

Original Research Article

ISOLATION OF NON-FERMENTERS AND DETECTION OF METALLO BETA LACTAMASE PRODUCTION IN THE ISOLATES

ABSTRACT:

Aims: To know the prevalence of Non-fermentative Gram negative bacilli from various clinical samples, age and sex wise incidence, to evaluate the source of infection and to identify the isolated bacteria by conventional techniques. The study also aimed to know the antibiotic sensitivity pattern of the isolated non-fermenters and prevalence of metallo beta lactamase (MBL) producing non-fermenters among the carbapenem resistant isolates.

Methodology: A total of 250 samples were collected from the patients suffering with various infections. The samples were analyzed by using different standard microbiological techniques like microscopy, culture methods and biochemical reactions. The isolates were tested for antibiotic susceptibility using Kirby-Bauer method. For MBL detection in the present study, Imipenem-EDTA combined disc test and Imipenem-EDTA double disc synergy test (DDST) were used. **Results:** The isolation rate of non-fermenters was observed as 24% from different clinical samples. The highest rate of non-fermentative isolates was observed in patients with hospital acquired infections (93.33%). The incidence of various species of non-fermenters isolated in the order of prevalence was *Pseudomonas aeruginosa* (66.67%), *Acinetobacter baumannii* (16.67%), *Alcaligenes faecalis* (10%) and *Stenotrophomonas maltophilia* (6.66%). The isolates showed high sensitivity to imipenem (91.67%) and the incidence of MBL producing non-fermenters among the imipenem resistant organisms was found to be 100%. **Conclusion:** The use of broad spectrum antibiotics should be avoided and quick detection and prompt infection control measures are important to prevent further spread of MBLs to other Gram negative bacilli.

Key words: 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. In hospitals they also isolated from nebulizers, Dialysate fluids, saline and catheter devices. Some can cause serious infections in immune-compromised hosts [2]. 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. The most important members are *Pseudomonas* followed by *Acinetobacter*, *Stenotrophomonas*, *Moraxella*, *Alcaligenes*, *Flavobacterium*, *Achromobacterium* 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]. Among them, *Pseudomonas aeruginosa* is the most common isolate followed by *Acinetobacter* and *Alcaligenes*. Bacteraemia due to *Pseudomonas* and *Acinetobacter* has become an important cause of morbidity and mortality particularly in hospitalized patients with debilitating diseases. Antibiotic resistance pattern shows that many isolates were multi-drug resistant. This is due to more and more invasive procedures for diagnosis and therapy during long stay of patients in the hospital.

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. As some of the non-fermenting organisms show multidrug resistance, the nosocomial outbreaks of carbapenem resistant non-fermenters is due to metallo betalactamase enzyme production. So this enzyme is responsible for multidrug resistance, and they possess high hydrolytic activity that leads to degradation of higher generation cephalosporins. Plasmid mediated MBL genes spread rapidly to other species of gram negative bacilli, therefore rapid detection of metallo betalactamase production is necessary to modify therapy

and to initiate effective infection control to prevent their dissemination [4]. So identification of metallo
betalactamase producing non-fermenters was also included in this study.

2. MATERIALS AND METHODS:

Different clinical samples collected from both IP and OP patients for a period of one year, constituted
the material for this study. A total of 60 non-fermenters isolated from different clinical specimens
including Pus, Urine, Endotracheal aspirates, Sputum, Blood, Catheter tips, Ascitic fluid, Pleural fluid
and CSF from 250 patients. The cases included individuals of both sexes and all age groups. 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 37°C for 24h and then examined for growth. The wound swabs are inoculated into
nutrient broth and incubated for 3-4h at 37°C and then after noting the turbidity due to growth sub
cultured onto Mac-conkey, 5% blood agar and incubated for 12-18h at a temperature of 37°C. After
studying the colony morphology, Non-lactose fermenting colonies suspected to be *Pseudomonas*
aeruginosa and *Alcaligenes faecalis* were taken from Mac-conkey agar medium and inoculated into
peptone water and incubated for 3-4h at a temperature of 37°C. In case of Acinetobacter growth on
Mac-conkey 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 42°C etc. In addition to the biochemical reactions,

84 strains of *Pseudomonas aeruginosa* were further tested for their ability to grow on ceftrimide (0.3%)
 85 agar. *Pseudomonas aeruginosa* was identified by its colony characters, pigment production and its
 86 characteristic odour, growth on ceftrimide agar with 6% NaCl.

