

Detection of oxacillanase and metallobeta lactamase in carbapenem resistant isolates of *Acinetobacter* spp., from various clinical samples by phenotypic and genotypic methods in a tertiary care hospital

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Abstract

Background: Non fermenting Gram Negative Bacilli (NFGNB) once considered as contaminants, now emerged as a major cause of life threatening nosocomial infections and as multidrug resistant pathogens. *Acinetobacter* species are the opportunistic pathogens with increasing prevalence in the nosocomial infections. Community acquired infections are also common in *Acinetobacter*. It accounts for 10% of all community-acquired bacteraemic pneumonias.

Aim: To isolate, identify and detect Carbapenem resistance producing *Acinetobacter* spp., and confirm Oxacillanase (OXA) and Metallo Beta Lactamase (MBL) production by phenotypic & genotypic methods.

Materials and Methods: This cross sectional study conducted in a tertiary care hospital for a period of one year and samples collected like pus, urine, endotracheal aspirates, blood, sputum and body fluids were identified using standard protocol, which includes Gram staining, test for motility, catalase test, oxidase test, OF test and various biochemical reactions. The resistant strains of *Acinetobacter* species as identified by phenotyping were subjected to molecular analysis of OXA-51, VIM and IMP genes.

Results: Out of 110 isolates of non-fermenters, *Acinetobacter baumannii* accounted for 36 (81.8%) and *Acinetobacter lwoffii* 8 (18.2%). The antimicrobial susceptibility pattern revealed maximum resistance to Gentamycin (50%), Cotrimoxazole (47%), followed by Ciprofloxacin (50%) and Cefotaxime (32.2%). Sensitivity to Polymyxin B (100%) followed by Imipenem and Meropenem (75.5%). MBL production was 20.5%. Molecular characterization of MBL of *Acinetobacter* species revealed, OXA-51 (33.3%), 3 (33.3%) for bla IMP. and 2(22.2%) isolates blaVIM positive.

Conclusion: *Acinetobacter baumannii* were the most common isolate in this study. Differences in antimicrobial susceptibility pose a great problem in treating these infections. MBL production by these organisms leads to high morbidity and mortality and left with the only option of treating them by potentially toxic drugs like Colistin and Polymyxin B.

Keywords: *Acinetobacter*, Metallo Beta Lactamase, OXA, IMP, VIM Gene.

Introduction

Non Fermenting Gram Negative Bacilli (NFGNB) are aerobic, non-spore forming organisms that do not utilise carbohydrates as a source of energy (or) degrade them through metabolic pathways other than fermentation.⁽¹⁻³⁾ These are ubiquitous in nature and frequently considered as contaminants, most of them have emerged as important nosocomial pathogens causing opportunistic infections in immunocompromised hosts which accounts for about 15% of all bacterial isolates from a clinical microbiology laboratory⁽³⁾ This group includes organisms from genera like *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Burkholderia*, *Alcaligenes*, *Weeksella* and many more. Currently, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the most commonly isolated nonfermenters pathogenic for humans whereas infections caused by other species are relatively infrequent.⁽³⁾

In the National Nosocomial Infection Surveillance (NNIS) survey from the Centre for Disease Control and Prevention (CDC), infections caused by nonfermenters is the fourth most common cause of nosocomial infection and leading cause of hospital acquired infections which includes wound infections up to a range of 62%⁽⁴⁾ and

urinary tract infections up to 12%-30%. It causes life threatening bacteraemia especially in intensive care settings at a rate of 10%. *Acinetobacter* species are the opportunistic pathogens with increasing prevalence in the nosocomial infections.⁽⁵⁾ It accounts for 10% of all community-acquired bacteraemic pneumonias.⁽⁶⁾ *Acinetobacter* spp., have been reported to cause high mortality rate of 32% to 52% in blood stream infections. Similarly mortality rate up to 70% have been reported in ICU acquired pneumonia.⁽⁷⁾ Hence the identification of *Acinetobacter* spp., from clinical specimens is very essential. Resistance to carbapenems in non-fermenters can be intrinsic or acquired. These acquired MBL genes (IMP, VIM, SPM, GIM types) are usually clustered with other resistance determinants on mobile DNA elements and their presence is virtually constant marker for multidrug resistance. Different *Acinetobacter* species have differences in their antimicrobial susceptibility pattern, hence it is important to identify *Acinetobacter* isolates at species level.⁽⁸⁾ *A.baumannii* is the most common species isolated from clinical specimens and they developed 70% of resistance to third generation cephalosporins, aminoglycosides and quinolones. 87% of *Acinetobacter* isolates were Multidrug resistant.⁽⁹⁾ For

