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Original research article

Phenotypic identification of non-fermenting gram-negative bacilli and their antimicrobial susceptibility pattern

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Abstract

Background: Non-Fermenting Gram-Negative Bacilli (NFGNB) are defined as strict aerobic and non-spore forming group of bacteria that do not ferment carbohydrates but generate energy required for their metabolic activities by oxidative pathway. NFGNB are known saprophytes, resilient in nature which allows them to survive even in the harshest hospital environment making them an apt etiological agent for nosocomial opportunistic infections. Urinary tract infections, ventilator-associated pneumonia, septicemia, and surgical site infection are some of the important hospital acquired infection associated with these agents.

Aims & Objectives:

1. To isolate and speciate the non-fermenting Gram negative bacilli from various clinical specimens.
2. To find out the antimicrobial susceptibility pattern of isolated, non-fermenting Gram negative bacilli.

Material and Methods: The present cross sectional study was conducted in the central laboratory of Sri Ramachandra medical college of higher education and research a tertiary care hospital. This study was undertaken after obtaining institutional ethical committee clearance. (REF: CSP/19/May/77/154) and lasted for a period of 3 months (October 2019 to December 2019). During our study period a total of 15838 clinical specimens were received in our laboratory for processing from OPD as well as hospitalized patients from various wards. The samples were processed according to standard procedures and were first subjected to direct Gram stain and then all specimens were inoculated onto routine culture medium (Blood agar, McConkey agar) except Urine specimens which was inoculated onto Cystine Lactose Electrolyte Deficient agar (CLED) and all plates were incubated at 37 ^{o}C. for 18-24 hours.

Results and Observations: Out of 15838 clinical specimens that were collected in the time period of 3months (October 2019-December 2019), 5148 different types of organisms were isolated and in that 846 NFGNB. Which is 5% out of total number. Out of 846 isolates, 529 (62.52%) were isolated from Males and 317 (37.47%) were isolated from females.

Conclusion: From this study we can conclude that NFGNB mainly causes wound infections followed by respiratory infections. Emergence of resistance to multiple anti-microbial agents is a problem and this study showed *Acinetobacter baumannii* as the most common multi drug resistant agent in comparison to others. MDR NFGNB was detected most commonly form urinary tract infection. Identification of NFGNB and monitoring their susceptibility patterns are important especially in those isolated from urine samples as highlighted by our study.

Keywords: NFGNB, *Acinetobacter baumannii*, MDR NFGNB, Antimicrobial susceptibility pattern, non-fermenting gram-negative bacilli (NFGNB), acinetobacter lwoffii, burkholderia pseudomallei, pseudomonas aeruginosa

Introduction

Non-Fermenting Gram-Negative Bacilli (NFGNB) are defined as strict aerobic and non-spore forming group of bacteria that do not ferment carbohydrates but generate energy required for their metabolic activities by oxidative pathway Adane Bitew (2019) NFGNB are known saprophytes, resilient in nature which allows them to survive even in the harshest hospital environment making them an apt etiological agent for nosocomial opportunistic infections. Urinary tract infections, ventilator-associated pneumonia, septicemia, and surgical site infection are some of the important hospital acquired infection associated with these agents Atit Dineshchandra Shah *et al.*, (2021). Due to the difficulty in Identification of these agents, studies undertaken have shown a wide range in their rate of isolation. Indian studies in the last

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five years has shown an isolation rate of 3% to 29%. Atit Dineshchandra Shah *et al.*, (2021), Prudhivi Sumana *et al.*, (2017), Mandira Sarkar *et al.*, (2018), Anshu Shastri *et al.*, (2019) Rajeev Kumar (2020), Navin Kumar Chaudhary *et al.*, (2021), Kirtilaxmi K. Benachinmardi (2021) Among the NFGNB, *Pseudomonas* and *Acinetobacter* are established pathogens, with high isolation rates. Infections by other species were relatively uncommon however last few years have shown a rise in these agents, especially causing pathogenic infections in immune compromised patients Kiran Chawla *et al.*, Stenotrophomonas maltophilia and *Burkholderia cepacia* complex have risen to become important pathogens, others such as *Sphingomonas paucimobilis*, *Ochrobactrum anthropic*, Moraxella, Alcaligenes, Flavobacterium and *Achromobacter xylosoxidans* have also been associated with infections.

