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Epidemiological Pattern of Non-fermenting Metallo Beta Lactamase Producing Bacterial Pathogens Isolated from Clinical Specimens in a Tertiary Care Hospital in Kakinada, India

Purimitla Usha Rani[1] and Payala Vijayalakshmi[1*]

[1]Department of Microbiology, GITAM Institute of Medical Sciences and Research, GITAM University, Visakhapatnam-530045, Andhra Pradesh, India.

Authors' contributions

This work was carried out in collaboration between the both authors. Author PUR designed the study, wrote the protocol, literature survey and performed the bench work. Author PV wrote the first draft of the manuscript and managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Non-fermentative Gram negative bacilli are known causative pathogens responsible for hospital associated infection and responsible high morbidity and mortality rate across all genders and ages.
Objectives: This study examined the prevalence of Non-fermentative Gram negative isolates expressing Metallo beta-lactamase isolates (NFGNB-MBL) among carbapenem-resistant isolated from clinical specimens and the epidemiological pattern (Age, Sex and Source of infection).
Methodology: A total of 250 clinical specimens from diverse infections were collected and analyzed, using standard microbiological methods. For MBL detection, two previously described methods were employed i.e. Imipenem-EDTA combined disc test and Imipenem-EDTA double disc synergy test (DDST).

**Corresponding author: E-mail: bavisettyvijayalakshmi2@gmail.com;*

Results: Sixty (24%) NFGNB isolates were identified; highest of isolation was recovered from hospital associated infections (93.33%). *Pseudomonas aeruginosa* accounted for n=40 (66.67%) followed by *Acinetobacter baumannii* n=10 (16.67%), *Alcaligenes faecalis* n=6 (10%) and *Stenotrophomonas maltophilia* n=4 (6.66%) respectively. The isolates showed high sensitivity to imipenem and least sensitivity to Gentamicin. All the carbapenem resistant isolates of non-fermenters showed Metallo-beta lactamase production.
Conclusion: The prevalence of MBL producing isolates and association with hospital infection is of clinical concern and the therapeutic option in treatment and management of patients may be futhered worsen.

Keywords: Non-fermenters; gram negative bacilli; antibiotic sensitivity; imipenem; MBL production.

1. INTRODUCTION

Non-fermenting Gram negative bacilli are a group of aerobic, non-sporing organisms that either do not use carbohydrates as a source of energy or degrade them through metabolic pathways other than fermentation [1]. These organisms are abundant in nature like soil, water, plants, decaying vegetation, food stuffs and normal flora of humans. Although NFGNB are considered commensals or contaminants, their pathogenic potential has been well established by their frequent isolation from clinical samples and their association with clinical disease. Clinical significance of NFGNB isolates is furthered highten, because of their implication in hospital associated infections, acquisition and dissemination of resistant genes and has been known to worsen the treatment and management of patients. Out breaks of nosocomial infections, emerging antimicrobial resistance and epidemiology complexity have made NFGNB the remarkable organisms. NFGNB are innately resistant to many antibiotics and are known to produce extended spectrum beta-lactamase and expressing MBL. They account for around 15% of all bacterial isolates from clinical samples. Most of the Non-fermenting Gram negative bacilli have emerged as important nosocomial pathogens in hospitalized patients of severe burns, urinary tract infections, wound infections, septicaemia, pneumonia, osteomyelitis, peritonitis, meningitis, cirrhosis of liver etc. Some can cause serious infections in immuno-compromised hosts [2]. The most important members are *Pseudomonas* spp. followed by *Acinetobacter* spp*, Stenotrophomonas* spp*, Moraxella* spp*, Alcaligenes* spp*, Flavobacterium* spp*, Achrobacterium* spp etc. All these may be acquired nosocomially, so that rapid identification and epidemiological characterization are important in tracking hospital outbreaks. The rising incidence of Pseudomonas infection in hospital and its ubiquitous association in hospital environmental soil and water had led to an increased interest in identifying the strain by various bacteriological and biochemical properties by different research workers. Non-fermenting Gram negative bacilli were isolated from blood cultures of patients admitted to high risk units like oncology, nephrology, burns, NICU and cardiology [3]. With particular references to ICU and major superficial and systematic infections bacteraemia due to *Pseudomonas* spp. and *Acinetobacter* spp. has become an important cause of morbidity and mortality in hospital association infections. Prolonged hospital stay, more invasive procedures for diagnosis and therapy, indiscriminate use of broad spectrum antibiotics and underlying host factors also contribute to morbidity and mortality.

