The Phenotypic Detection Of Carbapenemase In Meropenem Resistant Acinetobacter Calcoaceticus–Baumannii Complex In A Tertiary Care Hospital In South India

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ABSTRACT

Background and objectives: The predominant Acinetobacter spp. in clinical settings are the members of the Acinetobacter calcoaceticus– baumannii complex which are multi drug resistant and are responsible for causing outbreaks. Carbapenem resistance due to metallo-β-lactamase production in Acinetobacter spp. is on the rise. We investigated the production of carbapenemase among the meropenem resistant Acinetobacter spp. which were further screened for metallo-β-lacatmase production. The co-resistance to other classes of antibiotics was also investigated.

Materials and Methods: Forty five non duplicate consecutive meropenem resistant Acinetobacter calcoaceticus– baumannii complex were investigated for carbapenemase production by the modified Hodge test. The carbapenemase producing isolates were further screened for metallo-β-lacatmase production by the combined disc diffusion test by using imipenem with EDTA as the chelator. The co-resistance to other classes of antibiotics was also investigated to identify the multi drug resistant isolates.

Results: Of the 45 non duplicate consecutive meropenem resistant Acinetobacter calcoaceticus– baumannii complex which were screened, 95% (43/45) of them were multi drug resistant and 71% (32/45) were found to be carbapenemase producers by the modified Hodge test, of which 21% (7/32) were found to be metallo-β-lacatmase producers phenotypically by the combined-disk test.

Conclusion: Carbapenem resistance in Acinetobacter calcoaceticus–baumannii complex is very high and is predominantly due to carbapenemase production. However, metallo-β-lactamase production among these isolates is not very high but is gradually increasing. Only 21% of our isolates were metallo-β-lactamase phenotypes, thus suggesting that the production of carbapenem hydrolyzing oxacillinase is still the most common mechanism of resistance to carbapenems in this species.

Key Words : Metallo-β-lactamase, Acinetobacter spp., Carbapenems

INTRODUCTION

Acinetobacter spp. is considered as the most common oxidase negative non fermenting gram negative bacilli which have been encountered in the clinical laboratory. It is widely distributed in nature and in the hospital environment, thus causing opportunistic infections in debilitated patients, especially in intensive care units. [1] *Acinetobacter spp.* has been included in the list of the six top priority dangerous drug resistant microbes, which has been released by the Infectious Disease Society of America. The predominant *Acinetobacter spp.* in clinical settings are members of the *Acinetobacter calcoaceticus– baumannii complex (Acb complex)*, which are multi drug resistant (MDR), thus leaving carbapenems as the only therapeutic option. [2] The *Acb complex* can frequently cause outbreaks and are very versatile, surviving in the hospital environment for long periods, thereby posing a difficult challenge for infection control. The first known carbapenem resistant *Acinetobacter spp.* was isolated in 1983 in Scotland and the carbapenem hydrolyzing β-lactamase was designated as oxacillinase-23 (OXA-23). [3] Though OXA is the predominant carbapenemase which is responsible for carbapenem resistance, reports on the IMP- or VIM-class metallo-β-lactamase (MBL) producing *Acinetobacter spp.* are also on the rise. [4] We investigated the production of carbapenemase among the meropenem resistant strains of the *Acinetobacter spp.* which were further phenotypically screened for MBL production. The co-resistance to other classes of antibiotics was also investigated.

MATERIALS AND METHODS

Forty five consecutive, non duplicate, meropenem resistant isolates of the *Acb complex* which were isolated from the samples of patients who were admitted to our hospital from August 2009 to January 2010 were included in our study. Only the *Acb complex* isolates which were obtained as a predominant growth in the culture, with relevant clinical history, were included in our study.

