

# Characterization and Antimicrobial Susceptibility Pattern of Non-fermenting Gram-negative Bacilli and Molecular Analysis of *Pseudomonas* Species from Respiratory Samples of Intensive Care Unit Patients in A Tertiary Care Hospital

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## Abstract

Among patients admitted to the intensive care unit (ICU) the most common cause of infection is lower respiratory tract infections, which account for 4.4% of hospital admissions. Among the non-fermenters, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus-baumannii* complex, cause many types of nosocomial infections. Ventilator-associated pneumonia is the most common emerging infection among them. The goal of this study was to isolate and speciate non-fermenting Gram-negative organisms from respiratory samples of ICU patients and to detect antimicrobial susceptibility patterns of the isolated non-fermenters. This cross-sectional study was conducted at the Institute of Microbiology in collaboration with Medical Intensive Care units in Rajiv Gandhi Government General Hospital. A total of 200 patients who satisfied the inclusion criteria were included in the study conducted between March 2019 and March 2020. Culture, sensitivity, and anti-microbial susceptibility tests were performed for the respiratory samples collected as per standard protocols. *Pseudomonas aeruginosa* (49%) was the most commonly isolated non-fermenter followed by *Acinetobacter baumannii* (24.3%). Among *Pseudomonas aeruginosa* isolates, the highest percentage were ESBL producers (44.4%). Carbapenem resistance among *Pseudomonas* isolates was 33%. The study showed increased isolation of MDR non-fermenters from the ICU causing Ventilator-associated pneumonia (VAP). To prevent VAP caused by these MDR pathogens, clinicians should follow strict infection control practices, use invasive devices on a short-term basis, and use antibiotics judiciously.

**Keywords:** Non-fermenters, Ventilator Associated Pneumonia(VAP), *Pseudomonas aeruginosa*, *Acinetobacter baumannii*

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## INTRODUCTION

Among patients admitted to the intensive care unit (ICU), the most common cause of infection is lower respiratory tract infections, which account for 4.4% of hospital admissions and occur in 10-25% of all intensive care patients, leading to high overall mortality (22.71%).<sup>1</sup>

Gram-negative followed by Gram-positive organisms are usually implicated in the etiologies of respiratory tract infections in ICU patients. Multi-drug resistant non-fermenting Gram-negative organisms<sup>1</sup> are commonly the most notorious among them.

Non-fermenting Gram-negative organisms are ubiquitous, prefer moist environments, and in hospital ICUs can be isolated from nebulizers, dialysate fluids, saline, catheters, and other devices.<sup>2</sup> Among the emerging infections wide variety of nosocomial infections such as bloodstream infections, ventilator-associated pneumonia (VAP), urinary tract infections, and wound infections<sup>1</sup> are caused by non-fermenters such as *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus-baumannii* complex, and *Stenotrophomonas maltophilia*.

*Pseudomonas aeruginosa* is a common pathogen present in hospital ICUs involved in causing various life-threatening infections due to various innate and acquired drug resistance.<sup>3</sup> Metallo beta-lactamase-producing *Pseudomonas* has been documented with increasing mortality rates in ICU patients.<sup>4</sup> Beta lactamases which cause carbapenem hydrolysis, with elevated carbapenem MICs, belong to molecular classes A, B, and D.<sup>5</sup> *Acinetobacter* species is an emerging opportunistic infection in ICUs. There is an increasing trend of aminoglycoside resistance and carbapenems resistance in nosocomial outbreaks.<sup>6,7</sup>

*Pseudomonas* resists a wide range of antipseudomonal agents<sup>8</sup> and develops resistance via both intrinsic and extrinsic mechanisms. Intrinsic genes cause over-expression of efflux pumps (*mexAB*, *mexCD*, *mexEF*, and *mexXY*), development of chromosomal hyper-Amp C producers, and loss of porins (*opr D*). ESBL (*bla<sub>SHV</sub>*, *bla<sub>TEM</sub>*, *bla<sub>VEB</sub>*, *bla<sub>PER</sub>*, and *bla<sub>OXA</sub>* types) carbapenems (*bla<sub>GES</sub>*, *bla<sub>KPC</sub>*, *bla<sub>IMP</sub>*, *bla<sub>SPM</sub>*, *bla<sub>VIM</sub>* and *bla<sub>NDM</sub>*) are the extrinsic genes.