NFGNB exhibiting MBL enzymes have demonstrated MDR pattern and are associated with hospital associated infections. The enzyme possesses hydrolytic activity responsible for degradation of cephalosporins. In addition the plasmid mediated MBL genes can easily be acquired by other GNB. Therefore, periodic surveillance and early detection will serve as an effective control mechanism and interruption of dissemination into the hospital environment [4].

In the present study an attempt has been made for isolation and identification of various non-fermenting gram negative bacilli and to study the various biochemical characters of isolated non-fermenting organisms from various clinical materials. The identification of metallo- beta-lactamase producing non-fermenters was also included in this study.

2. MATERIALS AND METHODS

The study was conducted in a tertiary care hospital, Rangaraya Medical college, Kakinada, India for a period of one year involving clinical samples from out/in patients. Clinical specimens collected included Pus, Urine, Endotracheal aspirates, Sputum, Blood, Catheter tips, Ascitic

fluid, Pleural fluid and CSF from 250 patients. Demographic variables of the patient were recorded age, gender and type of patient. Gram staining was performed initially to study the morphological characteristics of the clinical isolates [5].

2.1 Culture and Biochemical Reactions

All the clinical specimens were initially processed to separate the non-fermenters from the other Gram negative bacilli.

Pus, respiratory secretions and other specimens were inoculated onto 5% blood agar, Mac-conkey agar and Nutrient agar and urine samples were inoculated into CLED agar and incubated aerobically at 37ºC for 24 h and then examined for growth. The wound swabs are inoculated into nutrient broth and incubated for 3-4 h at 37ºC and then after noting the turbidity due to growth sub cultured onto MacConkey, 5% blood agar and incubated for 12-18 h at a temperature of 37ºC. In case of Acinetobacter growth on MacConkey medium showed a faint pink tint, while no pigmentation was seen on blood agar. After observing the motility of organisms, Gram stain was done and confirmed that the organisms are gram negative.

Non fermenting organisms are provisionally identified by colonial morphology and pigment production and these were subjected to various biochemical and biological tests for confirmation [5]. The tests include carbohydrate fermentation tests with glucose, lactose, sucrose, xylose, mannitol and maltose. The organisms were also tested for Indole production, Methyl red test, Voges-proskauer test, Citrate utilization, Urease test, Oxidase test, Catalase test, Nitrate reduction test, Triple sugar iron agar test, Hughs Leifsons test (O/F test), Gelatin hydrolysis, Amino acid decarboxylation test, Growth on nutrient agar containing 6% NaCl, growth at 42ºC etc. In addition to the biochemical reactions, strains of *Pseudomonas aeruginosa* were further tested for their ability to grow on cetrimide (0.3%) agar. *Pseudomonas aeruginosa* was identified by its colony characters, pigment production and its characteristic odour, growth on cetrimide agar with 6% NaCl.

2.2 Antibiotic Sensitivity Testing of Isolated Non-fermenters Using Disc Diffusion Method

Overnight broth culture having semi-confluent growth of the isolated bacterial strain was used as inoculums. Antibiotic susceptibility testing was carried out by disc diffusion method according to CLSI guidelines on Muller- Hinton agar. The following antibiotic discs were tested Amikacin (30 µg), Gentamicin (10 µg), Cefoperazone (75 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Piperacillin/ Tazobactum (100/10 µg), Polymyxin B (300 U). The Zone of inhibition can be compared according to CLSI guidelines.