There are 17 recognized species within the genus, Acinetobacter. The major genus characteristics include the inability to ferment glucose (non-fermenter), lack of oxidase production (oxidase negative), and non-motility. The specific phenotypic characteristics include the appearance as cocci or coccobacilli on gram staining, the ability to grow on MacConkey's agar and the resistance to penicillin. Many of the species within this genus are difficult to separate reliably by phenotypic methods alone and frequently are placed into groups or complexes based on the biochemical test results. The predominant *Acinetobacter spp.* in clinical settings is identified as the *Acb complex* as all of them have the ability to oxidize glucose and are therefore described as the saccharolytic species of this genus. [2], [6] Antibiotic susceptibility was assessed by the Kirby Bauer disc diffusion method. [7] The antibiotics which were included were ciprofloxacin (5μg), cefipime (30μg), ceftazidime (30μg) and gentamycin (10μg), piperacillin (100μg), amikacin (30μg), piperacillin /tazobactam (100/10μg), cefoperazone/sulbactam (75/30μg) and meropenem

(10µg). Meropenem resistance was used as the indication for carbapenemase production. A total of 45 *Acb complex* isolates with a reduced susceptibility to meropenem were further screened for carbapenemase and MBL productions by the modified Hodge test (MHT) and the combined disc test (CDT) respectively.

The Clinical Laboratory Standard Institute (CLSI) has not yet included any standardized phenotypic detection method for screening MBL positive strains in the *Acb complex*, though it has included screening and confirmatory tests for suspected carbapenemase production in Enterobacteriaceae. [7]

THE MODIFIED HODGE TEST

Lee et. al had modified the Hodge test which was developed to detect penicillinase producing Neisseria gonorrhoea by substituting Escherichia coli ATCC 25922 for penicillin susceptible Staphylococcus aureus ATCC 25923, and a 10µg imipenem disc for a 10 U penicillin disk. [5] An overnight culture suspension of E.coli coli ATCC 25922 which was adjusted to one tenth turbidity of the McFarland no.0.5 tube was inoculated evenly on the surface of a Muller-Hinton agar plate containing 70µg/ml of zinc sulfate. After a brief drying at room temperature, an imipenem disk was placed in the center of the plate. Meropenem resistant test strains from an overnight culture were streaked heavily from the edge of the disk to the periphery of the plate. The presence of a distorted or clover leaf shaped inhibition zone was interpreted as positive for carbapenemase producing isolates. [5]

THE COMBINED DISC TEST

The combined disc test was carried out for 32 MHT positive isolates. The test strain was inoculated on plates with Mueller Hinton agar as recommended by CLSI for antibiotic sensitivity testing by the disk diffusion method. The presence of MBL was determined by placing two imipenem disks on the inoculated plate, in which 10 µl of 0.1 M EDTA (292µg) was added to one of the imipenem disks. After overnight incubation at 37°C, the inhibition zones of imipenem and imipenem with EDTA were compared. A zone difference of >4mm between the imipenem and the imipenem-EDTA inhibition zones confirmed the isolate to be MBL positive. [4]