Over the years, the interest in NFGNB stems more from the fact that they are highly intrinsically resistant to a lot of antimicrobial agents and have also developed acquired resistance to many drugs due to the indiscriminate and injudicious use of broad spectrum antibiotics Anshu Shastri *et al.*, (2019). Resistance to antimicrobial agent developing in NFGNB can be attributed to mutation in genes encoding porins, efflux pump mechanisms, due to chromosomal beta lactamases or due to alteration in penicillin binding proteins. Amandeep Kaur *et al.*, (2018).

In view of the wide range of rate of isolation of NFGNBs, its development as multidrug resistant organisms, its pathogenic clinical significance, and the advent rise in other uncommon non fermenters as pathogens, it warrants close monitoring of these agents frequently. The present study was undertaken to isolate, identify, characterize non fermenting Gram negative bacilli from various clinical samples upto genus and species level along with study of their antimicrobial susceptibility/resistance pattern.

Aims & Objectives

1. To isolate and speciate the non- fermenting Gram negative bacilli from various clinical specimens.
2. To find out the antimicrobial susceptibility pattern of isolated, non-fermenting Gram-negative bacilli.

Material and Methods

The present cross sectional study was conducted in the central laboratory of Sri Ramachandra medical college of higher education and research a tertiary care hospital. This study was undertaken after obtaining institutional ethical committee clearance. (REF: CSP/19/May/77/154) and lasted for a period of 3 months (October 2019 to December 2019). During our study period a total of 15838 clinical specimens were received in our laboratory for processing from OPD as well as hospitalized patients from various wards.

The samples were processed according to standard procedures and were first subjected to direct Gram stain and then all specimens were inoculated onto routine culture medium (Blood agar, McConkey agar) except Urine specimens which was inoculated onto Cystine Lactose Electrolyte Deficient agar (CLED) and all plates were incubated at 37 ^{o}C. for 18-24 hours.

To rule out chances of contamination, specimens from non-sterile sites like urine and respiratory samples (bronchoalveolar lavage or endotracheal aspirate) were subjected to quantitative cultures and only those showing significant colony forming units were taken into consideration.

All non-lactose fermenting colonies on MacConkey agar were subjected to preliminary biochemical tests (Oxidase test, Indole test, Triple sugar iron agar test, Urease test, Citrate utilization test and mannitol motility test) and those colonies which failed to acidify the TSI agar and mannitol were considered as non-fermenters and subjected for further speciation and identification by the following tests. Motility Test, Pigment Production, Oxidative fermentation (OF) of (Hugh-Leifson)-, Arginine hydrolase, growth at 42 ^{o}C, 1% sugars, Conformation of the organism was conducted by MALDI-TOF.

Antimicrobial susceptibility testing was performed by using Kirby Bauer's disc diffusion method as per CLSI guidelines 2019. Escherichia coli ATCC 25922 & Pseudomonas aeruginosa ATCC 27853 were used as control organisms. Organism were labelled as Multi drug resistance (MDR) if it displayed resistance to at least one antibiotic in three or more group of Antimicrobial agents.

Results

Out of 15838 clinical specimens that were collected in the time period of 3 months (October 2019-December 2019), 5148 different types of organisms were isolated and in that 846 NFGNB. Which is 5% out of total number Out of 846 isolates, 529 (62.52%) were isolated from Males and 317 (37.47%) were isolated from females.

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No. of NFGNB from total clinical specimens