2.3 Detection of Metallo Betalactamase (MBL) Production

Screening for MBL production was done on NFGNB demonstrating MDR pattern. MDR isolate was defined as resistant to 2 or more drugs or drug classes of therapeutic relevance [6]. The following methods were used for the MBL detection in the present study which includes Imipenem-EDTA combined disc test and Imipenem-EDTA double disc synergy test (DDST). InImipenem-EDTA combined disc test, the test organisms were inoculated onto plates with Muller-Hinton agar. Two 10 µg imipenem discs were placed on the plate, an appropriate amount of 10 µl of EDTA solution were added to one of them to obtain the desired concentration (750 µg). The inhibition zones of the imipenem-imipenem and EDTA discs were compared after 16-18 h incubation at 37ºC. In the combined disc test, if the increase in inhibition zone with the imipenem and EDTA was >7 mm than the imipenem disc alone, it was considered as MBL positive. Discs were prepared by adding EDTA solution to 10 µg imipenem discs to obtain a concentration of 750 µg. The discs were dried immediately in an incubator and stored at 4ºC or at -20ºC in an airtight vial without dessicant. Test strains were adjusted to the Mc Farland 0.5 standard and were inoculated to MH agar. A 10 µg imipenem discs and an imipenem + EDTA 750 µg were placed on MH agar. Another disc containing only 750 µg EDTA was also placed as a control. After overnight incubation, the established zone diameter difference of >7 mm between imipenem discs and imipenem + EDTA was interpreted as EDTA synergy positive. These were compared with ATCC 27853 strains of *Pseudomonas aeruginosa* as a negative control [6,7].

The Imipenem-EDTA double disc synergy test was performed as described by Lee et al. Test organisms were inoculated onto plates with MH agar as recommended by the CLSI. An imipenem (10 µg) disc was placed 20 mm centre

to centre from a blank disc containing 10 µl of 0.5 M EDTA (750 µg). Enhancement of the zone of the inhibition in the area between imipenem and the EDTA disc in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result [8].

3. RESULTS

Various age groups ranging from 1 and 79 years of both sexes were from 250 patients were included in the present study. It was noticed that, the highest number of cases were reported in the age group 20-29, that is 58 (23.2%) and a lower number of cases in the age group 70-79, which is 10 (4%). The rate of infection was more in males 160 (64%) while comparing with females 90 (36%) who attended the hospital from various infections (Fig. 1). Of the 250 clinical specimens analyzed, 60 (24%) were identified as Non-fermentative Gram negative bacilli. These 60 were used for further processing.

Clinical infections of the NFGNB positive isolates, 56 (28%) were recovered from hospital associated infections and 4 (8%) from community associated infections. The predominant non-fermenter was *Pseudomonas aeruginosa* 40 (66.67%), followed by *Acinetobacter baumannii* 10 (16.67%), *Alcaligenes faecalis* 6 (10%) and *Stenotrophomonas maltophilia* 4 (6.66%). Among 40 *Pseudomonas aeruginosa* isolates, the highest rate of *Pseudomonas aeruginosa* isolated from pus exudates 12 (30%) and lowest rate isolated from ascitic fluid and other body fluids 2 (5%) (Fig. 2).

Out of 10 *Acinetobacter baumannii* isolates, the highest rates of isolates were from urine 4 (40%) and lowest rate of isolates sputum and catheter tips 1 (10%) (Fig. 3).

The highest rate of *Alcaligenes faecalis* isolates was isolated from Blood 3 (50%) out of 6 isolates (Fig. 4) whereas urine, sputum, ascitic fluid and CSF were culture negative.

Among 4 *Stenotrophomonas maltophilia,* the highest rate of the isolates were reported from sputum (50%) (Fig. 5). No growth was seen from the specimens like endotracheal aspirates, catheter tips, urine, ascitic fluid and CSF.