ESBL and AmpC producers, carbapenem remain the drug of choice, whereas in carbapenem resistant strains we are left with Tigecycline and polymyxins which have started developing resistance to many GNBs.⁽¹⁰⁾ Hence the detection of carbapenem resistance is important in the treatment of patients and also preventing the spread of resistant strains. Carbapenem resistance in *Acinetobacter* may be due to oxacillinases, metallobeta lactamases, AmpC beta lactamases or due to porin deficiency.^(11,12)

Since there are no CLSI guidelines for the detection of Metallobetalactamases, different studies used different methods. Despite PCR being highly accurate and reliable, its accessibility is limited only to reference laboratories. The present study was therefore taken to identify the *Acinetobacter spp.*, from various clinical specimens and to determine their antimicrobial susceptibility pattern and also to detect the carbapenem resistance by different phenotypic methods and molecular detection of resistant genes (blaVIM and IMP and OXA – 51) among *Acinetobacter species.*, by PCR in the same isolates.

Materials and Methods

This Cross sectional study was conducted in the Department of Microbiology in a tertiary care hospital over a period of one year from July 2014 to June 2015. A total of 110 clinical isolates of NFGNB from various clinical specimens were included in this study. Samples were collected from patients attending OPD and wards who satisfied the inclusion criteria. Inclusion Criteria included were hospitalized patients of all age groups undergoing treatment in ICU, medical, surgical and paediatric ward, patients affected with burn wounds, Patients with non-healing ulcer, diabetic patients with ulcers, septicaemia and pneumonia, peritonitis, patients with indwelling urinary catheter and on ventilators. Exclusion criteria included patients on prior antibiotic therapy, isolates of repeated samples from the same patient, patient who do not give consent.

Isolation and identification is mainly based on the Gram staining, motility, colony morphology on Nutrient Agar, MacConkey Agar and Blood Agar. All the catalase positive, oxidase positive and negative, non-lactose fermenting colonies on MacConkey agar were provisionally identified by colony morphology and pigment production. They were inoculated in Triple sugar iron (TSI) agar slope. The colonies which failed to acidify the TSI agar were considered as non-fermenters and subjected to the following tests. Indole, Citrate, Urease, Nitrate reduction, grow that 42°C, Sensitivity to Polymyxin B and following special biochemical tests and grouped according to P.C. Schreckenberger scheme.⁽¹⁾

Antimicrobial susceptibility testing

Disc diffusion method: Antimicrobial susceptibility was performed for all the isolates by modified Kirby -

Bauer disc diffusion method. The panel of drugs used for antimicrobial sensitivity testing was as follows: Cefotaxime(30 µg), Ceftazidime (30 µg), Amikacin (30µg), Gentamycin (10µg), Ciprofloxacin(5µg), Ofloxacin (5µg), Piperazillin /Tazobactam 100/10µg), Trimethoprim/Sulfamethoxazole(1.25/23.75µg), Imipenem (10µg), Meropenem (10µg), Polymyxin B (300U). Interpretations were made using the Clinical and Laboratory Standards Institute, USA guidelines (January 2014, M100-S24- Volume 34 No.1, Table 2B-2, Page 62/63). Journal reference was used for Polymyxin B and Colistin Disc diffusion standards as no CLSI guidelines exist for the same.^(9,13)