87 **2.2 Antibiotic sensitivity testing of isolated Non-fermenters:** Overnight broth culture having
 88 semi-confluent growth of the isolated bacterial strain was used as inoculums. Muller-Hinton agar
 89 plates were used and subjected to antibiotic susceptibility test using Disc-diffusion method of Kirby-
 90 Bauer (1996), using the concentration of antibiotics per discs, recommended by the WHO experts
 91 committee on biological standardization. The antibiotics used were Amikacin (30µg), Gentamicin
 92 (10µg), Cefoperazone (75µg), Ceftazidime (30µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Imipenem
 93 (10µg), Piperacillin/Tazobactam (100/10µg), Polymyxin B (300U). Results were interpreted in Zone of
 94 inhibition and comparing with the standard zone size given by Kirby-Bauer chart [5].

95 **2.3 Detection of Metallo betalactamase (MBL) Production:** Screening for MBL production
 96 was done in Multidrug resistant isolates of the non-fermenters. MDR isolate was defined as resistant
 97 to 2 or more drugs or drug classes of therapeutic relevance. Among these some carbapenem
 98 resistant isolates MBL producers [6]. Resistance to carbapenem is due to decreased outer membrane
 99 permeability, increased efflux systems, alteration of penicillin binding proteins and the production of
 100 carbapenem hydrolyzing enzymes. The resistance may also be due to the production of MBLs, such
 101 resistance can be chromosomal or plasmid mediated [7]. The following methods were used for the
 102 MBL detection in the present study which includes Imipenem-EDTA combined disc test and
 103 Imipenem-EDTA double disc synergy test (DDST). In Imipenem-EDTA combined disc test, the test
 104 organisms were inoculated onto plates with Muller-Hinton agar. Two 10µg imipenem discs were
 105 placed on the plate, an appropriate amount of 10µl of EDTA solution were added to one of them to
 106 obtain the desired concentration (750µg). The inhibition zones of the imipenem-imipenem and EDTA
 107 discs were compared after 16-18h incubation at 37°C. In the combined disc test, if the increase in
 108 inhibition zone with the imipenem and EDTA was >7mm than the imipenem disc alone, it was
 109 considered as MBL positive [8]. Discs were prepared by adding EDTA solution to 10µg imipenem
 110 discs to obtain a concentration of 750µg. The discs were dried immediately in an incubator and stored
 111 at 4°C or at -20°C in an airtight vial without dessicant. Test strains were adjusted to the Mc Farland
 112 0.5 standard and were inoculated to MH agar. A 10µg imipenem discs and an imipenem + EDTA

750µg were placed on MH agar. Another disc containing only 750µg EDTA was also placed as a control. After overnight incubation, the established zone diameter difference of >7mm 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 (10µg) disc was placed 20mm centre to centre from a blank disc containing 10µl of 0.5M EDTA (750µg). 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: A total of 250 clinical samples were investigated for the Non-fermentative gram negative bacilli. The specimens were collected from the patients suffering from burns or wound infections, urinary tract infections, cirrhosis of liver, septicaemia, pneumonia, meningitis etc. Among the 250 clinical samples, 60 were found to be as non-fermenter isolates, which was 24% of the total cases. These 60 were used for further processing (Figure 1).