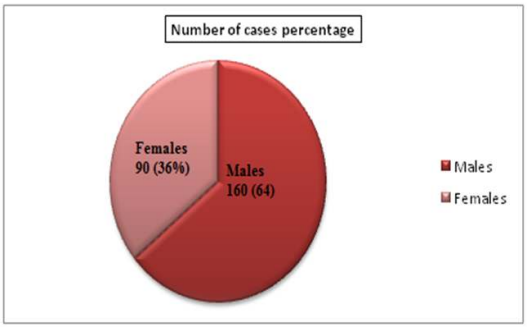

Fig. 1. Distribution of cases from various types of infections according to gender

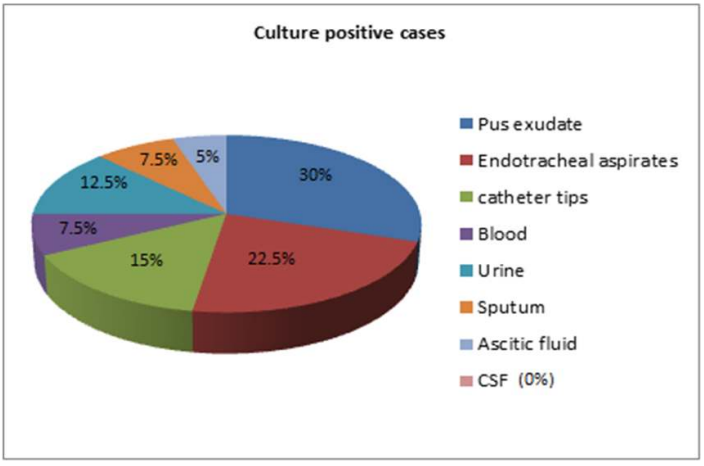

Fig. 2. Distribution of isolates of *Pseudomonas aeruginosa* from various clinical samples

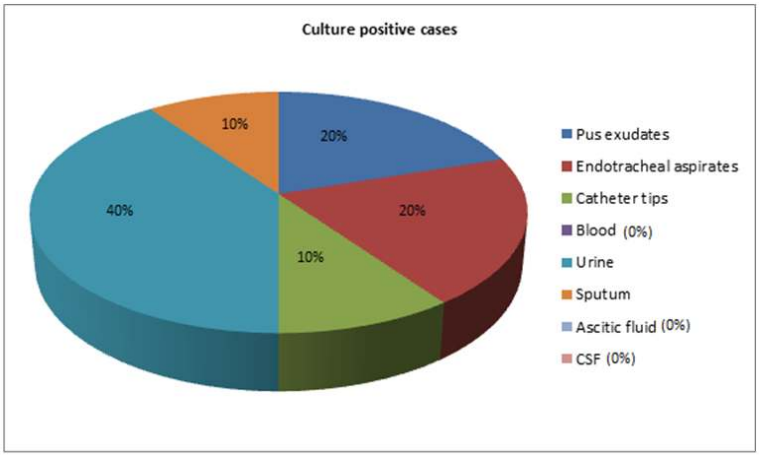

Fig. 3. Distribution of isolates of *Acinetobacter baumannii* from various clinical samples

