



Original Article

Phenotypic detection of ESBL and MBL production among Non-Fermenters isolated from various clinical specimens

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OPEN ACCESS

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Received: 13-01-2026

Accepted: 30-01-2026

Available online: 16-02-2026

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ABSTRACT

Introduction: The emergence of antimicrobial resistance is a major public health challenge globally. Non fermenters are emerging now-a-days and posing a critical threat to hospitalized patients. They also have become one of the most common etiological agents of hospital acquired infections. The aim is to isolate and identify the various non fermenters from patients admitted in our tertiary care hospital and their antimicrobial susceptibility pattern and carry out phenotypic tests to identify ESBL and MBL producers.

Materials and methods: A descriptive cross sectional study was conducted for a period of 7 months by collecting the clinical samples as per the standard protocols. The Identification of bacteria was performed by conventional microbial culture and biochemical tests according to the standard recommended techniques. All data were entered into a Data Collection proforma Sheet and were entered into Microsoft excel data sheet.

Results: Out of 1654 clinical samples we have isolated 100 (6.04%) non fermenters bacteria. Out of 100 samples majority were *Pseudomonas aeruginosa* it was 96% and remaining 4% were *Acinetobacter baumannii*. Majority of the isolates were from pyogenic infections and respiratory tract infections. Both *Pseudomonas aeruginosa* and *Acinetobacter baumannii* showed high susceptibility to amikacin (93%), meropenem (86%), imipenem (81%), moderate susceptibility to piperacilin+tazobactam (79%), levofloxacin (73%). Among non fermenters 23% were ESBL producers and 13% were MBL producers in the present study.

Conclusion: Non fermenter Gram Negative bacilli are becoming resistant to beta lactam and beta lactam inhibitors and also emerging as carbapenem resistant. To prevent the spread of carbapenem resistant Non fermenters it is critically important to implement policies stringently by state and local health authorities by framing a strong antimicrobial stewardship programme and practicing infection control measures.

Keywords: Non fermenters, Antibiotic Resistance ESBL, MBL.

INTRODUCTION

The Non fermenters are Gram negative, aerobic, motile or non motile, non sporing bacilli. Many non fermenters are widely distributed, taxonomic placement of these still remains unresolved [1]. The major genera of Non fermenters Gram Negative Bacilli (NFGNB) were classified into at least 15 families, including Alcaligenaceae, Alteromonadaceae, Burkholderiaceae, Flavobacteriaceae, Pseudomonadaceae and others.

They are the habitants of soil and environment; causing humans infections as an opportunistic pathogen. Risk factors include immunosuppression, trauma, foreign body, infused body fluids like saline irrigations, indwelling catheters, invasive diagnostic as well as therapeutic procedures, prolonged hospital stay and using broad spectrum antibiotics [2,3].

NFGNB accounts for nearly 12-16% of all bacterial isolates in a clinical microbiology laboratory [4]. They cause various infections such as septicemia, meningitis, pneumonia, urinary tract infections, and surgical site infections (SSI) [5].

Antimicrobial resistance of non fermenters is emerging globally mainly due to inherent resistant to most of the antibiotics available, this is a naturally possessed trait which is often encoded by their own DNA that shows resistance to certain antibiotics and during the therapy or due to selective pressure the strains get mutate become more resistant. The most common mechanism of resistance is mutation of porin genes. *Pseudomonas aeruginosa* expresses their antibiotic resistance by various mechanisms such as beta lactamase hydrolysis, decreased permeability, altered binding proteins, enzymatic hydrolysis by acetylation, adenylation, phosphorylation and altered ribosomal targets [6].

The emergence of antimicrobial resistance is a major public health challenge globally. Non fermenters are emerging now-a-days and posing a critical threat to hospitalized patients. They have become one of the most common hospital acquired infections. Extended-spectrum beta-lactamases (ESBL) producing pathogens offers resistance to penicillins, aztreonam, and first-, second-, and third-generation cephalosporins—but are unable to hydrolyze cephamycin or carbapenems [7]. For the treatment of ESBL producing pathogens penems are the best choice of management. In the latest decades this scenario has been changing due to emergence of metallo-beta-lactamases (MBL) in gram negative bacilli. It is very necessary to know the mechanisms of resistance acquisition of their corresponding genes and nature as well as the rate of their genetic exchange.