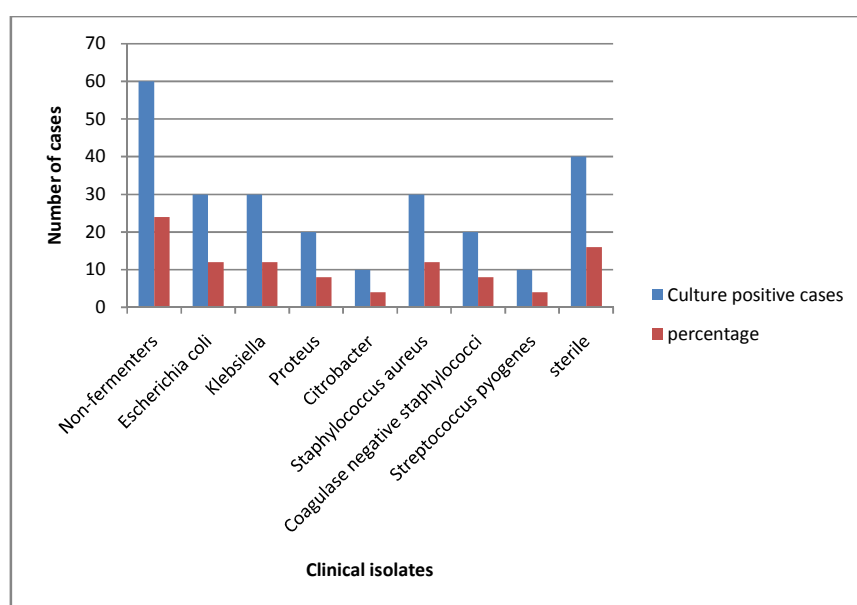


Figure 1: Different types of bacterial isolates in 250 clinical samples

Various age groups ranging from 1 and 79 years of both sexes were included in the present study. Out of 250 patients, 160 were males and 90 were females. The analysis of these cases in different age group is represented in (Table 1).

Table 1: Distribution of cases from different types of infections according to the age

S.NO	Age	Number of cases	Percentage
1.	1-9	15	6
2.	10-19	24	9.6
3.	20-29	58	23.2
4.	30-39	52	20.8
5.	40-49	27	10.8
6.	50-59	39	15.6
7.	60-60	25	10
8.	70-79	10	4
	Total	250	

From the table it was noticed that, different types of infections 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%). High number of males 160 (64%) and a lower number of females 90 (36%) were seen who attended the hospital from various infections (Figure 2).

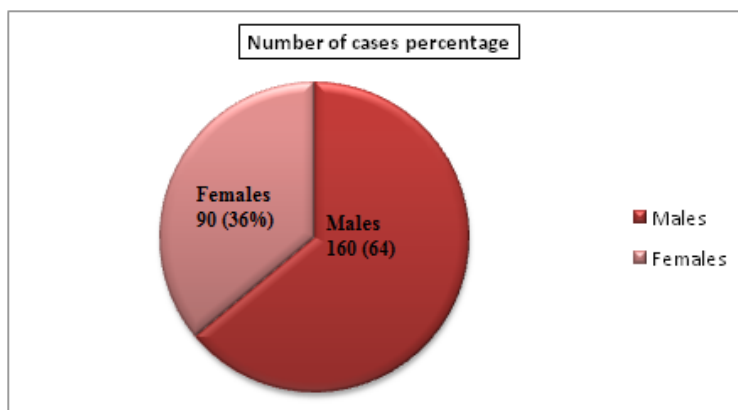


Figure 2: Distribution of cases from various types of infections according to sex

Depending on the source, the infections were categorized into Hospital acquired and Community acquired. The highest rate of non-fermenters (93.33%) isolated from the patients admitted into the hospital. The lowest prevalence was seen in patients attended to OPD (6.67%)(Table 2).

Table 2: Categories of cases based on the source of infection

Source of infection	Number of cases	Culture positive cases	Percentage
Hospital-acquired	200	56	93.33%
Community acquired	50	4	6.67%
Total	250	60	

Among 60 different non-fermenters were isolated, 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%) (Figure 3).

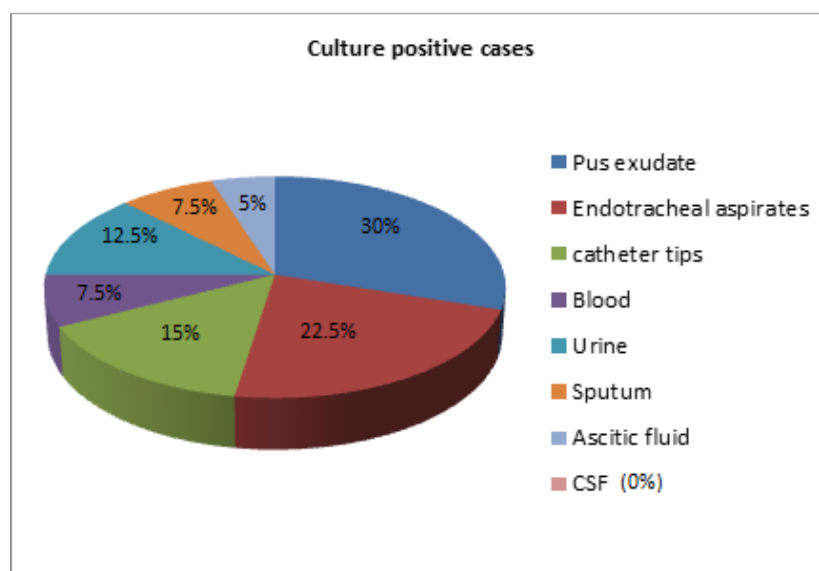


Figure 3: Distribution of isolates of *Pseudomonas aeruginosa* from various clinical samples

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%) (Figure 4).

