* spp (Table 1).

## DISCUSSION

In this study, 74 isolates were resistant to imipenem (10 mcg) and meropenem (10 mcg). These isolates were presumed to be probable carbapenemase producers. Phenotypic confirmation of carbapenemase was determined by MHT and CDT. Researchers previously showed MHT and CDT to be highly sensitive and specific phenotypic methods for the detection of carbapenemase production in *Pseudomonas* spp and *Acinetobacter* spp.[11,12] In this study, carbapenemase production detected by MHT was among 79.72% and that detected by CDT was in 83.78% of isolates. Abouelfetouh and colleagues recently reported carbapenemase production by 68.9% and 95.9% of *Acinetobacter baumanii* clinical isolates when compared with PCR.[13] Recent CLSI guidelines suggested Carba NP and carbapenem inactivation method (CIM) as recommended procedures for detecting carbapenemase production by *Enterobacteriaceae* and *Pseudomonas* spp. MHT and CDT though are not CLSI-recommended procedures for carbapenemase detection, yet many researchers showed that these tests were reliable and the results were comparable with other standard techniques.[12] Moreover, MHT and CDT were user-friendly, could easily be performed with minimal laboratory setup, and were cost-effective when compared with other recommended procedures.[14]

This study showed CDT as an effective method to differentiate carbapenemase types. With the use of CDT, it was found that out of 74 carbapenem-resistant isolates, 20(27.02%) were molecular class A producers, 33(44.59%) were molecular class B producers, 9(12.16%) isolates were identified as co-producing both molecular class A and B. These findings can guide clinicians to choose appropriate antibiotics. On the patient management aspect, infection by isolates producing only molecular

class A carbapenemase can be managed by the additional use of beta-lactamase inhibitors like clavulanic acid, avibactam, and relebactam.[15-17] These inhibitors are also effective against most of the Molecular Class D carbapenemases.[15] Similarly, for pure MBL-producing isolates, Aztreonam can be the best treatment of choice. MBL carbapenemases are also inhibited by EDTA but its clinical use is not approved as it is known to cause more harm in patients.[18] Moreover, identifying the type of carbapenemase is important in epidemiological studies, for tracing and control of outbreaks within wards, hospitals or communities. These things could work if the laboratory could provide an AST report along with the type of carbapenemase involved. So, this emphasizes the need for robust, cost-effective and easier methods like CDT for detection and identification and carbapenemase type determination.

In this study, CDT was found to be the best for *Pseudomonas* spp and *Klebsiella pneumoniae* with detection rate of 95.83% and 92.85% respectively, followed by *E coli* at 88.88%, *Acinetobacter* spp at 66.66%. Similarly, MHT had the highest detection rate in *Klebsiella pneumoniae* (85.71%), followed by *Acinetobacter* spp (85.15%), *E coli* (77.77%) and *Pseudomonas spp*. 70.83%. Notwithstanding the above, CDT could miss molecular class D, as this group of enzymes are not inhibited by EDTA and PBA which were used in the test. On the other hand, Class D beta-lactamases like OXA-24, OXA-23, and OXA-58 were commonly associated with MDR *Acinetobacter* spp. This could be the reason for the least detection of carbapenemase by CDT among *Acinetobacter* spp.[19] Detection rate by MHT was the lowest for *Pseudomonas* spp when compared with other isolates. Out of 7 MHT-negative *Pseudomonas* spp, 3 (5.8%) showed indeterminate MHT results. The indicator strain did not grow near to test strain**.** This observation was not found with other isolates. This could be because of the production of pyocyanin or pyoverdin which has an antibacterial effect.[20] Similar antagonistic effects of clinical and environmental isolates of *Pseudomona*s spp against coliforms were already been defined and studied in the past.[21]

In a nutshell, early detection and differentiation of carbapenemase-producing isolates is necessary for patient management, epidemiological studies, and infection control measures. Tests like MHT and CDT were found cost-effective, easy to perform, can be performed in minimal laboratory setup and have good sensitivity. Moreover, CDT can simultaneously detect and identify carbapenemase types.

## CONCLUSION

MHT and CDT are acceptable phenotypic methods for the detection of carbapenemase production and identification of carbapenemase types in terms of cost and sensitivity. The burden of carbapenem resistance is high in most of the low- and middle-income countries. These methods are recommended to be employed in routine AST methods if isolates are carbapenem-resistant. Timely detection of the mechanism of resistance to carbapenem can help in narrowing the treatment choice, planning for suitable measures for its control and prevention of the spread of resistant strains at different levels.