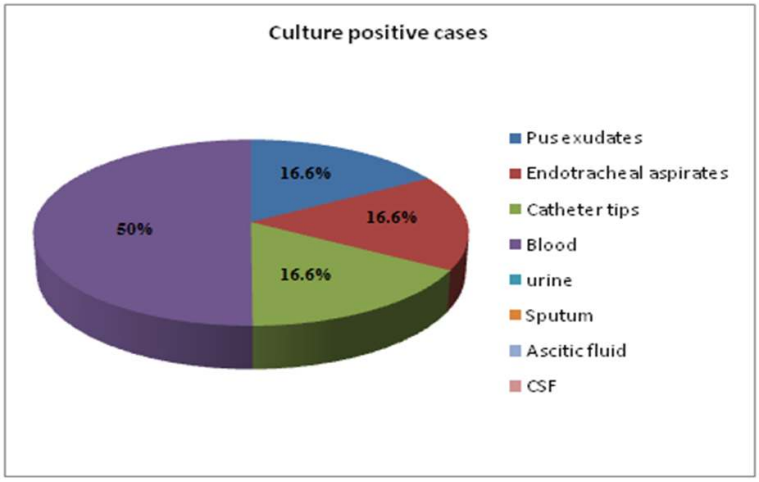

Fig. 4. Distribution of isolates of *Alcaligenes faecalis* from various clinical samples

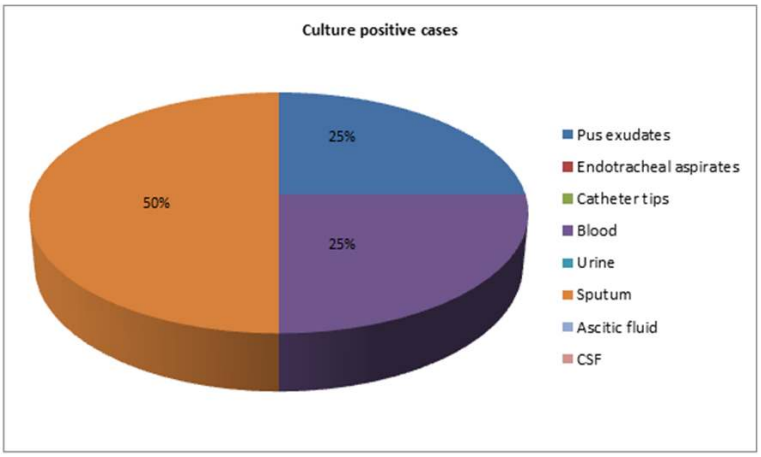

Fig. 5. Distribution of isolates of *Stenotrophomonas maltophilia* from various clinical samples

The isolated *Pseudomonas aeruginosa* differentiated from other species like *P. fluorescens, P. putida, P. stutzeri* and *P. luteola* by some characteristic features like Oxidase positive, fruity grape like odour, production of large colonies with metallic sheen, mucoid, pigmented (pyocyanin) colonies showing β-hemolysis. All the isolated non-fermenters were differentiated by using various cultural and biochemical tests (Table 1) (Figs. 6 and 7).

The overall antibiotic sensitivity pattern of Non-fermenters isolated from various clinical samples revealed that imipenem was most effective (Fig. 8), followed by ceftazidime, piperacillin/ tazobactum, ceftriaxone, cefoperazone, ciprofloxacin, amikacin and gentamicin. Imipenem resistant isolates showed sensitive to polymyxin-B (300 U) (Table 2). Among carbapenem resistant isolates of non-fermenters, 100% showed Metallo-betalactamase production (Fig. 9).

Fig. 6. Cetrimide agar plate showing yellow fluorescence of the colonies of *Pseudomonas aeruginosa*