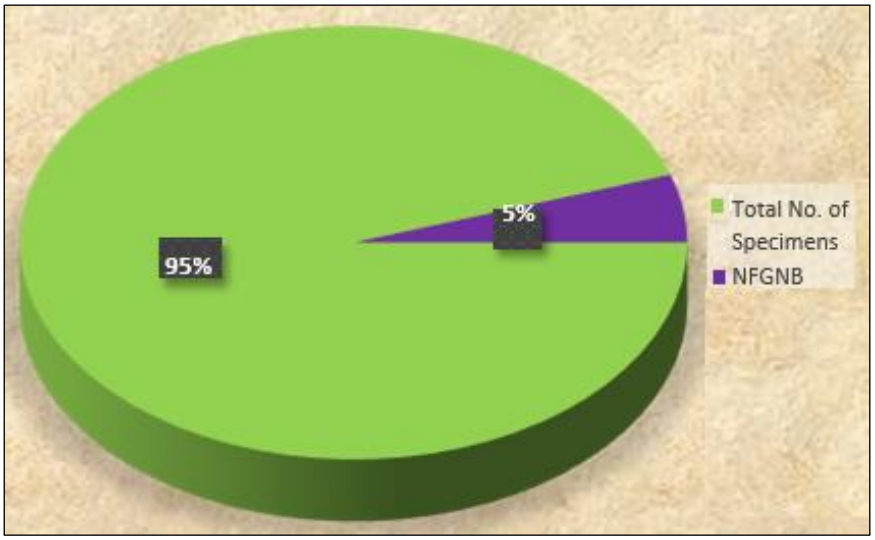

Fig 1: Total number of NFGNB from clinical specimens

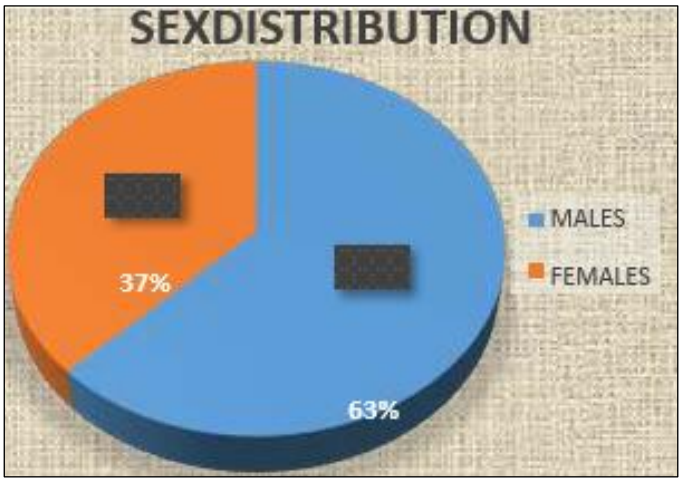

Fig 2: Sex Distribution

The age of the study population ranged from 2 years to 68 years. Age group 41-50 ranged higher with 28. 48% and age group 2-10 was low with 4%.

Table 1: Age Distribution

Age group	No. of Persons (n=846)
2-10	34(4)
11-20	98(11.58)
21-30	79(9.3)
31-40	113(11.35)
41-50	241(28.48)
51-60	196(23.16)
61-68	85(10)

The various clinical specimens from which NFGNB were isolated are Blood (57), Respiratory specimens (pleural fluids, bronchial wash, BAL, non-BAL and sputum = (146), Exudates (pus and tissue = 510), urine (133). (Figure-3 and Table-2)

Table 2: Total number of NFGNB in different clinical sections (n=846)

Specimens	Total No. of Specimens	Total No. of Isolates	Total No. of NFGNB (%)
Pus	4868	2422	510(10.47)
Blood	932	164	57(6.11)
Respiratory Specimens	1401	432	146(10.42)
Urine	8637	2130	133(1.53)
Total	15838	5148	846(5.34)

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Table 3: Distribution of clinical specimens and NFGNB

Organism	Blood	Body Fluids	PUS	Sputum	Tissue	Ear Swab	Urine	Total
Acinetobacter baumannii	16	0	94	0	9	31	23	173
Acinetobacter lwoffii	16	0	21	13	0	0	0	50
Burkholderia pseudomallei	0	2	0	0	0	0	0	2
Pseudomonas aeruginosa	25	101	187	30	49	119	110	621

Out of 846 NFGNB isolates, *Pseudomonas aeruginosa* was the predominant isolate 621 (73.40%) Followed by *Acinetobacter baumannii* 173(20.44%) and *Acinetobacter lwoffii* 50 (5.91%). Also 2 *Burkholderia pseudomallei* was also isolated from bronchial wash. (Figure-5)

