

CHARACTERISATION OF NONFERMENTING GRAM NEGATIVE
BACILLI IN CLINICAL SPECIMENS AND MOLECULAR TYPING OF
DRUG RESISTANCE

Dissertation Submitted to
The Tamil Nadu Dr.M.G.R Medical University

*in partial fulfillment of the regulations
for the award of the degree of*

**M.D MICROBIOLOGY
BRANCH - IV**



**MADRAS MEDICAL COLLEGE
THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY
CHENNAI, INDIA**

MARCH 2010

DECLARATION

I declare that the dissertation entitled **CHARACTERISATION OF NONFERMENTING GRAM NEGATIVE BACILLI IN CLINICAL SPECIMENS AND MOLECULAR TYPING OF DRUG RESISTANCE** submitted by me for the degree of M.D. is the record work carried out by me during the period of May 2008 to June 2009 under the guidance of Director & Professor **Dr.G.SUMATHI M.D., Ph.D.**, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D. Microbiology (Branch IV) examination to be held in March 2010.

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ACKNOWLEDGEMENTS

*I humbly submit this note to the “**Almighty**” who has given the health and ability to successfully complete the compilation and proclamation of this blue print.*

*I wish to express my sincere thanks to our Dean, **Dr.J.Mohanasundaram, M.D.,D.N.B., Ph.D.**, Madras Medical College for permitting me to use the resources of this institution for my study.*

*I feel indebted to **Dr.G.Sumathi M.D.,Ph.D.**, Director and Professor, Institute of Microbiology, for her constant support, invaluable suggestions, erudite guidance in my study and for being a source of inspiration in my endeavours. and thrusting her wholehearted support all along this study.*

*I owe special thanks to **Prof Dr.S.Geethalakshmi. M.D., Ph.D**, Vice Principal and Professor, Institute of Microbiology for her constant encouragements, innovative ideas and timely suggestions.for my study.*

*I express my thanks and gratitude to our former Directors **Dr.A.Lalitha M.D.,D.C.P.**, and **Dr.S.Shantha M.D.,Ph.D.**, and former AdditionalProfessors **Dr.G.Sasirekha M.D.,D.G.O.**, and **Dr.H.Kalavathy Victor M.D.**, for their ideas and support.*

*I owe my thanks to Additional Professors **Dr.G.Jayalakshmi M.D., D.T.C.D**, **Dr.Kamatchi M.D.**, **Dr.S.Thasneem Banu M.D.**, **Dr.Devasena M.D.**, for their valuable guidance for my study.*

*I extend my whole hearted gratitude to our Assistant Professor **Dr.Lata Sriram M.Sc., Ph.D.**, for her invaluable guidance in my study.*

*I also extend my sincere thanks to our Assistant Professors **Dr.J.Euphrasia Lata M.D., Dr.R.Deepa M.D., Dr.Balapriya M.D.,D.A., Dr.T.Sabeetha,M.D., D.G.O., Dr.T.V.Ratnapriya M.D., Dr.G.Venkatesh M.D., Dr. Lakshmi Priya M.D and Dr. Sripriya M.D.**, for their support in my study.*

*I would like to thank our former Assistant Professors **Dr.Sujatha Varadharajan M.D., Dr. K Kaveri M.D., D.C.H, Dr. M. Indumathy M.D.,D.G.O.**, for their valuable assistance in my study.*

*I extend my thanks to **Prof. Dr. Padma Krishnan, M.Sc., Ph.D.**, Institute of Basic Medical Sciences , Taramani, Chennai.*

*I also thank **Mrs. Bacilia**, Statistician, ICMR for her assistance in statistical analysis.*

I hereby express my gratitude to all the technical staff for their help throughout my study.

I would like to thank all my colleagues and all the staffs of Institute Of Microbiology, Madras Medical College, Chennai -3 for their help and support

I would like to thank Institutional Ethical Committee for approving my study.

Finally I am indebted to my family members who have been solid pillars of everlasting support and encouragements and for their heartfelt blessings.