Table 1. The differentiating features utilized for the isolates

S. no	Test	*P. aeruginosa*	*A. baumannii*	*A. faecalis*	*S. maltophilia*
1.	Gram stain	Gram negative rod	Gram negative coccobacilli	Gram negative rod	Gram negative rod
2.	Motility	Motile	Non-motile	Motile	Motile
3.	Catalase	Positive	Positive	Positive	Positive
4.	Oxidase	Positive	Negative	Positive	Negative
5.	Growth on MacConkey agar	Non-lactose fermenter (NLF)	Faint pink tint	NLF	NLF
6.	Haemolysis on 5% blood agar	Positive	Negative	Variable	Positive
7.	Pigment production on nutrient agar	Positive	Negative	Negative	Positive
8.	Growth at 37ºC	Positive	Positive	Positive	Positive
9.	Growth at 42ºC	Positive	Positive	Positive	Negative
10.	Indole test	Negative	Negative	Negative	Negative
11.	Methyl red test	Negative	Negative	Negative	Negative
12.	Voges-proskauer test	Negative	Negative	Negative	Negative
13.	Citrate utilization test	Positive	Negative	Positive	Positive
14.	Urea hydrolysis	Variable	Variable	Negative	Negative
15.	Nitrate reduction test	Positive	Negative	Negative	Variable
16.	$NO_3$to gas	Positive	Negative	Negative	Negative
17.	O/F glucose	Positive	Positive	Negative	Positive
18.	O/F lactose	Negative	Positive	Negative	Variable
19.	O/F Maltose	Positive	Negative	Negative	Positive
20.	Arginine decarboxylation	Positive	Positive	Variable	Negative
21.	Lysine decarboxylation	Negative	Negative	Variable	Positive
22.	Gelatin hydrolysis	Positive	Variable	Negative	Positive

Table 2. Antibiotic sensitivity pattern of the isolates

Type of the organism	Total number of strains	Amikacin	Gentamicin	Ciprofloxacin	Cefoperazone	Ceftazidime	Ceftriaxone	Piperacillin/ Tazobactum	Imipenem
P. aeruginosa	40	11	9	15	18	34	22	26	37
A. baumanii	10	4	4	5	4	7	6	8	10
A. faecalis	6	2	2	4	3	4	4	3	4
S. maltophilia	4	2	1	3	2	2	1	3	4
Total	60	19	16	27	27	47	33	40	55
Percentage of organism sensitive to each antibiotic		31.66	26.66	45	45	78.33	55	66.6	91.67