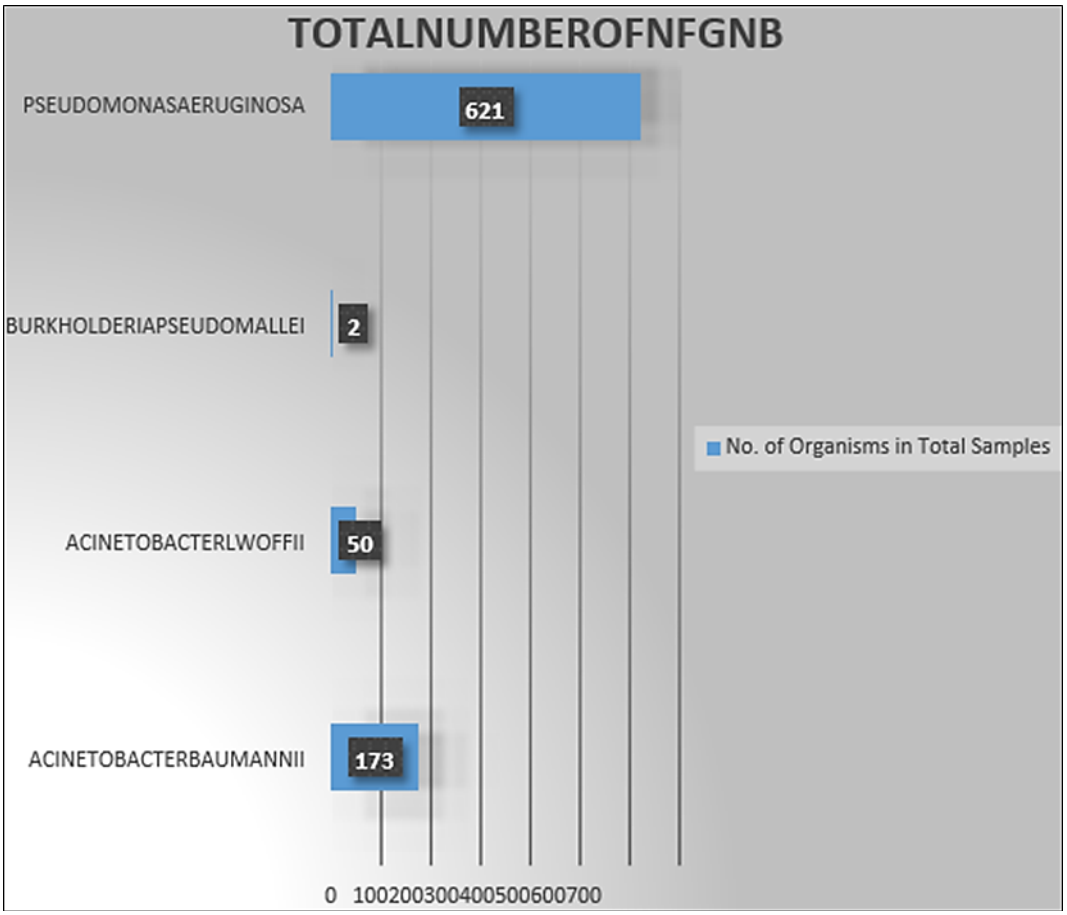

Fig 3: Isolated NFGNB

The sensitivity pattern of different isolated NFGNB is shown below.
Pseudomonas aeruginosa isolates were 100% sensitive to polymyxinB, 76. 32% and 78. 09% susceptibility towards meropenem and imipenem respectively. It had 60% susceptibility towards cephalosporins and better susceptibility (75%) towards beta lactam with β-lactamase inhibitors. Both Amikacin and ciprofloxacin showed 61% susceptibility.

Table 4: Sample Wise Distribution of Common NFGNB

Drug	Susceptible (%) n=621
Amikacin	382(61.51)
Ciprofloxacin	382(61.51)
Cefoperazone/Sulbactam	449(72.3)
Ceftazidime	393(63.28)
Piperacillin/Tazobactam	467(75.2)
Cefepime	422(67.95)
Levofloxacin	384(61.83)
Imipenem	485(78.09)
Meropenem	474(76.32)
Polymyxin-B	621(100)

Acinetobacter baumannii showed higher level of resistance to most of the antibiotics in comparison to pseudomonas aeruginosa. The lowest susceptibility rate was for cephalosporins. Cefotaxime, ceftazidime

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and cefepime with only a susceptibility rate of 13.29%, 28.32% and 25.43% respectively. Likewise, it showed a susceptible rate of 45.66% and 37.57% for beta lactam with Beta lactam inhibitors. However, no resistance was recorded for polymyxin B.