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INTRODUCTION

Nonfermentative Gram negative bacilli (NFGNB) are a group of aerobic, non spore forming organisms that either do not use carbohydrates as a source of energy or degrade them through metabolic pathways other than fermentation.^{57, 106, 107}

These bacteria are ubiquitous in nature particularly in soil and water. Although frequently considered as contaminants, most of the nonfermenting gram negative bacilli have emerged as important nosocomial pathogens causing opportunistic infections in immunocompromised hosts. Humidifiers, ventilator machines, dialysate fluids and catheter devices in the hospital environment have provided opportunities for these organisms to establish infection.^{74, 90}

Non-fermenting Gram negative bacilli cause variety of infections including urinary tract infections, wound infections, septicemia, pneumonia, osteomyelitis, meningitis etc. They are being recovered with increasing frequency from clinical specimens. Chronic infection, longer duration of hospitalization and prolonged antibiotic therapy are the predisposing factors for infection with nonfermenters. This group includes organisms from diverse genera like *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Burkholderia*, *Alcaligenes*, *Weeksella* etc, with *Pseudomonas aeruginosa* being the predominant species recovered from clinical specimens.^{49, 90, 107}

Nonfermenters generally require more effort for their identification as they have metabolic pathways requiring the use of special biochemical tests. In most of the laboratories they are not identified upto species level as it is routinely not possible. For this reason, reports of diseases due to these organisms are rare. The rise in incidence of

these infections necessitates their characterization upto species level.^{38,39}

Members of nonfermenting gram negative bacteria show resistance to a wide range of commonly used antibiotics leading to serious infections like sepsis. Multidrug resistance exhibited by them especially the organisms of great current importance like *Acinetobacter baumannii* and *Ps.aeruginosa* pose a major clinical problem in treating infections caused by them. Therefore early identification and institution of appropriate treatment is necessary to reduce the morbidity and mortality due to these organisms in hospitalized patients.⁴⁷

Nonfermenters often use several different mechanisms of resistance. One among them is production of Extended Spectrum of BetaLactamases (ESBL). ESBL are a group of betalactamases which share the ability to hydrolyse third generation cephalosporins and are inhibited by clavulanic acid. They are plasmid coded.²³

Production of carbapenamases is the other mechanism of resistance to antibiotics by the nonfermenters. These enzymes have high hydrolytic activity against penicillins, cephalosporins, and carbapenams. Resistance to carbapenams in nonfermenters can be intrinsic or acquired. Intrinsic resistance is seen in *S.maltophilia* while acquired Class B metallobeta lactamases (MBL) and class D serine carbapenamases are frequently found in *Ps.aeruginosa* and *Acinetobacter* respectively. These acquired MBL genes (IMP, VIM, SPM, GIM types) are usually clustered with other resistance determinants on mobile DNA elements and their presence is virtually constant marker for multidrug resistance. They have the potential to spread rapidly to other species causing nosocomial outbreaks of carbapenam resistance. Regular monitoring and documentation of carbapenam resistance is therefore crucial in developing strategies to control infections

due to these bacteria.^{40,48,69, 89, 92,102,109}

Within hospitals, the unnecessary use or overuse of antibiotics encourages the selection and proliferation of resistant strains of bacteria. It is not possible to completely eliminate this evolutionary phenomenon, but it can be modified by prudent antibiotic use. This requires the inclusion of an antibiotic policy in the infection control programme.

The present study was therefore undertaken to identify the nonfermenters from various clinical specimens, to analyse the risk factors associated with their infections, to determine the multidrug resistance and to guide initial empiric therapy for infections caused by them.

AIMS AND OBJECTIVES

- ❖ To isolate and speciate the nonfermenters from various clinical specimens.
- ❖ To study the sensitivity patterns of the isolates with common antimicrobials.
- ❖ To detect the incidence of multidrug resistance among nonfermenters.
- ❖ To detect the production of extended spectrum of betalactamases.
- ❖ To detect the acquired resistance to carbapenam antibiotics and production of acquired metallobetalactamases.(MBL)
- ❖ To identify the genes responsible for acquired MBL production
- ❖ To formulate antibiotic therapy for the infections caused by nonfermenters.