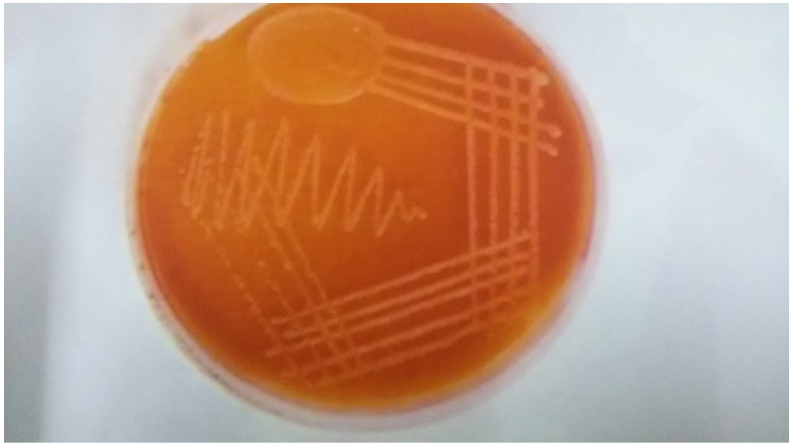

Fig. 7. MacConkey agar plate showing growth of *Acinetobacter baumannii*

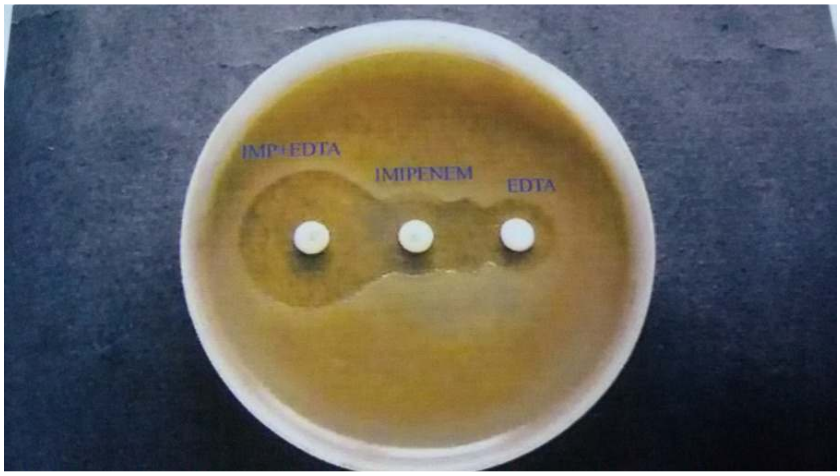

Fig. 8. Combined disc test showing MBL production by *Alcaligenes faecalis*

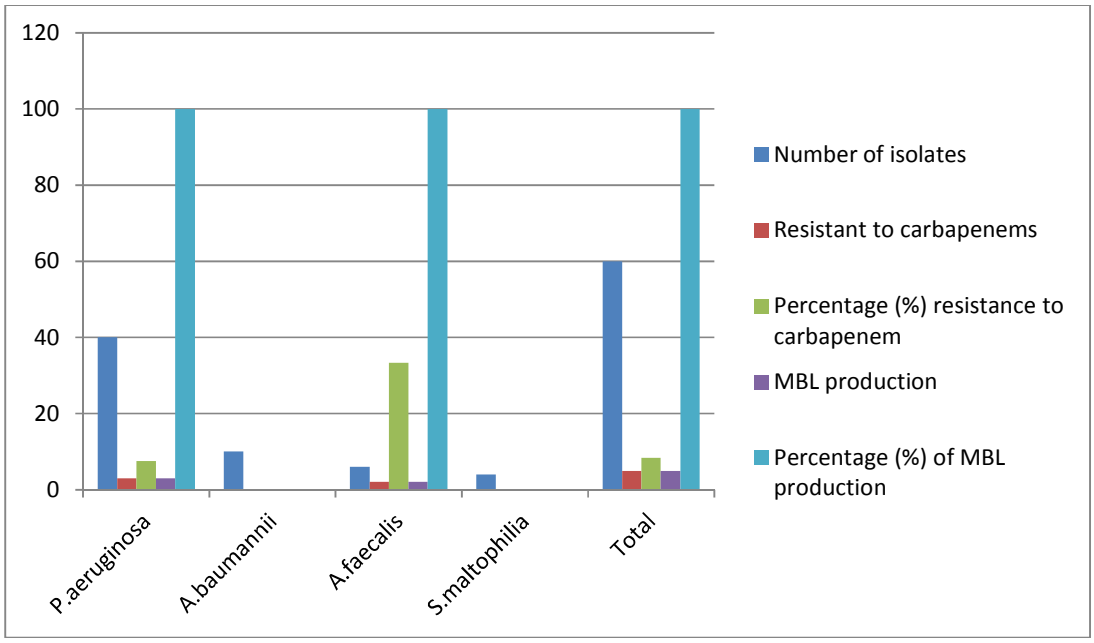

Fig. 9. Susceptibility of isolates to carbapenem in relation to MBL production

4. DISCUSSION

Preamble NFGNB MBL as major cause of hospital associated infections, high morbidity and mortality rate. The study examined the prevalence, demographic variable and resistance pattern. Five NFGNB MBL (8.33%) isolated from 60 clinical specimens. Out of 250 different samples, rate of recovery of non-fermenters is 24% which is comparable with the study of Pathmanathan et al. [9] (24.8%) and Shail Preet Sidhu et al. [10] (28%). Mahajan et al. [11] (80.78%), Dey and Bairy [12] (74.9%) reported a higher rate of isolates, whereas Lahiri et al. [13] reported a lower rate of isolates (12.9%). In the current study, males were found to be more commonly affected (64%) with different infections caused by non-fermenters than females (36%) which is comparable to the findings of Varaiya et al. [14] who observed that 66.66% were males and 33.3% were females. Nan-Yao Lee et al. [15] reported 67.4% were males and 32.6% were females. Prashanth et al. [16] also found that males were more commonly affected (58%) than females (42%). Different types of infections seems to be more common in males than