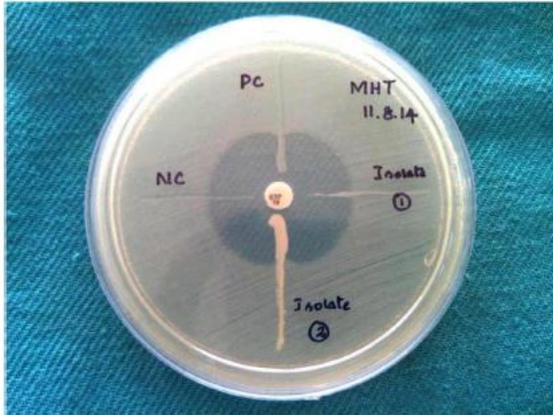
Detection of antimicrobial resistance mechanisms

Detection of Metallo Beta Lactamase (MBL) production in *Acinetobacter spp.*, by phenotypic methods: The *Acinetobacter* isolates which were found to be resistant to Imipenem, Meropenem were subjected to various phenotypic detection methods such as Combined disc diffusion Test, Double disc synergy test and Modified Hodge Test and confirmed by genotypic method i.e., PCR.⁽¹²⁾

Combined disc diffusion test (CDDT): The strain to be tested was inoculated onto MHA plate as suggested by the CLSI. Two (10µg) Imipenem or Meropenem discs were placed on the plate at the distance of 20mm and 10 µl of 0.5 M EDTA solution was added to one of them to obtain the desired concentration (750 µg). After 18 hours of incubation, the increase in inhibition zone with Imipenem EDTA, Meropenem with EDTA disc ≥ 5 mm than the Imipenem, Meropenem disc alone was considered as MBL positive.

Double disc synergy test (DDST): Lawn culture of the test organism was prepared over Mueller-Hinton agar plate as per CLSI guidelines. A plain sterile disc was kept 20 mm apart from either Imipenem or Meropenem (10µg) disc. 5 µl of EDTA was added to plain disc and incubation was done at 37°C overnight. Presence of an extended zone from Imipenem or Meropenem disc towards EDTA was interpreted as positive.

Modified hodge test (MHT): ATCC E.coli 25922 inoculum was prepared in 0.5 McFarland standards & lawn culture made on Mueller-Hinton agar plate. Meropenam disc kept in the centre of the lawn. Colonies were taken & streaked from edge of the disc to edge of the plate & incubation was done at 37°C overnight. The formation of a clover leaf like indentation along the test strains indicates carbapenemase production.^(14,15)



Meropenem resistant Isolate 2 – MHT Positive, Isolate 1 – Negative
Fig. 1

Minimum inhibitory concentration (MIC) for detecting meropenem resistance using macro broth dilution method: MIC was determined for the isolates which were showing resistance to Imipenem and Meropenem (<18mm) by disc diffusion method. MIC was determined by using Mueller Hinton broth as the medium in test tubes. Serial dilutions of Meropenam were prepared in distilled water. The concentrations used were 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 µg/ml. A young peptone water culture of the organisms corresponding to the concentration of 5×10^5 / ml is used as inoculum. A quality control strain of *Pseudomonas aeruginosa* ATCC 27853 was included. The plate was incubated at 37°C for 16-18 hrs. MIC is expressed as the highest dilution which inhibited the growth as judged by the lack of turbidity in the tube. The MIC ranges are ≤ 2 µg/ml – Susceptible: 4 µg/ml – Intermediate: ≥ 8 µg/ml – Resistant.

