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Research article

PHENOTYPIC DETECTION OF MBL, AMPC BETA-LACTAMASE AND CARBAPENEMASES IN MULTI DRUG RESISTANT ISOLATES OF *ACINETOBACTER BAUMANNII*

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ABSTRACT

Introduction: *Acinetobacter baumannii* is one of the major pathogens causing nosocomial infections due to emergence of resistance to various antimicrobial agents. Resistance due to antimicrobial degrading enzymes is now a worldwide problem and a major reason of concern for the treating physicians. Keeping this in mind, the present study was designed to isolate *Acinetobacter baumannii* and study various antimicrobial resistance mechanisms in them. **Materials and methods**: A total of 50 *A.baumannii* isolates from various clinical samples were screened for meropenem resistance for the detection of Carbapenemase and MBL production. Carbapenemase production was confirmed by Modified Hodge Test whereas MBL by Disk Potentiation Test. Cefoxitin resistance was used as a screening test for AmpC beta-lactamase production which was confirmed by AmpC disk test. **Results**: Maximum isolation of *A.baumannii* was found in patients admitted in the Intensive care unit with respiratory tract infection. Among the 50 *A.baumannii* strains, Carbapenemase production was observed in 26.4%, MBL production in 52.9% and AmpC beta lactamase production in 56%. **Conclusion**: Our study emphasizes on multi-drug resistant *A.baumannii* highlighting the antibiotic crisis as a result of emergence of various bacteria that show resistance to various antibiotics. *Acinetobacter* epitomises this trend, as it is an important nosocomial pathogen with a capability of cross-infection particularly in ICUs and a grave limitation of treatment options, thus, requiring an urgent need to control the spread of MDR strains in the hospitals.

Keywords: Acinetobacter, Modified Hodge Test, Metallo-beta-lactamse, AmpC beta-lactamse

INTRODUCTION

For many years, *Acinetobacter* species were considered to be saprophytic in the environment, found as a major constituent of the flora of soil, water and sewage and within the hospital environment. However, due to a number of agents as well as host factors, they have now emerged as important nosocomial pathogens predominantly in ICU settings most commonly affecting immuno-compromised patients, although they have also been isolated as the etiological agent of pneumonia in healthy individuals [1]. Multi drug resistance in *A.baumannii* is not a new phenomenon. They are known to be intrinsically resistant to various antibiotics along with the ability to acquire genes that encode for resistance determinants.[2]Production of beta-lactamases, aminoglycoside-modifying enzymes, diminished expression of outer membrane proteins [OMP] and up-regulation of efflux pumps play a crucial role in antibiotic resistance. Newer beta lactamases causing antimicrobial resistance include Extended-spectrum beta-lactamases (ESBL), AmpC beta-lactamses and Metallo-beta-lactamases (MBL). [3]

Beta-lactamases act on many penicillins, cephalosporins, carbapenems and monobactams.

Metallo-beta-lactamases also referred as Class B beta-lactamases act on penicillins, cephalosporins and carbapenems but not on monobactams.[4] MBLs have zinc as metal ion which is linked to cysteine or histidine residue and it reacts with the carbonyl group of the amide bond of penicillins, cephalosporins and carbapenems.[5]

AmpC beta-lactamases are known to bestow resistance to cephalosporins in the oxyimino group and are not affected by available available beta-lactamase inhibitors. [6] The presence of MBLs and AmpC beta lactamases in a single isolate confer resistance to carbapenems which are usually the drug of choice in *Acinetobacter* infections.

A. baumannii also possess an intrinsic class D oxacillinase belonging to the OXA-51-like group of enzyme. OXA-51-like enzymes are able to hydrolyze penicillins (benzylpenicillin, ampicillin, ticarcillin and piperacillin) and carbapenems (imipenem and meropenem). Accumulation of multiple resistance mechanism leads to the development of pan-resistant strains limiting the therapeutic options.

Many multidrug resistant bacteria including *A.baumannii* produce combinations of different enzymes responsible for drug resistance. With the increasing number of MBL, ESBL and AmpC producing bacteria along with porin loss and efflux mechanisms, an increase in carbapenem resistance has been observed. Carbapenems being the drug of choice for highly resistant *Acinetobacter* species, its increasing resistance pattern limits therapeutic options. Keeping this in mind, present study was undertaken to isolate *Acinetobacter baumannii* and study various antimicrobial resistance mechanisms prevalent amongst the isolates in one of the tertiary care hospitals in North India.