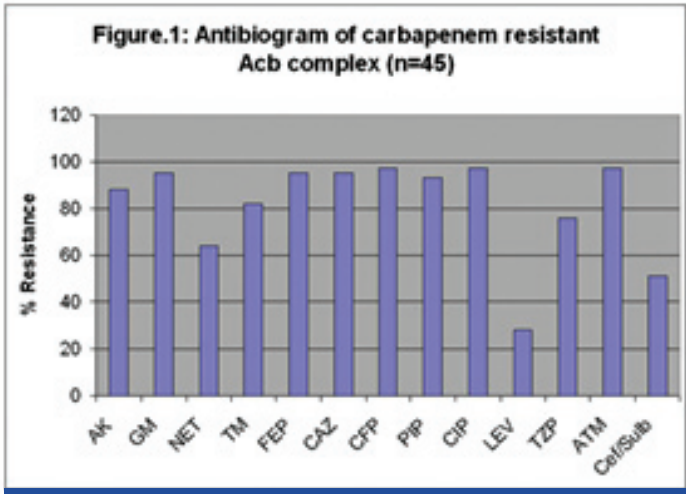
RESULTS

Antibiotic susceptibility testing of the 45 non duplicate carbapenem resistant isolates of the *Acb complex* isolates by the Kirby-Bauer disk diffusion method showed that 95% (43/45) of them were multi drug resistant (MDR). Multi drug resistance is defined as the resistance to three or more classes of antibiotics, including aminoglycosides, antipseudomonal penicillins, carbapenems, cephalosporins and quinolones. Co-resistance to other classes of antibiotics is shown in [Table/Fig 1]. The sample wise distribution of the 45 non duplicate carbapenem resistant isolates of the *Acb complex* is shown in [Table/Fig 2].

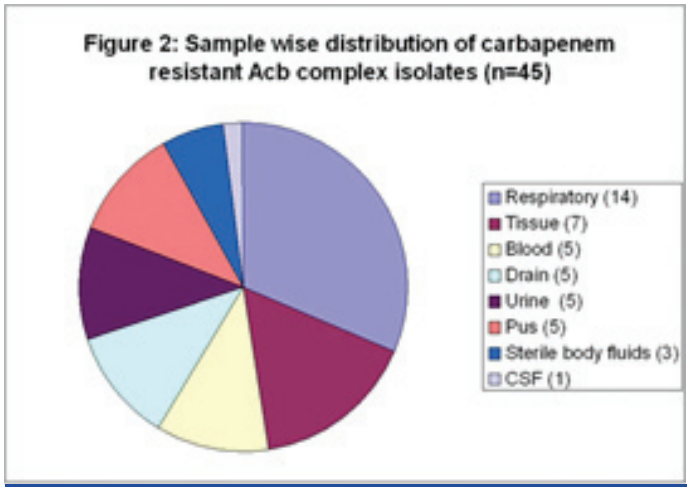
Of the 45 isolates of the *Acb complex* which were screened, 71% (32) were found to be carbapenemase positive by MHT. These positive isolates were further tested by CDT and 21% (7/32) were found to be MBL producers phenotypically.

DISCUSSION

Carbapenems are used as the last resort for treating MDR Gram-negative infections in any noscomial setting. Carbapenems have



[Table / Fig 1] : Antibiogram of carbapenem resistant Acb complex (n=45) AK, amikacin; GN, gentamicin; NET, netilmicin; TM, tobramycin ; FEP,cefipime; CAZ, ceftazidime; CFP, cefoperazone; PIP, piperacillin; CIP, ciprofloxacin; LEV, levofloxacin; TZP, piperacillin-tazobactam; ATM, aztreonam; Cef/Sulb, cefoperazone-sulbactam.



[Table / Fig 2] : Sample wise distribution of carbapenem resistant Acb complex isolates (n=45)

a broad spectrum activity and they are stable to hydrolysis by most of the β-lactamases, including the extended spectrum β lactamases (ESBLs) and the AmpC β-lactamases. However, there has been an alarming increase in the reports on carbapenem resistant *Acinetobacter spp.* over the last decade. [8]

Carbapenem resistance in the *Acb complex* is attributed to various causes such as the reduced expression of the outer membrane proteins (29 kDa, 33-36kDa), the modification of the penicillin binding proteins, the specific drug efflux and carbapenemase production. The carbapenemases which are found in A. baumannii belong to either the OXA class D family of serine-β-lactamases (OXA-23 to 27, 40, 51 ,64 to 71) or to the IMP/VIM class B family of MBLs (IMP-1, 2, 4 and 5 and VIM-1 and 2). [9] Carbapenem resistant *Acb complex* which produce carbapenemases are being increasingly isolated in recent times. [10] Recently, a new type of MBL called SIM-1 has also been reported. [11]