REVIEW OF LITERATURE

Nonfermenting Gram Negative Bacilli (NFGNB) are a group of taxonomically diverse organisms growing significantly under aerobic conditions. They all share the common phenotypic feature of failing to acidify the butt of Triple sugar iron agar (TSI) or Kligler iron agar (KIA) agar or of oxidative-fermentative (OF) media.⁷⁴

Aerobic nonfermenters are cosmopolitan in their distribution inhabiting soil, water, plants and animals. Their medical importance derives principally from their being opportunistic pathogens and clinical diseases they cause are nosocomial in origin. Approximately 15% of all gram negative clinical isolates are nonglucose fermenting gram negative rods. Of these, more than 2/3rds are *Pseudomonas aeruginosa*.^{49, 92}

Large group of these nonfermenters have undergone confusing taxonomic changes for many years. New definitions of species and genera using modern genotyping analysis, together with reliable identification methods have resulted in a better knowledge of these bacteria and a significantly increased awareness of their pathogenic role in hospitals and in rare cases of community acquired infections.^{49, 57}

The major genera of nonfermenting Gram negative bacilli have been classified into at least 15 families in addition to a number of clinically important nonfermenters with uncertain taxonomic positions. Medically important nonfermenters can be grouped on the basis of presence / absence of motility and the type of flagella present in strains

that are motile.⁵⁷

MOTILE WITH POLAR FLAGELLA	
<p>Family <i>Pseudomonadaceae</i> (rRNA group I) Genus <i>Pseudomonas</i></p> <p>Family <i>Burkholderiaceae</i> (rRNA group II) Genus <i>Burkholderia</i> Genus <i>Cupriavidus</i> Genus <i>Lautropia</i> Genus <i>Pandoraea</i> Genus <i>Ralstonia</i></p> <p>Family <i>Comamonadaceae</i> (rRNA group III) Genus <i>Comamonas</i> Genus <i>Acidovorax</i> Genus <i>Delftia</i></p> <p>Family <i>Caulobacteraceae</i> (rRNA Group IV) Genus <i>Brevundimonas</i></p> <p>Family <i>Xanthomonadaceae</i> (rRNA Group V) Genus <i>Stenotrophomonas</i></p>	<p>Family <i>Sphingomonadaceae</i> Genus <i>Sphingomonas</i></p> <p>Family <i>Oceanospirillaceae</i> Genus <i>Balneatrix</i></p> <p>Family <i>Alteromonadaceae</i> Genus <i>Alishewanella</i> Genus <i>Shewanella</i></p> <p>Family <i>Oxalobacteraceae</i> Genus <i>Herbaspirillum</i> Genus <i>Massilia</i></p> <p>Family <i>Methylobacteriaceae</i> Genus <i>Methylobacterium</i> Genus <i>Roseomonas</i></p> <p>Organisms Whose Taxonomic Position Is Uncertain CDC Groups Ic, O-1, O-2, O-3, Vb-3</p>

MOTILE WITH PERITRICHOUS FLAGELLA	NONMOTILE, OXIDASE NEGATIVE
<p>Family Alcaligenaceae Genus <i>Achromobacter</i> Genus <i>Alcaligenes</i> Genus <i>Bordetella</i> (<i>B. avium</i>, <i>B. . hinzii</i>, <i>B. bronchiseptica</i>, <i>B. trematumatum</i>) Genus <i>Kerstersia</i> Genus <i>Oligella</i> (<i>O. ureolytica</i>)</p> <p>Family Rhizobiaceae Genus <i>Rhizobium</i></p> <p>Family Brucellaceae Genus <i>Ochrobactrum</i></p> <p>Family Halomonadaceae Genus <i>Halomonas</i></p>	<p>Family Moraxellaceae Genus <i>Acinetobacter</i></p> <p>Family Alcaligenaceae Genus <i>Bordetella</i> (<i>B. pertussis</i>, <i>B. parapertussis</i>, <i>B. trematum</i>)</p> <p>Organisms Whose Taxonomlc Position Is Uncertain CDC group NO-1 CDC group EO-5</p>

NONMOTILE ,OXIDASE POSITIVE	
<p>Family Flavobacteriaceae Genus <i>Flavobacterium</i> Genus <i>Bergeyella</i> Genus <i>Chryseobacterium</i> Genus <i>Empedobacter</i> Genus <i>Myroides</i> Genus <i>Weeksella</i></p> <p>Family Sphingobacteriaceae Genus <i>Sphingobacterium</i> Genus <i>Pedobacter</i></p> <p>Family Moraxellaceae Genus <i>Moraxella</i> Genus <i>Psychrobacter</i></p>	<p>Family Neisseriaceae Genus <i>Neisseria</i></p> <p>Family Alcaligenaceae Genus <i>Oligella</i> (<i>O. urethralis</i>)</p> <p>Family Rhodobacteraceae Genus <i>Paracoccus</i> (EO-2)</p> <p>Organisms Whose Taxonomic Position Is Uncertain CDC groups EO-3, EO-4, EF-4b CDC groups IIc, IIe, IIg, IIh. IIi, <i>Gilardi</i> rod group 1</p>