The mortality rate of *Pseudomonas aeruginosa* causing pneumonia is up to 70%.<sup>8</sup> The present study focuses on the isolation, speciation, and drug susceptibility patterns of non-fermenting bacteria, with a specific emphasis on detecting multi-drug resistance in *Pseudomonas* species. This detection was achieved through both phenotypic and molecular detection methods, including PCR, to identify resistant genes in the isolated samples. This may emphasize the nosocomial spread of multi-drug-resistant non-fermenters and infection control practices in ICUs.

### Aim and objectives

1. To isolate and speciate non-fermenting Gram-negative organisms from respiratory samples of intensive care unit patients.
2. To detect antimicrobial susceptibility patterns of isolated non-fermenters.
3. To detect extended-spectrum beta-lactamases among isolated non-fermenters carbapenem resistance among *Pseudomonas* species by phenotypic methods.
4. To confirm the metallo-beta lactamase (MBL) production by phenotypic and genotypic methods among *Pseudomonas* species.

## MATERIALS AND METHODS

This cross-sectional study was conducted at the Institute of Microbiology in collaboration with Medical Intensive Care units in Rajiv Gandhi Government General Hospital. A total of 200 patients who satisfied the inclusion criteria were included in the study conducted between March 2019 and March 2020. Patients aged 18 years and above who were admitted to medical ICUs with signs and symptoms of respiratory infections, underwent intervention procedures, and had a prolonged stay were included in the study. A standard questionnaire was used to obtain the demographic and clinical profile of the patients involved in the study.

### Ethical consideration

The study (Ref – EC Reg. No. ECR/270/Inst/TN/2013) was approved by the Institutional ethical committee. The study proceeded after obtaining informed consent from the patients.

### **Sample collection, transport and processing**

Respiratory samples such as sputum, endotracheal aspirates, tracheal aspirates, and bronchoalveolar lavage were collected and immediately transported according to the standard operating procedures to the microbiology laboratory. The collected respiratory samples were homogenized mechanically by vortexing for 1 min and direct Gram' staining was done and examined for the presence of PMN cells, squamous cells, and bacteria.

### **Bacterial cultures**

Respiratory samples such as sputum, endotracheal aspirates, and Bronchoalveolar lavage were processed under aseptic precautions as per standard microbiological techniques.

### **Interpretation of cultures**

The interpretation was based on colony morphology, Gram stain, motility, and biochemical reactions. The non-fermenting Gram-negative bacilli were identified based on the catalase test, oxidase test, motility, nitrate reduction test, Hugh Leifson's oxidative fermentative test, test for indole production methyl red test, Voges Proskauer test, citrate utilization test, urease test growth at variable temperature, acetamide test, gelatin liquefaction test, 1% Sugar fermentation test, lysine decarboxylase test, ornithine decarboxylase test, and arginine dihydrolase test.

### **Antimicrobial susceptibility**

Antimicrobial susceptibility was carried on Muller Hinton Agar as per CLSI guidelines 2020, using Kirby Bauer's disc diffusion method and interpreted.

### **Detection of antimicrobial-resistant pattern Phenotypic method**

ESBL screening test using cefotaxime and ceftazidime discs, and carbapenem screening test using imipenem and meropenem were performed for the non-fermenters isolated from the respiratory samples. Positive screening tests for *Pseudomonas* species are subjected to confirmatory testing using the appropriate method outlined in CLSI 2020.

### **Extended spectrum $\beta$ lactamase detection<sup>9-12</sup>**

All the non-fermenters that were resistant to cefotaxime, or ceftazidime (screened) were tested for Extended spectrum of  $\beta$ -lactamases and confirmation was done using a cephalosporin/ clavulanate combination disk.<sup>9</sup>

### **Detection of metallo $\beta$ -lactamases production in pseudomonas species using the phenotypic method**

#### **Screening for MBL detection**

Production of MBL by *Pseudomonas* isolates was confirmed using a combined disk test (Imipenem with Imipenem-EDTA). Those isolates which were resistant to imipenem or meropenem were subjected to the confirmatory test. Enhancement of the zone of inhibition of the imipenem-EDTA combination disc of  $\geq 7$ mm when compared to the imipenem disc was interpreted as positive for MBL production.

### **Modified carbapenem inactivation method (mCIM)<sup>13</sup>**

Rapid Carba NP TEST<sup>14,15</sup> procedure done and interpreted as per kit guidelines.