MBL producing isolates have the ability to rapidly disseminate within an institution and lead to poor outcomes when infection ensues; therefore, it is immensely important to detect MBL in laboratory settings to avoid therapeutic failure. [4] Unfortunately, CLSI has not yet documented any standardized phenotypic

detection method for screening MBL positive strains in the *Acb complex*, though it has included screening and confirmatory tests for suspected carbapenemase production in Enterobacteriaceae. [7] However, MBL positive *Acinetobacter spp.* strains can phenotypically be detected by using 1) the Double disk synergy test (DDST) by using IPM (imipenem)-EDTA (ethylene diamine tetra acetic acid), IPM-SMA (sodium mercaptoacetic acid) and the CTZ (ceftazidime)-SMA combinations. [12] 2) the Modified Hodge test by using a 10 µg imipenem disk and zinc sulfate solution. [5] 3) the combined-disk test (CDT) by using the IPM-EDTA combination. [4] 4) the Etest by using the combinations of IPM-EDTA, IPM-MPA (mercaptopropionic acid) and CTZ-EDTA. [13] 5) the Agar dilution method to determine the MIC of the IPM-EDTA combination. [14] 6) the carbapenem hydrolysis assay by using crude cell sonicates with and without EDTA treatment at 300C by using a spectrophotometer. [15] Among these tests, we selected the MHT with zinc sulfate and the CDT methods for this study, as they were highly sensitive and specific, easy to carry out and the materials required were cost effective, non-toxic and were easily available and hence, they could be easily adapted in laboratories for routinely detecting MBL phenotypes.

Of the 45 carbapenem resistant isolates which were included in our study, only 32 isolates were found to produce the carbapenemase enzyme and the remaining 13 strains were found to be carbapenemase negative by MHT. They probably showed resistance to carbapenem due to the efflux mechanism. [16]

MHT is used as a screening method to detect carbapenemas producers and therefore, it gives a positive result with strains that produces carbapenemases like MBL, Klebsiella pneumonia carbapenemase (KPC) and OXA-type β lactamases. Carbapenem resistance can also occur as a result of the reduced expression of the outer membrane proteins combined with the AmpC-lactamases and the modification of the penicillin binding proteins and the efflux mechanisms. [17] In our study, of the 32 modified Hodge test positive strains, only 7 isolates were found to be MBL phenotypes; the remaining 25 (78%) strains presumably had the oxacillinase enzyme, as the OXA-23 encoding gene was found to be highly disseminated in the *Acb complex* from the Asia-Pacific nations, including India. [18]

Lee *et.al* reported that only 66% of the 39 MBL producing isolates of Pseudomonas aeruginosa and *Acinetobacter spp.* gave positive results by the Hodge test. He also noted that 10 more isolates with equivocal results became positive when the 10 µg IPM disks were supplemented with 10 µl of 50 mM zinc sulfate. Alternatively, the addition of zinc sulfate to Mueller-Hinton agar to get a final concentration of 70µg/ml also improved the test performance. [12]

Koh *et.al* in 2006, found that 30.5% of the 114 isolates of imipenem resistant *Acb complex* isolates showed carbapenemase activity; of which 4 isolates carried blaIMP-4, 5 carried blaOXA-58 and 40 carried blaOXA- 23. All four strains which were positive for blaIMP-4 also had blaOXA-58, while one strain with blaOXA-23 also had blaOXA-58. [17]

Many Indian studies have documented the presence of MBLs in Pseudomonas aeruginosa [6]; however, to our knowledge, only three studies have been carried out for the phenotypic detection of MBLs in A. baumannii in India. [6,19,20] Uma *et.al* in 2008, reported that 70% of the carbapenem resistant A. baumannii isolates were positive for MBL production by the EDTA-DDST, of which 42% harboured the blaIMP-1 gene, while none of them showed the presence of the blaVIM-2 gene. [6]

In our study, 71% (32/45) of the isolates of the *Acb complex* were found to be carbapenemase producers by the MHT. This was in concordance with the results which were obtained by Lee *et.al* in Korea, where 73% (59/81) of the isolates were found to be carbapenemase positive by the MHT, but was in disagreement with the other Korean studies using the same method, reporting a 25% and 14% prevalence of carbapenemase among the *Acb complex* isolates. Only one study in India used the MHT to detect carbapenemase production in the *Acb complex* and reported a very low prevalence of 2.2%. [20]