MATERIALS AND METHODS

A total of 50 consecutive non-duplicate *Acinetobacter baumannii* isolates from various clinical samples and patients from all age groups and both sexes from March 2013 till Feb 2014 were included. The samples comprised of sputum, urine, wound swabs, tracheal aspirates, blood, pus, bronchial lavage and endotracheal tubes. *A.baumannii* strains isolated were identified by standard microbiological methods.[7]The anti-microbial susceptibility testing was performed using antibiotics obtained from Hi-Media, Mumbai, by the Kirby Bauer disk diffusion method, as per the guidelines of the Clinical Laboratory Standards Institute (CLSI).[8]

Susceptibility to the following antibiotics (disc concentration) were tested: Ofloxacin(5 µg); Erythro mycin(15µg);Gentamcin(10µg);Cotrimoxazole(1.25+ 23.75 µg); Doxycycline(30 µg); Cefoxitin(30 µg); Ceftazidime(30µg); Piperacillin/Tazobactam (100+10 µg):Colistin(10µg);Tigecycline(15µg); Aztreonam(30 µg); Imipenem(10 µg) and Meropenem(10 µg).

Quality control strains used were *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Meropenem resistance was used to screen for beta-lactamase production and Cefoxitin resistance for AmpC beta lactamase production.

Detection of Carbapenemase

Modified Hodge Test: It was used to detect carbapenemase production in meropenem resistant strains. A culture suspension of *Escherichia coli* ATCC 25922 adjusted to 0.5 McFarland's standard was inoculated on the surface of Muller-Hinton Agar plate using a sterile cotton swab. After drying, 10µg meropenem disc was placed at the centre of the agar plate and test strains were streaked from the disc's edge to the periphery of the plate in four different directions. The plate was then incubated overnight at 37°C.

Presence of a clover leaf shaped zone of inhibition along the growth of test strain was considered as positive for carbapenemase production. [9] (Figure 1)

Quality control strains used were, *K. pneumoniae* ATCC® BAA-1705—MHT positive, *K. pneumoniae* ATCC® BAA-1706—MHT negative**.**

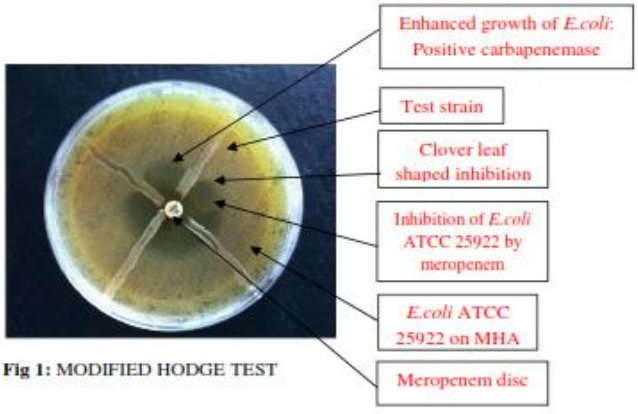
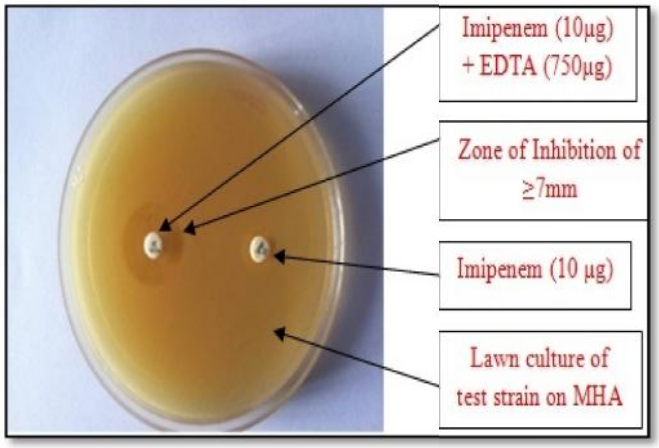