Hence this study was undertaken to isolate and identify the various non fermenters from patients admitted in our tertiary care hospital and their antimicrobial susceptibility pattern and carry out phenotypic tests to identify ESBL and MBL producers.

AIM & OBJECTIVES

1. To estimate the prevalence of non fermenters in various clinical samples in a tertiary care hospital.
2. To study the antimicrobial susceptibility pattern of the isolates & to estimate the antibiotic resistance rate.
3. To detect ESBL & MBL by phenotypic methods.

MATERIALS AND METHODS

Study Settings:

A descriptive cross sectional study was conducted for a period of 7 months by collecting the clinical samples from the Govt. Siddhartha Medical College and Hospital, Vijayawada which is a tertiary care hospital. Institutional ethical committee permission was obtained. An informed consent was taken from the study population.

Inclusion criteria:

Isolates from all clinical samples which were identified as Non-fermenting Gram negative bacilli as per recommended standard methods.

Exclusion criteria:

1. Mixed growth of > 3 types (probably contaminated sample)
2. Isolates from improperly collected samples.

Study population:

At Microbiology department we processed a total of 1654 clinical specimens from outpatients and inpatients who were attending Government General Hospital, Vijayawada as per the standard protocols. Out of which 100 non fermenters which were considered as pathogens were further processed in this study.

The procedure of the study and significance of the study was explained to the study participants before collecting a sample. The clinical samples collected were blood, urine, sputum, throat swab, endotracheal aspirate, broncho alveolar lavage, exudates, pus, and body fluids.

Study procedure:

The samples were collected into recommended sample collection containers under aseptic precautions. Samples were transported to the lab immediately after collection, if there is any delay in the transportation the samples were stored at 2-8°C and processed within 24 hours. Samples were inoculated on nutrient agar, blood agar and mac conkey agar which were incubated overnight at 37°C and observed for growth for 48 hrs. The identification of bacteria was performed by conventional microbial culture and biochemical tests according to the standard recommended techniques. As the NFGNB could be hospital contaminants and are opportunistic pathogens, their clinical significance was assessed by the time of microbiological detection of pathogen, inflammatory and infectious markers investigations, relevant radiological findings and clinical history and examination findings.

Antibiotic susceptibility testing:

Antimicrobial susceptibility testing was performed by Kirby Bauer disc diffusion technique according to latest CLSI guidelines. The following antibiotics were used for susceptibility testing: Ceftazidime, Cefepime, Levofloxacin, Amikacin, Piperacillin, Piperacillin-Tazobactam, Meropenem, Imipenem, and colistin. The *P.aeruginosa* ATCC 27853 strain was used as the quality control. All the discs were procured commercially (Hi-media laboratories limited). The diameter of the zone of inhibition was measured and interpreted according to the latest CLSI guidelines [8]. Colistin was tested by broth dilution.

Screening and confirmation for ESBL production:

All the isolates were screened for possible ESBL production using antibiotic discs of ceftazidime (30 µg). According to the guidelines, bacterial isolates showing ceftazidime < 22 mm were considered possible ESBL producers. The suspected ESBL producer strains were subjected to double disc synergy test (DDST) Fig 1 for the confirmation of ESBL production. ESBL producers were confirmed by using Ceftazidime disc alone and in combination with clavulanic acid by using disc diffusion method. Difference in zone of inhibition ≥ 5mm between Ceftazidime and clavulanic acid combination is suggestive of ESBL producers. *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as control for ESBL production.

Screening and confirmation for MBL producers:

Imipenem resistant isolates were tested for carbapenemase production. Further confirmation of MBL production was done by two methods - imipenem and imipenem EDTA E strip and mCIM method.