The carbapenemase positive isolates from our study were further tested by the combined disk test (CDT) method and 21% were found to be MBL producers phenotypically. This was in concordance with the results obtained by Lee *et.al* in 2005 by using DDST with imipenem and EDTA disks, who reported a 25% MBL prevalence. [11] Similar results were also obtained by Franklin *et.al* by using CDT (16%) and by Lee et.al (2003) by using DDST (14%). [14, 12] Our results were also in disagreement with the results of two Indian studies on the detection of MBL by DDST; Uma *et.al*, 70% (39/55) and Gupta *et.al*, 7.5% (4/200). [6, 19].

However, a study on the mechanism of resistance to carbapenems in meropenem resistant *Acinetobacter spp.* isolates from clinical samples, which was conducted by Sinha *et.al*; 2006, found that none of the meropenem resistant isolates produced MBLs .[8]

The most active agent against the carbapenem resistant isolates in our study was cefoperazone/sulbactam, with a susceptibility rate of 49%. Yun-Song Yu *et.al* studied 45 carbapenem isolates and found that cefoperazone/sulbactam and ampicillin/sulbactam were the only active agents, with a susceptibility of 63% and 43.5% respectively. [2] This may be due to the unique activity of sulbactam against *Acinetobacter spp.* Sulbactam acts synergistically with cephalosporins in the treatment of infections caused by such isolates.

CONCLUSION

This study confirms that majority of the currently prevalent carbapenem resistant *Acb complex* are multidrug-resistant. Carbapenemase production appears to be the most common mechanism of carbapenem resistance by the phenotype screening method. Since among these isolates only 21% were of the MBL phenotype, the production of carbapenem hydrolyzing oxacillinase is the most likely mechanism of resistance. Based on our study, the modified Hodge test with zinc sulfate and the combined-disk test, which are simple and highly sensitive methods, can be used for the routine phenotypic detection of MBL production in any laboratory settings.

REFERENCE

[1] Garnacho-Montero J, Ortiz-Leyba C, Fernández-Hinojosa E, Aldabó-Pallás T, Cayuela A, Marquez-Vácaro JA, Garcia-Curiel A, Jiménez-Jiménez FJ. *Acinetobacter spp.* baumannii ventilator-associated pneumonia: Epidemiological and clinical findings. Intensive Care Med 2005; 31:649-655.

[2] Yu, Yun-Song, Yang, Qing, Xu, Xiao-Wei, Kong, Hai-Shen, Xu, Gen-Yun, Zhong, Bu-Yun Typing and characterization of carbapenem-resistant