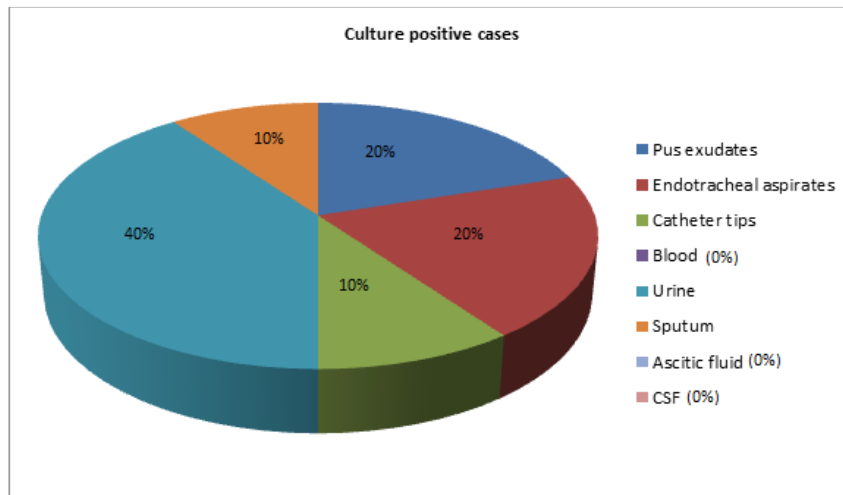


Figure 4: Distribution of isolates of *Acinetobacter baumannii* from various clinical samples

The highest rate of *Alcaligenes faecalis* isolates was isolated from Blood 3 (50%) out of 6 isolates (Figure 5) whereas urine, sputum, ascitic fluid and CSF were culture negative.

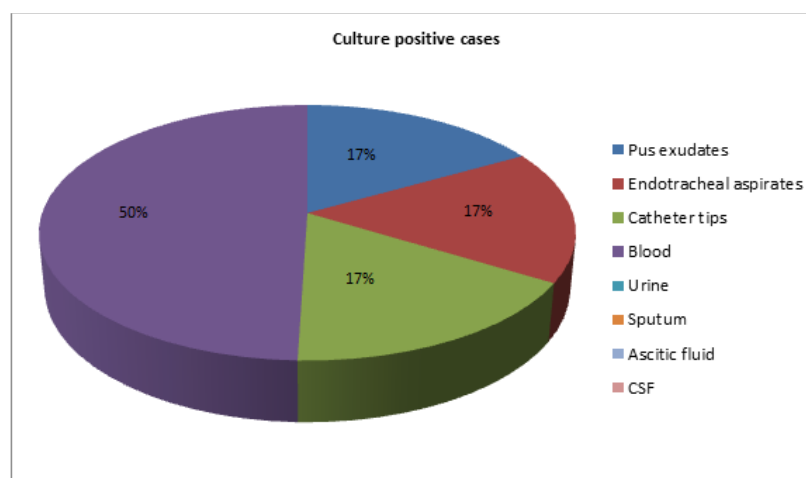
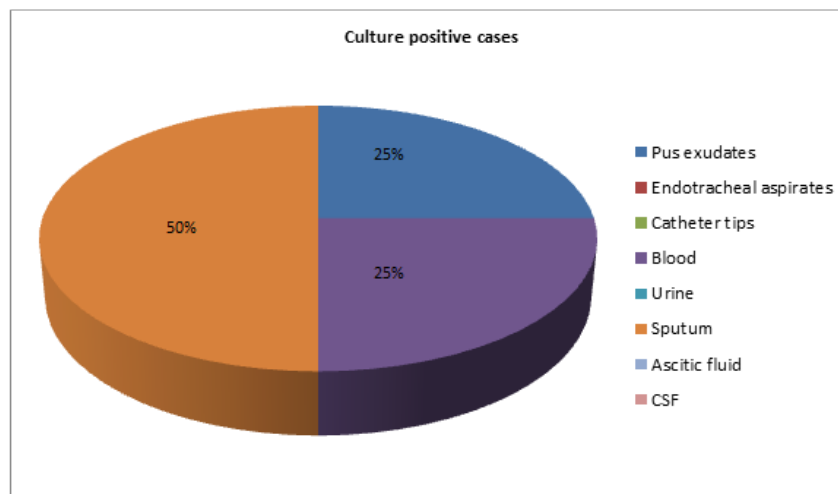