### **Colistin susceptibility for carbapenem-resistant isolates.<sup>16,17</sup>**

As per ICMR Standard Operating Procedures bacteriology,<sup>18</sup> Colistin MIC was determined using the Micro broth dilution method. Isolates having MIC of  $\leq 2\mu\text{g/mL}$  should be considered susceptible and  $> 2\mu\text{g/mL}$  considered resistant.

### **Molecular characterization**

The detection of bla VIM and bla NDM genes in *Pseudomonas aeruginosa* which were carbapenem-resistant and MBL-producing was done using conventional polymerase chain reaction (PCR).

## **RESULTS**

Among the 200 isolates, non-fermenters were 18.5% and other isolates were 21.5%. The remaining 60% were culture-negative samples. Maximum isolates were in the age groups of 39-48 years (35.1%) and 19-28 years (16.2%).

*Pseudomonas aeruginosa* (49%) was the predominant isolate among non-fermenters (Figure 1).

Sample distribution of isolated non-fermenters were predominantly from endotracheal aspirates (46%), followed by sputum (27%), Tracheal aspirates (5%) and Bronchoalveolar lavage (5%) (Figure 2).

ICU stay 59.4% was most common risk factor followed by mechanical ventilation 43.2%, Diabetes (27.0%), Prolonged antibiotic therapy & hypertension (21.6%), COPD (16.21%), Tracheostomy (10.8) and renal disease (5.4%) (Table 1).

Polymyxin B showed 100% sensitivity, while meropenem also showed 100% sensitivity against *P. fluorescence* and *stutzeri* and 61.1% sensitivity to *P. aeruginosa*. Piperacillin tazobactam showed 66.6% sensitivity to *P. aeruginosa* and

*A. baumannii*. Gentamicin (33.3%) had the lowest susceptibility among all isolates (Table 2).

Out of 21 *Pseudomonas* spp isolated 7(33.3%) were MBL producers. As per one proportion Z test, P value was less than 0.0001 (Table 3).

Presence of resistant genes VIM and NDM (Metallo beta-lactamase genes) for resistant isolated was tested by PCR. 3 were positive for VIM (42.8%) and 2 were positive for NDM (28.5%) production among 7 tested resistant isolates (Table 4).

Among different methods of MBL detection, Combined disc test was 100% sensitive and modified Carbapenemases inhibitor method was 71.5% sensitive and RAPID Carba NP was 85.7% sensitive and PCR was positive in 71.5% isolates (Table 5).

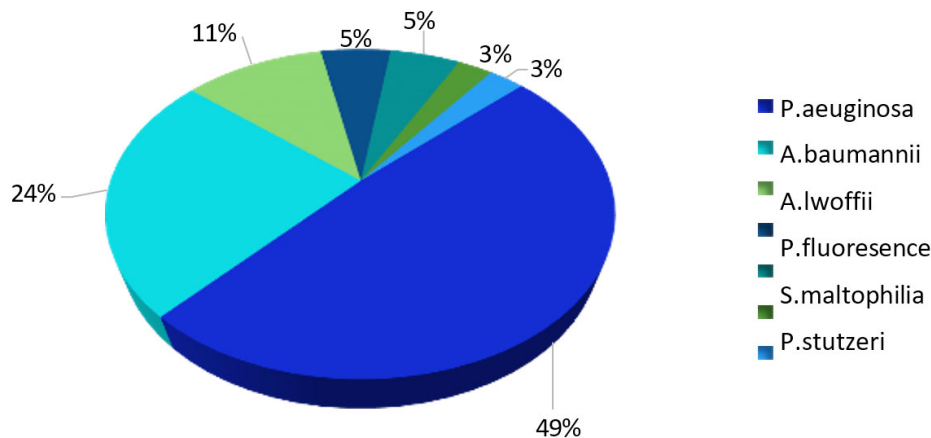
**DISCUSSION**

Non-fermenting respiratory pathogens have exhibited a dramatic increase in antimicrobial resistance due to prophylactic antibacterial therapy. Saad *et al.*<sup>19</sup> also identified prolonged ICU stay and mechanical ventilation as significant risk factors.

Predominant isolates among the non-fermenters were *Pseudomonas aeruginosa* (49%) followed by *Acinetobacter baumannii* (24.3%), *Acinetobacter lwoffii* (10.8%), *Pseudomonas fluorescence* (5.4%), *Stenotrophomonas maltophilia* (5.4%), *Pseudomonas stutzeri* (2.7%) and *Cryseobacterium indologenes* (2.7%).