E-test MBL strip method:

E-test MBL strip method was done by using imipenem+imipenem EDTA E strip which is a Ezy MIC strip procured from HiMedia, Mumbai. E-test MBL strips consist of a double-sided seven- dilution range of Imipenem IP (4–256 mcg/ml) and IP (1–64 mcg/ml) overlaid with a constant 36 gradient EDTA. Individual colonies were picked from overnight agar plates and suspended in 0.85% saline to a turbidity of 0.5 Mc Farland's. A lawn culture of the inoculum was performed on an MHA plate and E strip placed. The plates were incubated for 16–18 h at 37°C. The MIC endpoints were read where the inhibition ellipses intersected the strip.

Interpretation: When the ratio of the value obtained for Imipenem: the value of imipenem+EDTA is more than 8 or if the zone is observed on the imipenem+EDTA side and no zone is observed on the opposite side with imipenem, interpreted the isolates as MBL positive.

Carbapenemase detection by mCIM method:

Bacterial Isolate to be tested is emulsified in Trypticase soy broth, 10µg Meropenam disc is added to the broth and incubated 37°C for 4 hours. Meropenam disc was placed on MHA plate inoculated with ATCC E. coli, incubated at 37°C overnight and zone sizes were recorded. mCIM test was interpreted as follows: Positive: Carbapenemase detected- zone size 6-15mm or presence of pinpoint colonies within 16-18mm zone; Negative: Carbapenemase not detected - Zone diameter >19mm as per CLSI guidelines.

Data Collection:

All data were entered into a Data Collection proforma Sheet and were entered into Microsoft excel data sheet. The data were expressed as mean and SD and wherever necessary bar charts were also used. Statistical analysis was done in terms of numbers and percentages as quantitative data.

RESULTS

A total of 1654 clinical samples were processed as per the standard guidelines at Microbiology department to evaluate the exact pathogen causing infection and its antibiotic susceptibility pattern. Out of 1654 clinical samples we have isolated 100 (6.04%) non fermenters bacteria during the study period from various clinical specimens. Out of 100 samples majority were *Pseudomonas aeruginosa* i.e., 96% and remaining 4% were *Acinetobacter baumannii*.

Most of the Non fermenter infected patients were males (71%), with the male to female ratio being 2.4. On analyzing the non fermenters predominance in age group we found majority in 41-60 years of population, which accounted 40% (Fig 1).

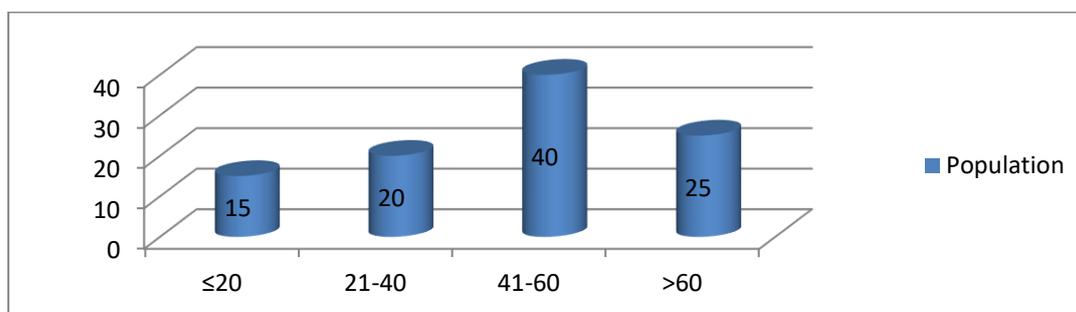
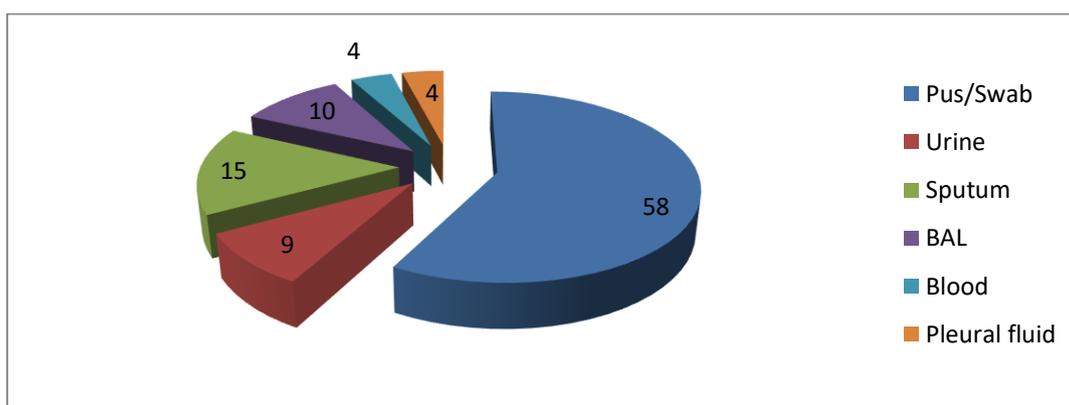