Figure 5: Distribution of isolates of *Alcaligenes faecalis* from various clinical samples

163 Among 4 *Stenotrophomonas maltophilia*, the highest rate of the isolates were reported from sputum
 164 (50%) (Figure 6). No growth was seen from the specimens like endotracheal aspirates, catheter tips,
 165 urine, ascitic fluid and CSF.



166
 167 **Figure 6: Distribution of isolates of *Stenotrophomonas maltophilia* from various**
 168 **clinical samples**

169 The isolated *Pseudomonas aeruginosa* differentiated from other species like *P.fluorescens*, *P.putida*,
 170 *P.stutzeri* and *P.luteolaby* some characteristic features (Table 3).

171 **Table 3: The differentiating characters of *Pseudomonas aeruginosa* from other**
 172 **species**

S.No	Property	<i>Pseudomonas aeruginosa</i>
1.	Oxidase test	Positive
2.	Odour	Fruity grape like odour
3.	On Blood agar (pyocyanin)	Large colonies with metallic sheen, mucoid, pigmented (pyocyanin) and beta type of hemolysis
4.	Growth at 42°C	Positive

5.	Arginine decarboxylation	Positive
6.	Lysine decarboxylation	Negative
7.	Nitrate reduction test	Positive
8.	Acetamide production	Positive
9.	Gelatin hydrolysis	Positive

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174 All the isolated non-fermenters were differentiated by using various cultural and biochemical tests
 175 (Table 4) (Figure 7) (Figure 8).

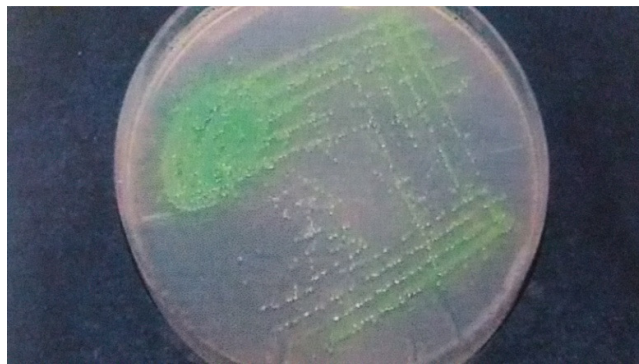
176 **Table 4: The differentiating features utilized for the isolates**

S.No	Test	<i>P.aeruginosa</i>	<i>A.baumannii</i>	<i>A.faecalis</i>	<i>S.maltophilia</i>
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 Mac-conkey 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 37°C	Positive	Positive	Positive	Positive
9.	Growth at 42°C	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

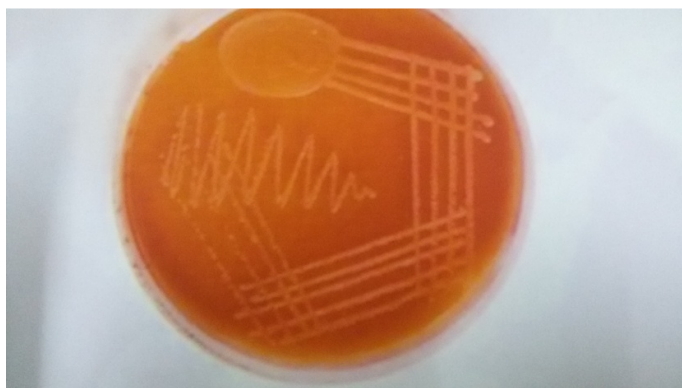
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180 **Figure 7: Cetrimide agar plate showing yellow fluorescence of the colonies of**
 181 ***Pseudomonas aeruginosa***