Fig 1: Modified Hodge Test

Detection of MBL

Disc Potentiation Test: The test organism adjusted to 0.5 McFarland's opacity standards was inoculated on Muller-Hinton agar plate. Two 10µg imipenem discs,

one containing 750 µg EDTA, obtained from Himedia, Mumbai were placed on the inoculated plate and incubated for 24hrs at 37°C. The zones of inhibition around the imipenem disc alone and imipenem-EDTA disc were recorded. An increase in the zone of inhibition of at least 7mm around the imipenem-EDTA disc as compared to imipenem



alone was considered as a positive result.[10] (Fig 2)

Fig 2: Disc potentiation test

Detection of AmpC beta-lactamases

The AmpC Disc Test: Cefoxitin resistant strains were subjected to AmpC disc test for the production of AmpC beta-lactamase production. A culture suspension of *Escherichia coli* ATCC 25922 adjusted to 0.5 McFarland's standard was lawn cultured on Muller-Hinton Agar plate. A cefoxitin disc (30µg) was placed on the surface of the agar and a blank disc moistened with sterile saline and inoculated with few colonies of test strain was placed besides cefoxtin disc in such a way that it was almost touching it. The plate was incubated overnight at 37ºC. Flattening or indentation of zone of inhibition around cefoxitin disc in the vicinity of the disc with the test strain was considered as positive for the AmpC beta lactamase production. An undistorted zone was considered as negative.

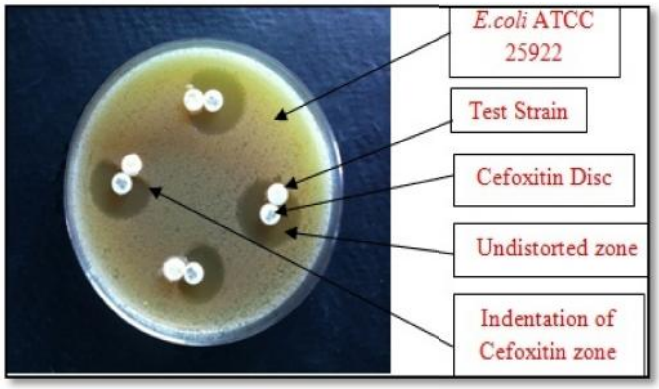


Fig 3: **AmpC Disc test**

RESULTS

Of the 50 isolates, 28 **(**56%) strains were isolated from the respiratory secretions (sputum, tracheal secretions, endotracheal tips and BAL), 12 (24%) from pus (including wound swabs) and 9 (18%) from blood samples and only 1 (2%) from urine. Intensive care unit (ICU) had the most isolation rate of 34 (74%) *A.baumannii* followed by medicine ward 5 (10%), surgery ward 4 (8%), paediatric ward 3 (6%) and ENT ward 1 (2%).

In our study, all the strains were found to be resistant to gentamicin, erythromycin, trimethoprim-sulphamethaxole,piperacillin/tazobactam,ceftazidime, cefoxitin and aztreonam. 40 (80%) of the isolates were resistant to ofloxacin while 34 (68%) to doxycycline. Colistin and tigecycline showed 50 (100%) sensitivity towards all the strains. Graph 1 depicts the antibiogram of *A.baumannii.*(Fig 4)

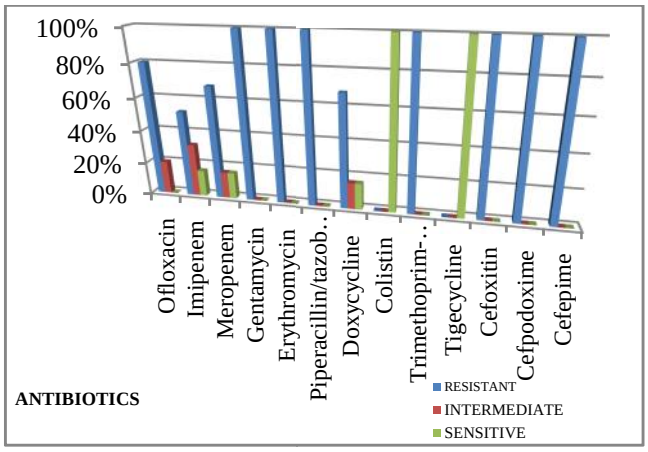


Fig 4: **Antibiogram of *A.baumannii***

Of the 50 isolates, 34 (68%) isolates expressed resistance to meropenem and only 26 (52%) to imipenem. Of these 34 meropenem resistant strains, 9 (26.4%) were positive for Carbapenemase production by Modified Hodge test and 16 (52.9%) were positive for MBL production by EDTA disc potentiation test (EDTA-DPT). Of the 50 cefoxitin resistant isolates, 28 (56%) were confirmed for AmpC beta lactamase production by AmpC disc test. Table 1 depicts the results for MHT, MBL and AmpC production in *A.baumannii* isolates.