Table 5: Drug Susceptibility of *Pseudomonas Aeruginosa* (figure-6)

Drug	Susceptible(%)n=173
Cefotaxime	23(13.29)
Amikacin	54(31.21)
Ciprofloxacin	57(32.94)
Cefoperazone/Sulbactam	79(45.66)
Ceftazidime	49(28.32)
Piperacillin/Tazobactam	65(37.57)
Cefepime	44(25.43)
Levofloxacin	80(46.24)
Imipenem	75(43.35)
Meropenem	73(42.19)
Polymyxin-B	173(100)

Antibiogram obtained for *Acinetobacter lwoffii* showed 100% sensitive to polymyxin B, 60% and 50% susceptibility towards meropenem and imipenem respectively. The lowest susceptibility rate was to cephalosporins which was 26-30%. It also showed 28% susceptibility to Amikacin and 60% to cefoperazone/sulbactam.

Table 6: Drug Susceptibility of *Acinetobacter baumannii* (figure-8)

Drug	Susceptible (%) n=50
Cefotaxime	15(30)
Amikacin	14(28)
Ciprofloxacin	27(54)
Cefoperazone/Sulbactam	30(60)
Ceftazidime	14(28)
Piperacillin/Tazobactam	22(44)
Cefepime	13(26)
Levofloxacin	48(96)
Imipenem	25(50)
Meropenem	32(64)
Polymyxin-B	50(100)

Burkholderia pseudomallei showed 100% susceptibility towards co-trimoxazole and Imipenem at the same time it was 100% resistance to Amikacin and Ceftazidime.

Table 7: Drug Susceptibility of *Acinetobacter lwoffii* (figure-9)

Drug	Susceptible (%) n=2
Amikacin	0
Ceftazidime	0
Co-trimoxazole	2(100)
Imipenem	2(100)

Table 8: Drug Susceptibility of *Burkholderia pseudomallei* (figure-7)

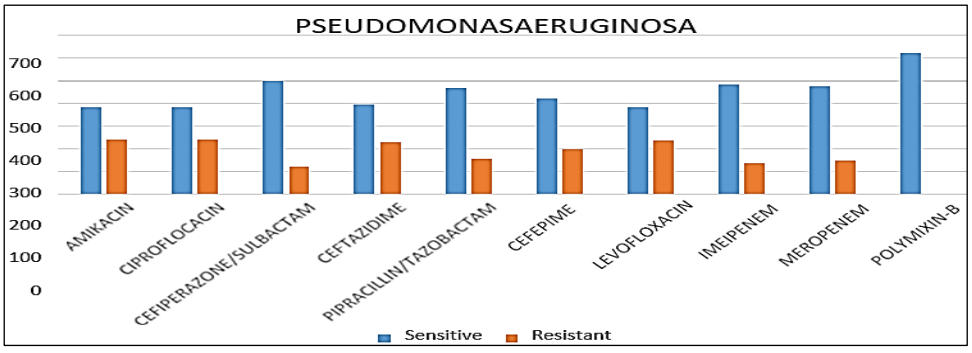

Fig 4: Sensitivity and Resistance pattern of *Pseudomonas aeruginosa*

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Fig 5: Sensitivity and Resistance pattern of *Burkholderia pseudomallei*