**Table 1.** Associated risk factors with isolated non-fermenters (n=37)

Risk Factors	Isolates	Percentage
ICU stay	22	59.4%
Mechanical ventilation	16	43.2%
Diabetes	10	27.0%
Prolonged antibiotic therapy	8	21.6%
Hypertension	8	21.6%
COPD	6	16.21%
Tracheostomy	4	10.8%
Renal disease	2	5.40%



**Figure 1.** Various species of non-fermenters (N=37)

**Table 2.** Antimicrobial susceptibility pattern of non-fermenters

Antibiotics	<i>P.aeruginosa</i> (n=18)		<i>P.fluorescence</i> (n=2)		<i>P.stutzeri</i> (n=1)		<i>A.baumannii</i> (n=9)		<i>A.lwoffii</i> (n=4)		<i>S.maltophilia</i> (n=2)		<i>C.indologenes</i> (n=1)	
	S	%	S	%	S	%	S	%	S	%	S	%	S	%
Gentamicin	6	33.3	1	50	1	100	3	33.3	1	25	-	-	1	100
Amikacin	8	44.4	2	100	1	100	5	55.5	2	50	-	-	0	0
Ciprofloxacin	8	44.4	1	50	0	0	3	33.3	1	25	-	-	1	100
Cotrimoxazole	-	-	-	-	-	-	4	44.4	3	75	2	100	1	100
Ceftazidime	8	44.4	1	50	1	100	4	44.4	1	25	-	-	1	100
Levofloxacin	12	66.6	2	100	1	100	6	66.6	3	75	2	100	-	-
Piperacillin tazobactam	12	66.6	2	100	1	100	6	66.6	4	100	-	-	1	100
Meropenem	11	61.1	2	100	1	100	5	55.5	3	75	-	-	0	0
Tetracycline	-	-	-	-	-	-	5	55.5	2	50	-	-	-	-
Polymyxin B	18	100	2	100	1	100	9	100	4	100	2	100	-	-

**Table 3.** MBL producers among *Pseudomonas* spp. (N=21)

Organisms	MBL Producers	One Proportion Z Test
<i>P. aeruginosa</i>	7(33.3%)	5.950
<i>P. fluorescence</i>	-	-
<i>P. stutzeri</i>	-	-
Total	7(33.3%)	

**Table 4.** Resistance gene distribution in *pseudomonas aeruginosa* (n=7)

Gene Tested	Positive	Percentage
<i>bla</i> <sub>VIM</sub>	3	42.8%
<i>bla</i> <sub>NDM</sub>	2	28.5%
Total	5	71.1%

*Pseudomonas aeruginosa* (56.7%), followed by *Acinetobacter* species (39.3%) were the predominant isolates (Figure 1) reported in the study by Chawla *et al.*, which correlates with the current study.<sup>20</sup>