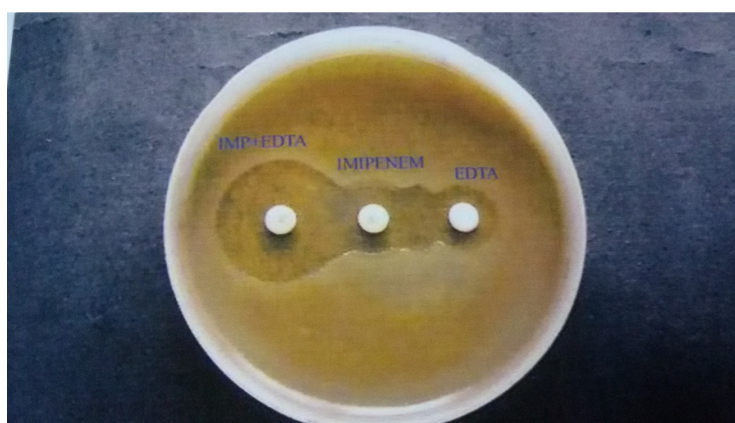


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183 **Figure 8: Mac-conkey agar plate showing growth of *Acinetobacter baumannii***

184 The overall antibiotic sensitivity pattern of Non-fermenters isolated from various clinical samples
 185 revealed that imipenem was most effective (Figure 9), followed by ceftazidime,
 186 piperacillin/tazobactam, ceftriaxone, cefoperazone, ciprofloxacin, amikacin and gentamicin. Imipenem
 187 resistant isolates showed sensitive to polymyxin-B (300U) (Figure10). Among carbapenem resistant
 188 isolates of non-fermenters, 100% showed Metallo-betalactamase production (Table 5).

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191 **Figure 9: Combined disc test showing MBL production by *Alcaligenes faecalis***

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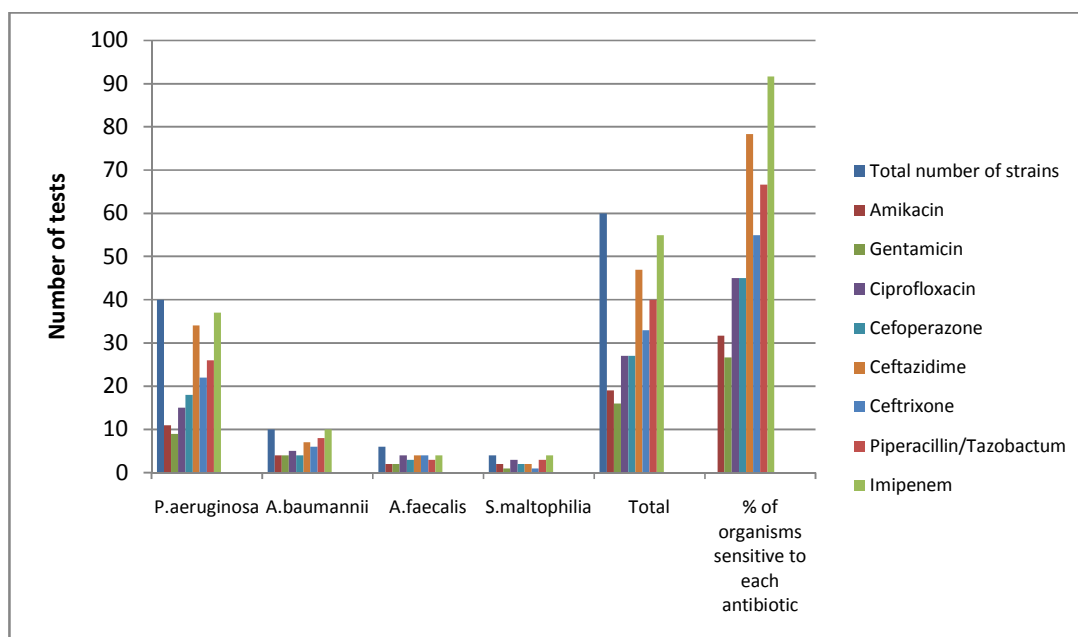


Figure 10: Antibiotic susceptibility pattern of the isolates

Table 5: Susceptibility of isolates to carbapenem in relation to MBL production

S.No	Type of organisms	Number of isolates	Resistant to carbapenem	Percentage	MBL production	Percentage
1.	<i>P.aeruginosa</i>	40	3	7.5	3	100
2.	<i>A.baumannii</i>	10	0	0	0	0
3.	<i>A.faecalis</i>	6	2	33.33	2	100
4.	<i>S.maltophilia</i>	4	0	0	0	0
5.	Total	60	5	8.33	5	100