Fig 1. Age wise distribution of pathogens

These 100 non fermenters were isolated from different specimens. Majority of the isolates were from pyogenic infections and respiratory tract infections. Non fermenters were predominantly noted in pus/swab samples (58%), followed by sputum (15%), bronchoalveolar lavage (10%), urine (9%), blood (4%) and pleural fluid (4%).



Both *Pseudomonas aeruginosa* and *Acinetobacter baumannii* showed high susceptibility to amikacin (93%), meropenem (86%), imipenem (81%), moderate susceptibility to piperacillin/tazobactam (79%), levofloxacin (73%). Other antibiotics susceptibility percentage is cefipime 62%, ceftazidime 63%. 100% of isolates were sensitive to colistin.

Table 1. Antibiotic susceptibility testing of Non fermenters

Antibiotics	<i>Pseudomonas aeruginosa</i> (n=96)			<i>Acinetobacter baumannii</i> (n=4)		
	Sensitive	Intermediate	Resistance	Sensitive	Intermediate	Resistance
Ceftazidime	60 (62.5%)	4 (4.1%)	32(33.3%)	3 (75%)	0	1 (25%)
Cefipime	58 (60.4%)	8 (8.3%)	30 (31.2%)	3 (75%)	0	1 (25%)
Levofloxacin	70 (72.9%)	4 (4.1%)	22 (22.9%)	3 (75%)	0	1 (25%)
Amikacin	89 (92.7%)	5 (5.2%)	2 (20.8%)	4 (100%)	0	0
Piperacillin	48(50%)	4 (4.1%)	44(45.8%)	3(75%)	0	1(25%)
Piperacillin/tazobactam	76 (79.1%)	6 (6.25%)	14 (14.5%)	3 (75%)	0	1 (25%)
Meropenem	83 (86.4%)	5 (5.2%)	8 (8.3%)	3 (75%)	0	1 (25%)
Imipenem	78 (81.2%)	8 (9.3%)	8 (9.3%)	3 (75%)	0	1 (25%)
Colistin	-	96 (100%)	0	-	4 (100%)	0

On assessing for the ESBL isolates, 22% were detected amongst *Pseudomonas aeruginosa*. Carbapenemase detection was noted in 18 (18.7%) *Pseudomonas* isolates and 1 (25%) *Acinetobacter* isolate.

The mCIM method and Imipenem+Imipenem EDTA e strip method were compared on Carbapenem resistant *Pseudomonas aeruginosa* isolates (n=18) to analyze the sensitivity and specificity of methods. 12 (12.5%) out of 18 carbapenemase resistant *Pseudomonas* isolates showed MBL detection by mCIM method and E test MBL strip method. Both mCIM method and E test MBL strip method showed positive on 12.5% *Pseudomonas* isolates, which conclude that either of these methods are significant to test MBL production in carbapenem resistant isolates.