Molecular method

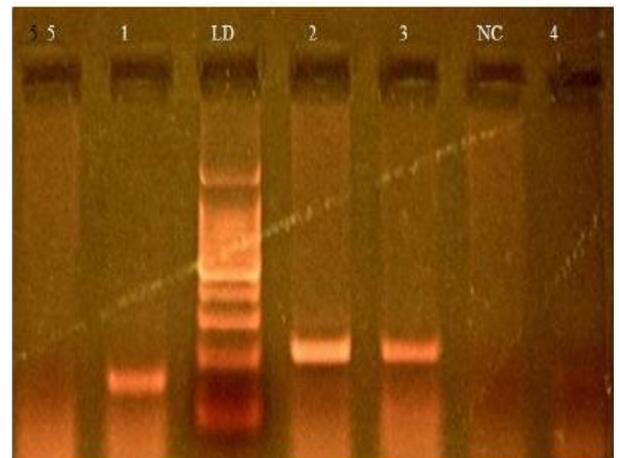
Irrespective of the phenotypic methods done for screening MBL, the isolates were subjected to conventional PCR for the detection of Oxacillinase gene OXA-51 and Metallo Beta Lactamases genes bla-IMP1 and bla-VIM1. DNA purification kit (PureFast® Bacterial Genomic DNA purification kit), PCR Master Mix, Agarose gel electrophoresis consumables and Primers from HELINI Biomolecules, Chennai, India were used for performing PCR.

Results

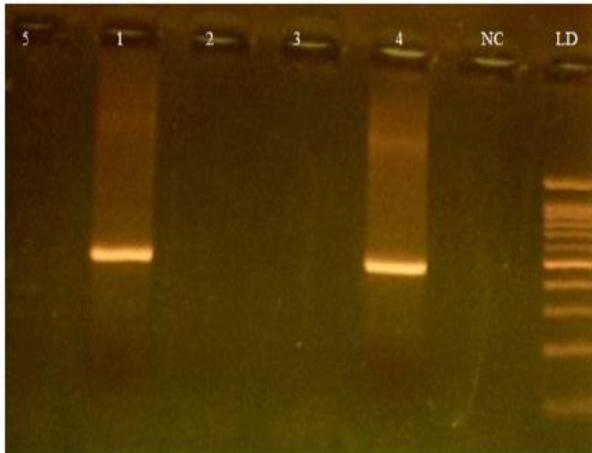
During our study period, out of 110 isolates of non-fermenter, the total number of *Acinetobacter* isolated was 44. Among them, *Acinetobacter baumannii* accounted for 36 (81.8%) and *Acinetobacter lwoffii* 8 (18.2%). Among the 44 isolates of *Acinetobacter species* screened for Meropenem resistance by Kirby -Bauer disc diffusion method, of which 9 isolates (20.5%) were found to be resistant to Meropenem which is significant.

(p value – 0.0015 as per one proportion Z-test). Out of 44 isolates of *Acinetobacter species*, 9 (20.5%) isolates of *A.baumannii*. were MBL producers. None of the isolates of *A.lwoffii* were MBL producers. Among the 9 isolates which were further tested for their meropenem minimum inhibitory concentration, all the 9 isolates have their MIC values greater than 8 µg/ml, hence they are resistant to meropenem. Among the 9 isolates, 3(33.3%) isolates have MIC 256 µg/ml, 2(22.2% isolates 128 µg/ml, another 2(22.2%) isolates 64 µg/ml and the remaining 2(22.2%) isolates have 32 µg/ml.

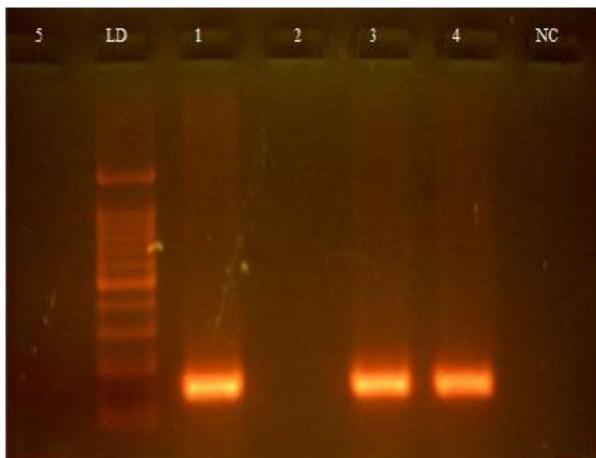
The Meropenem resistant isolates were tested for most common carbapenemase gene, OXA – 51 and metallobeta lactamase genes VIM and IMP by PCR. Among the 9 Meropenem resistant isolates, 4 isolates showed positivity by PCR. Among the four isolates, 3 isolates were positive for OXA-51, 2 isolates VIM and 3 isolates were positive for IMP. Sensitivity and specificity of Modified Hodge test for *A.baumannii* was 75% and 100% respectively and for the EDTA – disk synergy test sensitivity was 100% and specificity 80% whereas for the EDTA- combined disc test both were 100% The differences in sensitivity and specificity between these tests were statistically significant (p<0.01 by chi-square test).