Table 1: Modified Hodge Test, Disc Potentiation Test and AmpC Disc Test

MBL		CARBAPENEMASE		AMPC	
Screen Test (Meropenem resistance)	Confirmatory Test (EDTA- disk potentiating test)	Screen Test (Meropenem resistance)	Confirmatory Test (MHT)	Screen Test (Cefoxitin resistance)	Confirmatory Test (AmpC disk test)
34 (68%)	16 (47.05%)	34 (68%)	9 (26.4%)	50 (100%)	28 (56%)

Of the 50 *A.baumannii* isolates, co existence of Carbapenemase and MBL production was observed in 8 (16%) isolates. There were 2 isolates which expressed all three resistance mechanism whereas only 7 isolates expressed no resistance mechanism. Fig 5 depicts co-existence of various resistance mechanisms in MDR *A.baumannii.*

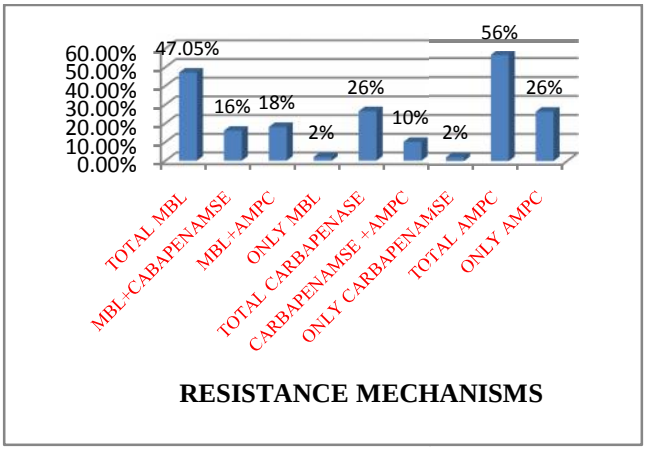


Fig 5: Existence of Multi Resistance Mechanisms in MDR *A.baumannii*

Of the 50 isolates, 47.05% were found to be MBL producers, out of which 16% also co-produced carbapenemase and 18% co-produced AmpC. However, there were 2% strains which only expressed MBL production. Carbapenemase production was found in 26% of the total strains, of which 10% also co-produced AmpC beta lactamase and 2% strains were positive only for carbapenemase production. However, 26% of the isolates were positive for only AmpC beta lactamase production, suggesting AmpC beta lactamase being a more expressed resistance mechanism in our study.

DISCUSSION

A.baumannii is an effective human colonizer in the hospital. Combination of its environmental flexibility and presence of multiple resistance determinants makes it a successful nosocomial pathogen. MDR *A.baumannii* infections tend to occur more frequently in immune-compromised individuals, patients on broad spectrum antibiotics and with underlying diseases and those subjected to invasive procedures.[12]According to Ambler Classification, Beta lactamases are grouped into 4 major molecular classes; A, B, C and D. A, C and D are referred as serine-beta-lactamases, whereas group B beta lactamases are called MBL. Newer beta lactamases that hydrolyse cephamycins, cephalosporins, monobactams and carbapenems are of increasing concern as they limit therapeutic options leading to treatment failures and poor prognosis. [13]

We observed that 56% of the *A.baumannii* isolates were from the respiratory secretions which included sputum samples, endotracheal secretions, bronchio-alveolar lavage and endotracheal tips, 24% from pus samples, 18% from blood and only 2% from urine. In a study by Muthusamy et al.[11] conducted in Coimbatore, South India isolation rate was found to be 73% from respiratory secretions. In yet another study by Jaggi et al. **[14]** in Gurgoan, Haryana, 57.4% of the *A.baumannii* isolates were from respiratory secretions, 23.8% from blood, 13.5% from pus and 2.5% from urine, an observation similar to ours, suggesting thereby that respiratory tract would be the most common site of isolation in our geographical area.