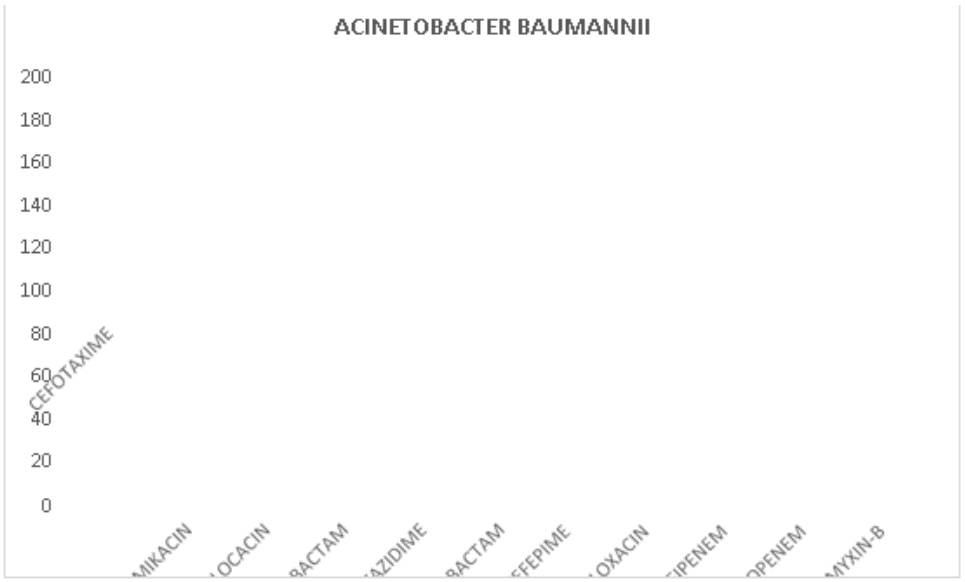

Fig 6: Sensitivity and Resistance pattern of *Acinetobacter baumannii*

Fig 7: Sensitivity and Resistance pattern of *Acinetobacter lwoffii*

The susceptibility pattern of all the Non-Fermenters that were isolated in our study is compared with each other. That is shown in the table-9.

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Table 8: Susceptibility Pattern of NFGNB

Antimicrobials	*A. baumannii* (%)	*A. lwoffii* (%)	*P. aeruginosa* (%)	*B. pseudomallei* (%)
Cefotaxime	23(13.29)	15(30)	-	-
Amikacin	54(31.21)	14(28)	382(61.51)	0
Ciprofloxacin	57(32.94)	27(54)	382(61.51)	-
Cefoperazone/sulbactam	79(45.66)	30(60)	449(72.36)	-
Ceftazidime	49(28.32)	14(28)	393(63.28)	0
Piperacillin/tazobactam	65(37.57)	22(44)	467(75.20)	-
Cefepime	44(25.43)	13(26)	422(67.95)	-
Levofloxacin	80(46.24)	48(96)	384(61.83)	-
Imipenem	75(43.35)	25(50)	485(78.09)	2(100)
Meropenem	73(42.19)	32(64)	474(76.32)	-
Polymyxin-b	173(100)	50(100)	621(100)	-
co-trimoxazole	-	-	-	2(100)

Multi drug resistance (MDR) is when an organism is resistance to more than three group of Antimicrobial agents. Among 621 *Pseudomonas aeruginosa* 187 organisms (30%) shows MDR. MDR in 173 *Acinetobacter baumannii* isolates is 89(51%). 20(40%) MDR in *Acinetobacter lwoffii*.

Table 9: Distribution of MDR organisms

Classification of Drugs	*Pseudomonas aeruginosa* (n=621)	*Acinetobacter baumannii* (n=173)	*Acinetobacter lwoffii* (n=50)	*Burkholderia pseudomallei* (n=2)
Cephalosporins	199	124	35	2
Aminoglycosides	238	119	36	2
Quinolones	236	93	2	0
B-Lactamase & B-Lactamase Inhibitors	154	94	20	0
Carbapenems	136	98	18	0
Polymyxin-B	0	0	0	0
MDR	187(30%)	89(51%)	20(40%)	0

Distribution of MDR organisms of different specimens are given in the table (Table-10). Urine shows the highest rate of MDR organism while Pus has the second highest rate of MDR. MDR *Pseudomonas aeruginosa* is found to be high in Urine with (97) 51.87% and MDR *Acinetobacter baumannii* in urine is 34(38.20%) while MDR *Acinetobacter lwoffii* in urine is 20(60%).

Table 10: MDR organism distribution in different specimen

Organism	Blood	PUS	Respiratory specimens	Urine	Total
Acinetobacter baumannii	11	26	18	34	89
Acinetobacter lwoffii	1	6	1	12	20
Burkholderia pseudomallei	0	0	0	0	0
Pseudomonas aeruginosa	13	56	21	97	187