Isolate No.-1, 2, 3 Positive LD-DNA Ladder NC- Negative Control
Fig. 2: PCR of IMP gene



Isolate No.-2, 4 Positive LD-DNA Ladder NC- Negative Control

Fig. 3: PCR of VIM gene

Isolate No.-1, 3, 4 Positive LD-DNA Ladder NC- Negative Control

Fig. 4: PCR of OXA 51

Discussion

In our study, we have isolated 44 *Acinetobacter* sp., over a period of one year. The antimicrobial susceptibility testing and screening for MBL production revealed that 9 isolates i.e., 20.5%. The higher percentage of resistance warrants the need for active screening for such resistance strains in order to prevent the outbreaks. The prevalence and sensitivity of non-fermenters often varies between communities, in the same community and hospitals, among different patient populations in the same hospital. Faced these variations, the physician in clinical practice has the responsibility of making clinical judgments and should access to recent data on the prevalence and antimicrobial resistance pattern of commonly encountered pathogens.

It is therefore important to institute a system for the surveillance of antimicrobial resistance that will involve the collection of both clinical and microbiological data.

The present study observed highest resistance of *Acinetobacter* sp., against first line antibiotics which are

the commonly used drugs. This necessitates the judicious use of these antibiotics in empirical therapy. Maximum sensitivity was observed with newer agents like carbapenems and piperacillin-tazobactam and Polymyxin. Moderately sensitive to Aminoglycosides and Fluroquinolones. Major risk of using monotherapy is the emergence of antibiotic resistance as observed in the present study which showed high rate of multidrug resistance and MBL producers.

Carbapenemase resistance was observed as emerging drug resistant mechanisms in the NFGNB from this hospital. Antibiotic therapy either empirical or documented is based upon antibiotic combination supplemented by the knowledge of local epidemiology of susceptibility pattern in choosing a suitable combination. Therefore combination therapy such as piperacillin-tazobactam, quinolones, amikacin, imipenam-amikacin would be an ideal choice of therapy on the basis of antimicrobial susceptibility testing as observed in this study along with an adequate infection control measures especially in the surgical and ICU units.^(16,17)

The treatment of *Acinetobacter* infections remains a great challenge because resistance to aminoglycosides, cephalosporins and quinolones has substantially increased worldwide. Carbapenems are the drug of choice for MDR *Acinetobacter* infections, for ESBL producing isolates, but resistance to carbapenems by the production of carbapenemases and various other mechanisms has limited the therapeutic options.⁽¹⁸⁾ Because of increasing carbapenem resistance and limited therapeutic options available, the old antibiotic colistin is being used more extensively nowadays, but resistance to colistin has also been reported.⁽¹⁹⁾ In my study all the isolates were sensitive to Polymyxin B. Hence currently combination therapy like meropenem with tigecycline and colistin with sulbactam or rifampicin are being tried in the treatment of *Acinetobacter* spp., infection.⁽²⁰⁾

Conclusion

Differences in antimicrobial susceptibility pose a great problem in treating these infections. MBL production by these organisms leads to high morbidity and mortality and left with the only option of treating them by potentially toxic drugs like Colistin and Polymyxin B. It is therefore important to institute a system for the surveillance of antimicrobial resistance that will involve the collection of both clinical and microbiological data. The present need is that all the health care institutions should have a coordinated effort to curtail inappropriate use of antibiotics, their own antimicrobial stewardship program, and vigilant detection of resistant non-fermenters, regular surveillance and infection control protocols to control the increasing incidence of highly resistant non-fermenters.

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