## CONFLICT OF INTEREST

None

## SOURCES OF FUNDING

None

## REFERENCES

1. Mauldin PD, Salgado CD, Hansen IS, Durup DT, Bosso JA. Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant

gram-negative bacteria. Antimicrob Agents Chemother. 2010;54(1):109–15. http://dx.doi.org/10.1128/aac.01041-09

2. Miriagou V, Cornaglia G, Edelstein M, et al. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin Microbiol Infect. 2010;16(2):112–22. http://dx.doi.org/10.1111/j.1469-0691.2009.03116.x
3. Bassetti M, Nicolini L, Esposito S, Righi E, Viscoli C. Current status of newer carbapenems. Curr Med Chem. 2009;16(5):564–75. http://dx.doi.org/10.2174/092986709787458498
4. Limansky AS, Mussi MA, Viale AM. Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. J Clin Microbiol.2002;40(12):4776–8. http://dx.doi.org/10.1128/jcm.40.12.4776-4778.2002
5. Rodríguez-Martínez J-M, Poirel L, Nordmann P. Molecular Epidemiology and Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemothe. 2009;53(11):4783–8. http://dx.doi.org/10.1128/aac.00574-09
6. Livermore DM, Woodford N. The β-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. Trends Microbiol. 2006;14(9):413–20. http://dx.doi.org/10.1016/j.tim.2006.07.008
7. 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. http://dx.doi.org/10.1111/1469-0691.12655
8. Collee JG, Mackie TJ, McCartney JE. Mackie & McCartney Practical Medical Microbiology. 14th ed. New York: Churchill Livingstone. 1996.
9. CLSI. M100-S25 performance standards for antimicrobial susceptibility testing; Twenty-fifth informational supplement; 2015.
10. Tsakris A, Poulou A, Pournaras S, et al. A simple phenotypic method for the differentiation of metallo- -lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. J Antimicrob Chemother. 2010;65(8):1664–71. http://dx.doi.org/10.1093/jac/dkq210
11. Noyal MJ, Menezes GA, Harish BN, Sujatha S, Parija SC. Simple screening tests for detection of carbapenemases in clinical isolates of nonfermentative Gram-negative bacteria. Indian J Med Res. 2009;129(6). https://pubmed.ncbi.nlm.nih.gov/19692754/
12. Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH. Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase-producing strains of Pseudomonas and Acinetobactet species. Clin Microbiol Infect. 2001;7(2):88–91. http://dx.doi.org/10.1046/j.1469-0691.2001.00204.x
13. Abouelfetouh A, Torky AS, Aboulmagd E. Phenotypic and genotypic characterization of carbapenem-resistant Acinetobacter baumannii isolates from Egypt. Antimicrob Resist Infect Control. 2019;8(1). http://dx.doi.org/10.1186/s13756-019-0611-6
14. Ramana KV, Rao R, Sharada CHV, Kareem MA, Reddy R, Ratna Mani MS. Modified Hodge test: A useful and the low-cost phenotypic method for detection of carbapenemase producers in Enterobacteriaceae members. J Nat Sci Biol Med.2013;4(2):346. http://dx.doi.org/10.4103/0976-9668.117009
15. Queenan AM, Bush K. Carbapenemases: The versatile β-lactamases. Clin Microbiol Rev. 2007;20(3):440–58. http://dx.doi.org/10.1128/cmr.00001-07
16. Shirley M. Ceftazidime-avibactam: A review in the treatment of serious gram-negative bacterial infections. Drugs. 2018;78(6):675–92. http://dx.doi.org/10.1007/s40265-018-0902-x
17. Hilbert DW, DeRyke CA, Motyl M, Hackel M, Young K. Relebactam restores susceptibility of resistant Pseudomonas

aeruginosa and Enterobacterales and enhances imipenem activity against chromosomal AmpC-producing species: analysis of global SMART 2018–2020. BMC Microbiol. 2023;23(1). http://dx.doi.org/10.1186/s12866-023-02864-3

18. Lanigan RS, Yamarik TA.Final report on the safety assessment of EDTA, calcium disodium EDTA, diammonium EDTA, dipotassium EDTA, disodium EDTA, TEA-EDTA, tetrasodium EDTA, tripotassium EDTA, trisodium EDTA, HEDTA, and trisodium HEDTA. Int J Toxicol. 2002;21(2):95–142. http://dx.doi.org/10.1080/10915810290096522
19. Poirel L, Naas T, Nordmann P. Diversity, epidemiology, and genetics of class D β-lactamases. Antimicrob Agents Chemother. 2010;54(1):24–38. http://dx.doi.org/10.1128/aac.01512-08
20. Baron SS, Rowe JJ. Antibiotic action of pyocyanin. Antimicrob Agents Chemother. 1981;20(6):814–20. http://dx.doi.org/10.1128/aac.20.6.814
21. Martins VV, Macarenco AC, Gradella DG, Stehling EG. Antagonism between clinical and environmental isolates of Pseudomonas aeruginosa against coliforms. Water Sci Technol Water Supply. 2014;14(1):99–106. http://dx.doi.org/10.2166/ws.2013.178