It is a well documented fact that a lot of risk factors associated with *Acinetobacter* infections exist in the ICU like potential environment reservoirs, opportunities for cross transmission, sick, immune-compromised patients who are colonized, patients with multiple wounds and indwelling devices, heavy use of broad spectrum antibiotics and frequent contamination of the hands of health care workers employed in patient care.

This fact was supported by our results, where 74% of the *A.baumannii* isolates were from the ICU followed by medicine ward (10%), 8% from surgery ward, 6% from paediatric and 2% from ENT ward. Sinha N et al. [15] also noted similar findings with maximum isolation from the ICU of 22.14% followed by

paediatrics (20.71%), neurosurgery (15.71%) and general surgery wards (12.85%).

In our study, high resistance of 68% was observed against meropenem, which was in contrast to a study conducted earlier by Sinha N et al.[15] where only 20% resistance was observed. Taneja et al,[16] observed 18.5% and Dheepa M et al [11] observed 35% resistance. However, in Brazil, the resistance to carbapenemase was found to be ranging from 71.4% to 100% in various hospitals of that region. [17]

Thus, the aforementioned observations could only suggest that Carbapenemase-producing *Acinetobacter* spp might be on a rise worldwide which could be due to indiscriminate carbapenemase usage and selection pressure in hospitals.

In our study, 26.4% organisms were carbapenemase producers as evidenced by the Modified Hodge Test. The prevalence of carbapenemases as reported by Noyal et al.[9] was 14.3% whereas another study by Kumar et al.[18] documented a very high prevalence of 71%. A very low prevalence of 2.96% was also reported by Patwardhan et al.[19]

No established phenotypic methods are available for detection of specific serine carbapenemases. However, for zinc based carbapenemases (MBL) various methods like EDTA-disc potentiation test, MBL E-test and EDTA based microbiological assay are available.

In our study, 47.05 % of meropenem resistant strains were confirmed to be MBL producers, whereas Dheepa Muthusamy et al [11] detected 10% of the strains to be MBL producers in her study conducted in South India and John S et al [20] detected 14.8%. Noyal MJC et al [9] did a similar study in Pondicherry, South India and identified 6.5% MBL producers. In a study done at AIIMS, New Delhi, 48.72% of *A.baumannii* strains were ascertained to be MBL-enzyme producers by the same method, thus implying rapid spread of resistance amongst this pathogen. [21]

Although there are no CLSI guidelines for the detection of AmpC beta lactamase production, but we followed AmpC disk test to detect AmpC production and observed 28 (56%) of 50 cefoxitin resistant isolates of *A.baumannii* showed production of AmpC beta-lactamase enzyme. Noyal et at [9] and Sinha et al[22] also reported 67.4% and 42.9% respectively, in their studies.

Although carbapenems are the drugs of choice for *A. baumannii* infections, such resistance profile limits therapeutic options to polymyxins and tigecycline, which showed 100% sensitivity to *A.baumannii* in our study. These drugs have their own grave side effects, limiting their routine usage for patients in hospitals.

Thus, it is recommended to perform these simple tests like Modified Hodge Test for carbapenemase, Disc Potentiation test for MBL and AmpC disk test for AmpC beta lactamase production in microbiology laboratories to determine resistance mechanisms and prevent indiscriminate use of antibiotics.

CONCLUSION

A.baumannii is becoming a global medical challenge due to the emergence of multi-drug resistance. Newer beta lactamase are a matter of concern as they are developing rapidly and lead to treatment failure. Carbapenems are known to be effective therapeutic agents for *A.baumannii* infections and its resistance limits the use to polymyxins and tigecycline. Disappointingly, there are limited antibiotics for the treatment of infections caused by MDR *A.baumannii* on the horizon. Several new medicines are still in research and combination of drug therapy is being currently used in the hospitals including ours to treat MDR A.baumannii infections.

Thus, due to such high prevalence of resistance, antibiotics must be used judiciously by the clinicians and appropriate infection control measures need to be implemented to control the spread of infections in hospitals.

ACKNOWLEDGMENT: None

Conflict of Interest: Nil

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