Discussion

Non-fermenting Gram Negative bacilli (NFGNB) occur as saprophytes in the environment and some are found as commensals in humans due to which most NFGNB when isolated were regarded in the past as just contaminants. As we gained more knowledge about their ability to survive in harsh hospital environments, their increased association with immune compromised patients and their rise as multi drug resistant organisms they have emerged now as important nosocomial pathogens. In the past decade, their global isolation rates and the significance we attribute to their pathogenic potential in infections have increased dramatically. Various studies from India (Neeraj Goel *et al*., 2011) shows an isolation rate varying as high as 22% to as low as 3.5% (Kirtilaxmi K *et al*., 2019) [4]. In south India the isolation rates have ranged from 4.1 and 3.5% Malini *et al*., (2007) [22] and KirtilaxmiK *et al*. (2019) from Karnataka respectively, 4.1% from Debosmitapaul *et al*., from Tripura (2020) [29] and 4.24% Prudhivesumana *et al*., (2017) [30] from Andhra Pradesh. However, it is said their prevalence rate and antibiotic susceptibility pattern varies with time and place. In our study, out of 15838 clinical samples, 846 yielded Nonfermenting Gram Negative bacilli (NFGNB). That is an isolation rate of 5.3%. Which correlates well with all other studies from in and around our state. Keeping in mind the nature of NFGNB as commensals and contaminants, their clinical significance when isolated were assessed by a combination of relevant laboratory and clinical criteria. A relevant clinical history of the patient, repeated isolation and a correlating Gram stain.

We had only one study (R. Saranya *et al*., (2018)) [38] which compared the age and sex with NFGNB, in our study, out of 846 isolates 529 (62.52%) were isolated from males and 317(37.47%) were from females. Males were more susceptible which was similar to that study and the age group of 41-50 is

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highest ranging 28.48% where 2-10 years is lowest ranging 4%, it is contradictory with their study because they isolated highest number of NFGNB from the age group 31-40.

Out of the 846 NFGNB isolated during the study period, pus sample (510 isolates, 10. 47%) and respiratory sample (146 isolates, 10.2%) yielded the maximum number of NFGNB. his was in controversy with other studies (Debosmitapaul *et al*., 2020) [29] who had reported very less isolation rates from respiratory samples. Most studies had urine as the second most common specimen of isolation, especially Amandeep Kaur *et al*., (2018) [46] who had reported a high isolation rate of 23.2%. Our rate of isolation from urine had been very less (1.5%). Though urine had one of the highest sample rate (8637) and total of 2130 were culture positive but only 133 had significant NFGNB. Frequent isolation of NFGNB from respiratory sample in this study is probably attributed to the fact that this study was conducted during the winter months when we expect more of respiratory infections. Very few NFGNB isolates from urine probably attributed to the fact that most of our samples were from wards or outpatient setup where there are less chances of patients who are immune compromised or are catheterized when compared to those in ICU setup. In this study, NFGNB isolated from blood samples were 6. 1%. Amandeep kaur *et al*., (2018) [46] and Jitendra *et al*., (2017) [18] have reported NFGNB isolates obtained from blood with an isolation rate 4. 9% and 5. 9% respectively thus showing similarity with the results of this study.

In the present study, *Pseudomonas aeruginosa* was the most frequently isolated NFGNB621 (73. 40%) followed by *Acinetobacter baumanii* 173 (20.44%).In our study, Pseudomonas was isolated as the predominant organism from all the samples thereby establishing again the versatile pathogenic nature of this pathogen. Many studies such as Jitendra *et al*., (2017) [18] have indicated similar pattern. Like Pseudomonas, *Acinetobacter baumannii* has also emerged in the last decade as an important nosocomial opportunistic pathogen. Many studies have reported Acinetobacter as the most frequently isolated NFGNB. In our study *Acinetobacter baumannii* was isolated maximum from pus sample followed by urine. Even though we had high NFGNB isolations from respiratory sample yet we had zero isolation rate of *Acinetobacter baumannii* from this specimen. On the other hand, Acinetobacter lwoffii which normally colonizes the oropharynx and skin and is mostly implicated in causing gastroenteritis, pneumonia and septicaemia was isolated from respiratory specimen, mainly sputum sample and had a high isolation rate from blood which was similar to that of *Acinetobacter baumannii*. A study by Prudhivi Sumana *et al*., (2017) [30] had similar finding to ours in which *Acinetobacter baumannii* and *Acinetobacter lwoffii* were one of the predominant species to be isolated from blood. In our study *Burkholderia pseudomallei* was isolated from bronchial wash repeatedly on 2separate occasions from same patient and identified in Micros can (Walk Away 96 Plus). This patient had come with history of pulmonary tuberculosis. It was susceptible to commonly used drugs co-trimoxazole, ceftazidime, tetracycline and chloramphenicol.