Acinetobacter spp. calcoaceticus-baumannii complex in a Chinese hospital J Med Microbiol 2004;53: 653-656
[3] Anton Y. Peleg, Harald Seifert and David L. Paterson *Acinetobacter spp.* baumannii: Emergence of a successful pathogen. Clin Microbiol Rev.2008;21: 538–582.
[4] Franklin C, Liolios L, Peleg AY.Phenotypic detection of Carbapenem-susceptible MBL-producing Gram negative bacilli in the Clinical laboratory. J Clin Microbiol 2006; 44:3139-44.
[5] Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH Modified Hodge and EDTA-disk synergy tests to screen metallo-□-lactamase-producing strains of Pseudomonas and *Acinetobacter spp.* species. Clin Microbiol Infect 2001; 7: 88-91.
[6] R. Uma Karthika, R. Srinivasa Rao, Suchismita Sahoo, P. Shashikala, Reba Kanungo, S. Jayachandran and K. Prashanth Phenotypic and genotypic assays for detecting the prevalence of MBL in clinical isolates of *Acinetobacter spp.* baumannii from a South Indian tertiary care hospital J Medical Microbiology 2009; 58: 430-435.
[7] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement, document M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute; 2010;45-110
[8] Sinha M, Srinivasa H. Mechanisms of resistance to carbapenems in meropenem- resistant *Acinetobacter spp.* isolates from clinical samples. Indian J Med Microbiol 2010 25:121-125
[9] Jones R N, Bledenbach, D J Sader, H S Fritsche, T R Toleman, M .A & Walsh, Jones RN, Biedenbach DJ, Sader HS, Fritsche TR, Toleman MA, Walsh TR. (2005) Emerging epidemic of metallo-beta-lactamase-mediated resistances Diagn Microbiol Infect Dis 2005;51:77-84.
[10] Jeong, S H Kwon Bae, K.O, Y.J Sohn, S G Jang, S J Sung, K H Yang, K S Lee, K & Other authors , Outbreaks of imipenem resistant *Acinetobacter spp.* baumanii producing carbapenemases in Korea. J Microbial 2006; 44, 423-431.
[11] Lee K, Yum JH, Yong D, Lee HM, Kim HD, Docquier JD, Rossolini GM, Chong Y. Novel acquired metallo-beta-lactamase gene, bla(SIM-1), in a class 1 integron from *Acinetobacter spp.* baumannii clinical isolates from Korea. Antimicrob Agents Chemother 2006;49:4485-4491.
[12] Lee, K., W. G. Lee, Y. Uh, G. Y. Ha, J. Cho, and Y. Chong VIM- and IMP-type metallo-ß-lactamase-producing Pseudomonas spp. and *Acinetobacter spp.* spp. in Korean hospitals. Emerg. Infect. Dis.2003; 9:868-871.
[13] Walsh, T. R., A. Bolmstrom, A. Qwarnstrom, and A. Gales Evaluation of a new Etest for detecting metallo-ß-lactamases in routine clinical testing. J. Clin. Microbiol.2002; 40:2755-2759
[14] Hemalatha V, Sekar U, Kamat V Detection of MBL producing Pseudomonas aeruginosa in hospitalized patients. Indian J Med Res 122:148-52.
[15] Dongeun Yong, Yeoung Seon Choi, Kyoung Ho Roh, Chang Kim, Youn Hee Park, Jong Hwa Yum, Kyungwon Lee and Yunsop Chong Increasing prevalence and diversity of MBLs in Pseudomonas species, *Acinetobacter spp.* species and Enterobacteriaceae from Korea Antimicrobial agents and Chemotherapy 2005;50:1884-1886.
[16] Wong E.W, Mohd Yousuf M.Y, B. F Masoor M, Anbazghan D, Ong S, Sekaran S D Disruption of ade B gene has a greater effect on resistance to meropenems than ade A gene in *Acinetobacter spp.* spp. isolated from University Malaya Medical Centre. Singapore Med J.2009; 50:822-6
[17] Koh TH, Sng LH, Wang GC, Hsu LY, Zhao Y IMP-4 and OXA β-lactamases in *Acinetobacter spp.* baumannii from Singapore. J Antimicrob Chemother 2007; 60:1173-4.
[18] Mendes RE, Bell JM, Turnidge JD, Castanheira M, Jones RN Emergence and widespread dissemination of OXA-23, -24/40 and -58 carbapenemases among *Acinetobacter spp.* spp. in Asia-Pacific nations: report from the SENTRY Surveillance Program. J Antimicrob Chemother 2009; 63:55-59.
[19] Gupta V, Datta P, Chander J. Prevalence of MBL (MBL) producing Pseudomonas spp and *Acinetobacter spp.* spp in a tertiary care hospital in India. J Inf.2006; 52:311-4.
[20] Noyal M.J.C, Menezes G.A, Harish B.N, Parija S.C Simple screening tests for detection of carbapenemases in clinical isolates of nonfermentative Gram-negative bacteria Indian J Med Res 2009;129: 707-712.

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