Fig 1 Double disc synergy test (DDST)

DISCUSSION

Non fermenters are nutritionally versatile, dangerous opportunistic pathogens because of their tolerance to physical, chemical and antibacterial compounds. The problem with the non fermenters is they are inherently resistant to many antibiotics including broad spectrum antibiotics. Antibiotics of Non fermenters have been draining out due to antimicrobial resistance. The drugs that are commonly used to treat the infections are carboxypenicillins (Carbenicillin, Ticarcillin) the ureidopenicillins (Piperacillin), the antipseudomonal cephalosporins (Ceftazidime), monobactams (Aztreonam), carbapenems (Imipenem and Meropenem) and aminoglycosides (Gentamicin, Tobramycin, Amikacin). *P.aeruginosa* has the capability of carrying multi-resistance plasmids and this feature has led to the emergence of some *P.aeruginosa* strains that are resistant to all antibiotics [9,10].

Out of 1654 clinical samples we have isolated 100 (6.04%) non fermenters bacteria during this study period from various clinical specimens. A study from Central India by Soni M et al [11] reported 10.01% of non fermenter gram negative bacilli (NF-GNB) and a study from Northwestern India by Grewal US et al [12] noted 11.6% of prevalence of NF-GNB, both these are in similar to this study. Sarkar M et al [13] did a study in Eastern India noted 13.18% of clinical samples were non fermenters. Benachinmardi K et al [14] noted male predominance during the isolation of non fermenters from clinical specimens which is similar finding as this study. On analyzing the non fermenters predominance in age group we found majority in 41-60 years of population, which accounted 40% in this study. Grewal S et al [12] noted 40% of the non fermenters were in the age group of 46-65 years. Variation in the age group and difference in the prevalence of non fermenters depends on various factors like health policies, localities, health care resources, patient factors and antibiotic usage in the community.

Non fermenters were predominantly noted in pus/swab samples (58%), followed by sputum (15%), bronchoalveolar lavage (10%), urine (9%), blood (4%) and pleural fluid (4%). Soni M et al reported the majority of MDR NF-GNB isolated were from pus (45.50%) followed by blood (20.50%). In similar to our study most of the isolates of NFGNB were from pus samples studied by others [15,16,17].

Out of 100 samples majority were *Pseudomonas aeruginosa* (96%) and remaining *Acinetobacter baumannii* (4%) in the present study. Sarkar M et al [13] noted among non fermenters, most were *Acinetobacter baumannii* (51.34%) followed by *Pseudomonas aeruginosa* (42.09%), *Burkholderia cepacia* complex (4.38%) and others (2.19%). Others included *Burkholderia pseudomallei*, *Acinetobacter lwoffii* and *Stenotrophomonas maltophilia*. Soni M et al [11] observed out of 743 non-duplicate MDR non-fermenters, the most common were *Pseudomonas aeruginosa* (51.7%), *Acinetobacter baumannii* (23.4%), and others (24.9%). Malini N et al [18] conclude that *Pseudomonas aeruginosa* was the most common nonfermenter, accounting for 53.8%, followed by *Acinetobacter baumannii* (22.2%), and *Pseudomonas fluorescens* (10.8%). Other significant NFGNB isolated were: *Sphingobacterium* species (5.2%), *Acinetobacter lwoffii* (3.1%), and *Stenotrophomonas maltophilia* (2.6%). In most of the studies *Pseudomonas* species were predominant non fermenter isolate among various infectious samples, as it is an environmental pathogen and resistant to few disinfectants and antibiotics the pathogen is spreading easily in the community and hospital.