females, and this male preponderance may be due to that males are more involved with outdoor activities and therefore chances of exposure to infections are more likely. An observation of the source of infection in the present study was indicated that the rate of isolation of Non-fermenters was higher among the hospital acquired infections (93.33%) than in community acquired infections (6.67%). Our observation is comparable with the study of Lahiri et al. [13] who showed higher incidence of non-fermenters (82.9%) in hospital acquired infections than in community acquired infections (17.1%). Among the isolated non-fermenters *Pseudomonas aeruginosa* was the commonest isolate (66.67%) which coincides with the study of Arjun Srinivasan et al. [17] (66.75%) and Mahajan et al. [11] (54.54%). Pathmanathan et al. [9] reported a lower rate of isolates (7.2%) and also by Neelam Kaistha et al. [18] (11.5%). *Acinetobacter baumannii* was the second most common non-fermenter (16.67%), in our study. This observation is similar to that of Pathmanathan et al. [9] (17.6%). The higher incidence of the isolate was reported by Neelam Kaistha et al. [18] (30.76%) and also by Dey and Bairy [12] (47.9%). 10% of *Alcaligenes faecalis* were obtained in the present study where as Shali Preet Sidhu et al. [10] reported only 2% and Seeta et al. (3) reported only 1.45%. 6.66% *Stenotrophomonas maltophilia* was reported in the present work but Shali Preet Sidhu et al. [10] reported only 1%. Regarding the antibiotic susceptibility pattern, the highest sensitivity rate was observed with Imipenem (91.67%) and it coincides with the study of Duggal et al. [19] (95.74%) and Agarwal et al. [7] (90.5%). The least sensitivity was observed to gentamicin (26.66%) in our study. In the present investigation, 7.5% of the *Pseudomonas aeruginosa* proved to be resistance to the carbapenems and all the carbapenem resistant strains were MBL producers (100%). Shali Preet Sidhu et al. [10] reported that 6.97% of the *Pseudomonas aeruginosa* showed resistance to the carbapenems, and it is coinciding with the present study. Irfan et al. [4] reported (100%) of the carbapenem resistant isolates of *Pseudomonas aeruginosa* were MBL producers, which are also coinciding with the study. All the pathogenic cultures which were isolated in the current investigation showed 100% sensitivity to Polymyxin-B. Antibiotic options are rather limited in multiple-resistant NFGNB infections; old but well known Polymyxins, which have become available again, are an option in the treatment of resistant strains and now-a-days widely used in clinical practices for NFGNB infections. Many unusual organisms were involved in the hospital association infections had numerous predisposing factors such as immunosuppression in the case of cancers, diabetes, chronic diseases, prolonged hospital stay, different surgical conditions and continuous antibiotics usage were also contribute to morbidity and mortality. Though they were not frequently isolated like other bacteria, they hoist significant therapy issues throughout intrinsic resistance to multiple antibiotics classes, and increased acquired rate of resistance factors that determine their rapid propagation in the hospital. Education of health care staff recommending medication, hospital infection control, antibiotic management, execution of an active surveillance system, limit of non-human use of antimicrobials and distribution of data about resistant strains at national and international level are suggested to control resistant bacteria. The world wide resistance pattern of nosocomial pathogens illustrates wide variation from country to country and within the same country over a period of time. Appropriate identification and detection of antibiotic susceptibility pattern is of huge value because of high intrinsic resistance of different NFGNB to various antimicrobial agents. Therefore, different international authorities put emphasis on every hospital should have antibiotic policy of its own [20].

5. CONCLUSION

Non-fermenters are emerging as nosocomial pathogens and they show Multidrug resistance, so the excess use of carbapenems and other broad spectrum antibiotics should be avoided and also early detection and prompt infection control measures are important to prevent further spread of MBLs to other Gram negative bacilli.

ETHICAL APPROVAL

The ethical clearance was obtained as per university policy for this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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