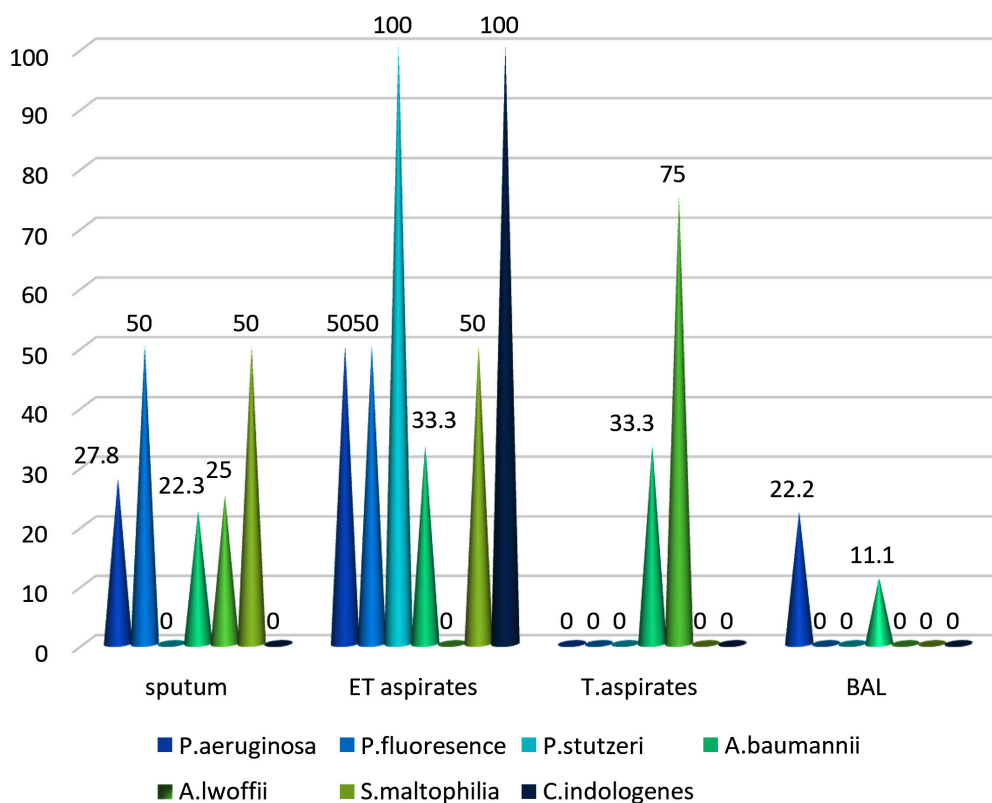
The present study showed that the sample distribution of isolated non-fermenters was predominantly from endotracheal aspirates (46%), which correlates with the study done by Vasundhra Devi P *et al.*, wherein non-fermenters were predominantly isolated from endotracheal aspirates (55%). Higher resistance was observed in ICU patients, mainly due to device-associated infections in the hospital environment. These pathogens are often highly resistant to hospital disinfectants and can easily spread from patient to patient.<sup>1</sup>

*Pseudomonas stutzeri* exhibited 100% sensitivity to gentamicin, amikacin, ciprofloxacin, ceftazidime, piperacillin tazobactam, meropenem, and polymyxin B. *Pseudomonas fluorescence* exhibited 100% sensitivity to amikacin, piperacillin tazobactam, and meropenem, whereas gentamicin, ciprofloxacin, and ceftazidime exhibited 50% sensitivity pattern. This is indifferent to the study done by Malini *et al.*<sup>21</sup> where it was 95% sensitive to imipenem, only 9% sensitive to piperacillin, 47% sensitive to amikacin, and 14% to ceftazidime.

*Acinetobacter lwoffii* showed various sensitivities, including 100% sensitivity to piperacillin tazobactam and polymyxin B, 75%

**Table 5.** Comparison of different methods for MBL detection

Organism	No. of isolates	CDT		mCIM		RAPID Carba NP		PCR	
		+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
<i>P. aeruginosa</i>	7	7	-	5	2	6	1	5	2
Percentage		100%	-	71.5%	28.5%	85.7%	14.3%	71.5%	28.5%



**Figure 2.** Sample wise distribution of non-fermenters (n=37)

to meropenem and cotrimoxazole, and 50% to amikacin and tetracycline. Its sensitivity was lower at 25% for gentamicin, ciprofloxacin, and ceftazidime, consistent with the findings by Malini *et al.*<sup>21</sup> In their study, amikacin, cotrimoxazole, and ciprofloxacin had 60% sensitivity, imipenem exhibited 100% sensitivity. *Stenotrophomonas maltophilia* exhibited 100% sensitivity to cotrimoxazole, levofloxacin, and minocycline, consistent with the findings of Malini *et al.*

Knowledge about the prevalence of ESBL is essential in initiating appropriate therapy. In this study, among the ESBL-producing non-fermenters (n=37), *Pseudomonas aeruginosa* is the highest ESBL producer (44.4%) followed by *baumannii*

(33.3%), *Acinetobacter lwoffii* (50%), and *Pseudomonas fluorescens* (50%). There is no ESBL production by *Stenotrophomonas maltophilia*, *Pseudomonas stutzeri*, and *Cryseobacterium indologenes*. Amirth Koirala *et al.*,<sup>22</sup> also reported that out of 158 (52%) confirmed ESBL producers, 46% were *Pseudomonas aeruginosa* and 63.3% were *Acinetobacter* spp.

In this study, the detection of meropenem-resistant *Pseudomonas* spp (n=21) showed sensitivity to 14 (66.7%) isolates and resistance to 7 (33.3%) isolates, which is analogous to the analysis done by Kali *et al.*,<sup>23</sup> wherein imipenem-resistant isolates were 11 (22.4%) according to their study.