Pseudomonas aeruginosa isolate were100% sensitive to polymyxin B,76. 32% and78. 09% susceptibility towards meropenem and imipenem respectively. It had 60% susceptibility towards cephalosporins and better susceptibility (75%) towards beta-lactam/β-lactamase inhibitors **(**Chitra Jayaprakash *et al*., 2016) [17]. Both Amikacin and ciprofloxacin showed 61% susceptibility. In view of the results our study shows 30% of strains of pseudomonas to be multi drug resistance. This is not similar to a study conducted by R. Saranya *et al*., (2018) [38].

Acinetobacter baumannii showed higher level of resistance to most of the antibiotics in comparison to pseudomonas aeruginosa. The lowest susceptibility rate was for cephalosporins. Cefotaxime, ceftazidime and cefepime with only a susceptibility rate of 13.29%, 28.32% and 25.43% respectively. Likewise, it showed a susceptibility rate of 45. 66% and 37. 57% for Beta lactam /Beta lactam inhibitors. It was shown to be resistant to amikacin, quinolones and to carbapenems. Showing only a 43% and 42% susceptibility to imipenem and meropenem respectively. Our study shows 51% of *Acinetobacter baumannii* to be multi drug resistant. However 100% susceptibility was recorded for polymyxin B. Studies such as Deepak Juyal *et al*., (2018) [20] Also showed *Acinetobacter baumannii* to be the more drug resistant among other NFGNB as was in concordance with our study (51%) Antibiogram obtained for *Acinetobacter lwoffii* revealed 40% of the isolates to be Multi drug resistance which was not in concordance to Kirti laxmi K *et al*., 2019 [4] (33%).The lowest susceptibility rate was to cephalosporins (26- 30%). 100% susceptibility to polymyxin B. It showed a high susceptibility to Levofloxacin (96%). 60% and50% susceptibility towards meropenem and imipenem respectively. This is contradictory to a Study conducted by Deepak Juyal *et al*., (2018) [20] since that study shows 80% susceptibility. It also showed only 28% susceptibility to Amikacin and it is not same as Amandeep kaur *et al*., (2018) [46] 80% and 60% to cefoperazone/sulbactam as Prudhivi Sumana *et al*., (2017) [30] 59%.

Multi drug resistant organism was isolated maximum from urine sample. Urine was the specimen where all the isolates showed highest rate of multi drug resistance uniformly. *Pseudomonas aeruginosa* showed maximum resistance in urine (51.87%) followed by *Acinetobacter baumanii* (38.20%). These organisms were resistant to Cephalosporins, Quinolones and Amikacin and *Acinetobacter baumannii* showed high resistance to Carbapenems. Studies such as R Saranya *et al*., (2018) [38] also showed similar findings.

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Summary and Conclusion

- 15, 838 clinical samples were included in this study, in which cultures were positive in 5148 samples. Out of 5148 culture positive samples, 846 yielded Non fermenting Gram Negative bacilli (NFGNB) which is an isolation rate of 5.3%.
- Males were more susceptible (62.5%).
- Age group: 2-68years.
- Most of the isolates of NFGNB were from pus (10. 47%) followed by respiratory samples (10.2%), blood (6.1%) and urine (1.5%).
- Most common NFGNB isolated was *Pseudomonas aeruginosa* (73. 40%) followed by *Acinetobacter baumannii* (20. 44), *Acinetobacter lwoffii (*5.91%*), Burkholderia pseudomallei*.
- Multidrug resistance was seen 30% of *Pseudomonas aeruginosa*, 51% with *Acinetobacter baumannii* and40% of *Acinetobacter lwoffii*.
- Highest rate of multi drug resistant bacteria was isolated from urine sample.

Though many studies have shown increasing rate of NFGNB, when taking into account other studies in south India and comparing it with our study, the prevalence rate shows a marginal rise of NFGNB in last few years. From this study we can conclude that NFGNB mainly causes wound infections followed by respiratory infections. Emergence of resistance to multiple anti-microbial agents is a problem and this study showed *Acinetobacter baumannii* as the most common multi drug resistant agent in comparison to others. MDRNFGNB was detected most commonly form urinary tract infection. Identification of NFGNB and monitoring their susceptibility patterns are important especially in those isolated from urine samples as highlighted by our study.

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