Both *Pseudomonas aeruginosa* and *Acinetobacter baumannii* showed high susceptibility to amikacin (93%), meropenem (86%), imipenem (81%), moderate susceptibility to piperacilin+tazobactam (79%), levofloxacin (73%). Other antibiotics susceptibility percentage is cefipime 62%, ceftazidime 63%. 100% of isolates were sensitive to colistin as per this study. Malini et al [18] reported almost similar to this study as *P. aeruginosa* isolates were highly susceptible to imipenem (94%), cefoperazone (71%), and amikacin (69%). Taneja N et al [19] showed 42% of isolates are resistant to imipenem and Veenakumari HB et al [20] showed that *P. aeruginosa* was 60-70% resistance to amikacin, and ciprofloxacin, which

was higher resistance than our study. In line with this study Sarkar M et al [13] reported highest sensitivity to gentamicin and amikacin shown by *A. baumannii* and *P. aeruginosa* respectively while both were mostly resistant to ceftriaxone. Nazir et al [21] showed that *Acinetobacter baumannii* was highly susceptible to colistin (100%), followed by tigecycline (84%), imipenem (40%), ciprofloxacin (28%), amikacin (26%), and ceftazidime (20%).

23% were ESBL producers and 13% were MBL producers in the present study which is similar to a study from Tamilnadu which noted 38.3% ESBL producers and 18% MBL producers among non fermenters isolated from various clinical samples [22]. ESBL producing *Pseudomonas aeruginosa* were 42.3% and 22.2% by Goel Varun et al [23] and Agarwal et al [24] and the MBL production was 24% and 28% by Nagaveni et al [25] and Anuradha et al [26] respectively. Taneja N et al reported 12% of *Acinetobacter* isolates being MBL producers.

Pseudomonas aeruginosa is emerging as a MDR pathogen due to its multiple complex resistance mechanisms, including decreased expression of outer membrane porins (OprD), hyperproduction of AmpC enzymes, upregulation of efflux pumps, carbapenemase production, and mutations in penicillin-binding protein targets [27].

In recent years *Acinetobacter* resistance increased towards aminoglycoside resistance and carbapenem resistant strains in nosocomial outbreaks [28]. For serious infections combined treatment with aminoglycoside and ticarcillin or piperacillin is synergistic and effective. The only antimicrobial agent which is proved active against MDR *Acinetobacter* is colistin [29]. Soni M et al [11] did an extensive study on MDR non fermenters from Central India, noted around 5.2% *Pseudomonas aeruginosa* and 2.3% *Acinetobacter baumannii* were resistant to colistin, and 88.2% were resistant to ceftazidime.

Both mCIM method and E test MBL strip method showed positive on 12.5% *Pseudomonas* isolates, which conclude that either of these methods were significant to test MBL production in carbapenem resistant isolates. Walsh TR et al noted Etest IP plus IP-EDTA with Mueller-Hinton agar had a sensitivity of 94% (79 of 84) and specificity of 95% (124 of 130) [30].

Isolation of medically important non fermenters from the hospitalized patient is worrisome but does not normally justify therapeutic intervention unless there is evidence of disease. Isolations of multi-drug –resistant, extensively drug resistant or even pan drug resistant gram negative pathogens are causing major therapeutic problems and at the same time are posing infection control issues in many health care centers. This results in increased morbidity and mortality of the patients, increased length of hospital stay and higher hospital costs [31]. Colistin is increasingly becoming the drug of choice for such pan-resistant organisms.

CONCLUSION

Pseudomonas species were predominantly isolated in various clinical samples. Non fermenters were significantly isolated in pyogenic infections followed by respiratory infections. Non fermenters are usually opportunistic infections responsible for respiratory infections, pyogenic infections, urinary tract infections, blood stream infections, ear infections, and skin infections. Non fermenters showed a good susceptibility to colistin, aminoglycosides, carbapenems, and fluoroquinolones.

Non fermenter Gram Negative bacilli are becoming resistant to beta lactam and beta lactam inhibitors and also emerging as carbapenem resistant; resistance to routinely used antibiotics is a major public health problem. To prevent this antibiotic resistant institute should frame a strong antimicrobial stewardship program and practice infection control measures on regular basis.

ACKNOWLEDGEMENT

We would like to express our gratitude to Multidisciplinary Research Unit, Government Siddhartha Medical College for their invaluable financial support in completing this project (LRAC reference no.LRAC/MRU/SMC/VJA/003/2023)

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