4. DISCUSSION: The present study was undertaken to evaluate the role of Non-fermentative gram negative bacilli in causing various infections especially in hospitalized patients and to know the prevalence of Metallo-betalactamase producing Non-fermenters. Out of 250 different samples, rate of recovery of non-fermenters is 24% which is comparable with the study of Pathmanathan et al. (24.8%) [9] and Shail Preet Sidhu et al. (28%) [10]. Mahajan et al. (80.78%) [11], Dey and Bairy (74.9%) [12]

202 reported a higher rate of isolates, whereas Lahiri et al. reported a lower rate of isolates (12.9%) [13].
 203 In the current study, males were found to be more commonly affected (64%) with different infections
 204 caused by non-fermenters than females (36%) which is comparable to the findings of Varaiya et al.
 205 who observed that 66.66% were males and 33.3% were females [14]. Nan-Yao Lee et al. reported
 206 67.4% were males and 32.6% were females [15]. Prashanth et al. also found that males were more
 207 commonly affected (58%) than females (42%) [16]. Different types of infections seems to be more
 208 common in males than females, and this male preponderance may be due to that males are more
 209 involved with outdoor activities and therefore chances of exposure to infections are more likely. An
 210 observation of the source of infection in the present study was indicated that the rate of isolation of
 211 Non-fermenters was higher among the hospital acquired infections (93.33%) than in community
 212 acquired infections (6.67%). Our observation is comparable with the study of Lahiri et al. [13] who
 213 showed higher incidence of non-fermenters (82.9%) in hospital acquired infections than in community
 214 acquired infections (17.1%). Among the isolated non-fermenters *Pseudomonas aeruginosa* was the
 215 commonest isolate (66.67%) which coincides with the study of Arjun srinivasan et al. (66.75%) [17]
 216 and Mahajan et al. (54.54%) [11]. Pathmanathan et al. [9] reported a lower rate of isolates (7.2%) and
 217 also by Neelam Kaistha et al. (11.5%) [18]. *Acinetobacter baumannii* was the second most common
 218 non-fermenter (16.67%), in our study. This observation is similar to that of Pathmanathan et al.
 219 (17.6%) [9]. The higher incidence of the isolate was reported by Neelam Kaistha et al. (30.76%) [18]
 220 and also by Dey and Bairy (47.9%) [12]. 10% of *Alcaligenes faecalis* were obtained in the present
 221 study where as Shali Preet Sidhu et al. [10] reported only 2% and Seeta et al. (3) reported only
 222 1.45%. 6.66% *Stenotrophomonas maltophilia* was reported in the present work but Shali Preet Sidhu
 223 et al. [10] reported only 1%. Regarding the antibiotic susceptibility pattern, the highest sensitivity rate
 224 was observed with Imipenem (91.67%) and it coincides with the study of Duggal et al. (95.74%) [19]
 225 and Agarwal et al. (90.5%) [7]. The least sensitivity was observed to gentamicin (26.66%) in our
 226 study. In the present investigation, 7.5% of the *Pseudomonas aeruginosa* proved to be resistance to
 227 the carbapenems and all the carbapenem resistant strains were MBL producers (100%). Shali Preet
 228 Sidhu et al. [10] reported that 6.97% of the *Pseudomonas aeruginosa* showed resistance to the
 229 carbapenems, and it is coinciding with the present study. Irfan et al. [4] reported (100%) of the
 230 carbapenem resistant isolates of *Pseudomonas aeruginosa* were MBL producers, which are also
 231 coinciding with the study.

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.

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Figure captions:

Figure 1: Different types of bacterial isolates in 250 clinical samples

Figure 2: Distribution of cases from various types of infections according to sex

Figure 3: Distribution of isolates of *Pseudomonas aeruginosa* from various clinical samples

287 **Figure 4: Distribution of isolates of *Acinetobacter baumannii* from various clinical**
 288 **samples**

289 **Figure 5: Distribution of isolates of *Alcaligenes faecalis* from various clinical samples**

290 **Figure 6: Distribution of isolates of *Stenotrophomonas maltophilia* from various**
 291 **clinical samples**

292 **Figure 7: Ceftrimide agar plate showing yellow fluorescence of the colonies of**
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294 **Figure 8: Mac-conkey agar plate showing growth of *Acinetobacter baumannii***

295 **Figure 9: Combined disc test showing MBL production by *Alcaligenes faecalis***

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