Choudry *et al.*<sup>24</sup> reported multi-drug resistant and MBL-producing *Pseudomonas aeruginosa* to be 44.4% which was higher compared to the present study.

Additionally, carbapenem resistance in *Pseudomonas aeruginosa* among respiratory isolates of ICU patients in our hospital was determined. We phenotypically characterized the resistance mechanisms and also evaluated the in vitro activity of colistin against the isolates. A susceptibility of 100% to colistin was observed among MBL-producing *Pseudomonas aeruginosa* (n=7). This did not correlate with the study done by Mohanty *et al.*<sup>25</sup> in India, who reported an 8% colistin resistance in *Pseudomonas aeruginosa*.

Colistin resistance variation may be due to different antibiotic policies among different hospitals and geographic variations. One isolate of MBL-producing *Pseudomonas aeruginosa* showed MIC of 2, which indicates that colistin should be mandatorily tested for MIC before administration to patients which will prevent the emergence of colistin resistance in a community. This is of utmost importance because the treatment of carbapenem-resistant infections relies on colistin, which is the only available effective antibiotic.

Molecular detection of MBL-producing *Pseudomonas aeruginosa* revealed that among the 7 isolates, 3 tested positive for bla VIM, and 2 tested positive for bla NDM. This finding correlates with the study conducted by Mohanam & Menon,<sup>26</sup> where, out of 20 isolates, 7 carried the bla VIM gene, and 6 carried the bla NDM gene. The primary mechanism of resistance among *Pseudomonas aeruginosa*<sup>27</sup> is due to the loss of OprD porin expression, without the expression of a carbapenemase.

Metallo  $\beta$ -lactamase-producing non-fermenters were phenotypically detected using a combined disc test (CDT), modified carbapenem inactivation method (mCIM), and Rapid Carba NP test. Among different methods, the combined disc test was 100% sensitive, modified Carbapenem inactivation method (mCIM) was 71.5% sensitive, RAPID Carba NP was 85.7% sensitive, and PCR was positive in 71.5% of isolates. These findings are consistent with Behra *et al.*,<sup>28</sup> where CDT showed 100% sensitivity. However, in a study conducted by Huang *et al.*<sup>29</sup> Rapid carba NP showed 100%

sensitivity, which contrasts with the results of this study.

*Pseudomonas* species showed maximum sensitivity to polymyxin B and colistin. The use of these antibiotics must be strictly restricted to critically ill ICU patients with severe infections, to avoid rapid emergence of resistance.

Rapid Carba NP test proves to be an effective screening test in addition to the phenotypic test with 85% sensitivity and a turnaround time of 30 minutes to two hours. Although the gold standard for carbapenemase detection is the genotypic method, the costs associated with these tests in a resource-poor laboratory limit its use. Rapid Carba NP, mCIM, and combined disc test were the most satisfactory simple, easy, rapid, and reliable methods among all the phenotypic methods for detecting carbapenemase-producing non-fermenting GNB.

Carbapenem resistance is considered an emerging drug-resistant mechanism in the NFGNB although not highly reported. Rapid intra-institutional spread of such resistant strains is common and hence must be notified to infection control teams. Antibiotic therapy, whether empirical or documented, should be based on a combination of antibiotics, taking into consideration the local epidemiology of sensitivity patterns to choose an appropriate combination.

## CONCLUSION

The study revealed that infections spread by non-fermenters represent a considerable health problem in ICUs in tertiary care hospitals. Their capacity to survive in a hospital environment underscores the necessity for accurate species-level identification and the detection of resistant strains to control their spread.

The most common organisms isolated were *Pseudomonas* and *Acinetobacter*. The infections caused by multi-drug-resistant  $\beta$ -lactamases producing *Pseudomonas* are capable of causing significant morbidity and mortality.

The present study showed increased isolation of MDR non-fermenters from ICUs causing ventilator-associated pneumonia. VAP caused by these MDR pathogens can be prevented by using invasive devices for a short-term, judicious

use of antibiotics, and by following strict infection control practices.

#### ACKNOWLEDGEMENT

None.

#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

#### FUNDING

None.

#### Data Availability

All datasets generated or analyzed during this study are included in the manuscript.

#### ETHICS STATEMENT

This study was approved by the Institutional Ethical Committee, Madras Medical College, Chennai, India, with reference number ECR/270/Inst/ TN/2013).

#### INFORM CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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