Among these the most commonly isolated organisms in clinical specimens in descending order of importance are:

1. *Pseudomonas aeruginosa*
2. *Acinetobacter baumannii*
3. *Stenotrophomonas maltophilia*
4. *Burkholderia cepacia*

with species of *Flavobacteriaceae* family and *Alcaligenes* groups recently been recognized as potential pathogens^{49, 90}

RISK FACTORS FOR THE DISEASES CAUSED BY NONFERMENTING GRAM NEGATIVE BACILLI^{4, 92, 90, 75}

1. Immunosuppression – Diabetes mellitus, malignancy, steroids / antibiotic treatment and transplantation.
2. Trauma – gunshot, knife wounds, punctures, surgical wounds and burns.
3. Foreign body implantation – catheters(urinary / blood stream), Prosthetic devices – joints, valves, corneal implants, contact lenses.
4. Infused fluids – dialysate, saline irrigations.
5. Prolonged hospitalization especially in Intensive Care Units.(ICUs)

PSEUDOMONAS AERUGINOSA

Ps.aeruginosa is the most common organism isolated among the nonfermenters from the clinical specimens, more often than all other *Pseudomonas* species especially in teaching hospitals with more than 500 beds.^{96, 82} They are ubiquitous organisms widely distributed in nature. They have emerged as a major hospital pathogens because of their

ability to grow in a variety of environments with minimal nutritional requirements.³⁸ Intensive care units, Immunosuppressants, invasive procedures and antibiotic usage have provided opportunities for emergence, persistence and transmission of *Pseudomonas* between patients, from patients to staff and to inanimate reservoirs.¹⁰⁵ Many carriage sites like respiratory tract, genitourinary tract and skin serve as source of dissemination.⁶⁷

The virulence is multifactorial including loss of host defence mechanisms like immunosuppression, loss of mucosal barrier, cellular factors, toxins elaborated by *Ps aeruginosa* like endotoxins, exotoxin A, enzymes like elastases, alkaline protease and hemolysins are responsible for many of the systemic manifestations of *Pseudomonas* disease.^{67,96} .In addition, the colonies of the organism form biofilms within which they are protected from host defenses and antimicrobial agents and communicate with each other through complex system of cell to cell signaling called Quorum sensing.^{15, 96} .The production of alginate and epithelial cell tropism in cystic fibrosis is associated with poor prognosis and high mortality.⁷⁵

In the National Nosocomial Infection Surveillance (NNIS) survey from the Centres for Disease Control and Prevention (CDC), it is the fourth most common cause of nosocomial infection and leading cause of hospital acquired infections.⁹⁰ It is the most common cause of wound infection caused by gram negative bacteria with an isolation rate of upto 62%.⁹⁶ Urinary tract infections caused by these organisms are mostly hospital acquired and isolations range from 12%-30%.⁹² It causes life threatening bacteremia especially in intensive care settings at a rate of 10%. *Ps aeruginosa* is the leading cause of pneumonia in ICU patients with a mortality of 80 -100%⁴⁹

Other infections caused by *Ps aeruginosa* are osteochondritis, chronic suppurative otitis media, external ear infections, meningitis following trauma and surgery, endochondritis and peritonitis^{74, 110}

IDENTIFICATION⁵⁷

Ps aeruginosa produces large flat colonies with spreading and serrated edges with a metallic sheen. Various diffusible pigments are produced like pyoverdinin and pyocyanin. It is beta-hemolytic on blood agar. It produces nonlactose fermenting colonies on MacConkey agar. They are motile organisms. It is oxidase positive, catalase positive, indole negative, citrate and urease variable. It oxidizes glucose in OF media, reduces nitrates to nitrites, arginine is decarboxylated, acetamide positive, ONPG negative, sensitive to Polymyxin B and grows at 42°C which differentiates it from *Pseudomonas fluorescens* and *Pseudomonas putida*.

ANTIBIOTIC SENSITIVITY

They are sensitive to semisynthetic penicillins like Piperacillin/Ticarcillin, third generation cephalosporins (ceftazidime), carbapenams (imipenem and meropenem), monobactams, aminoglycosides and fluoroquinolones.^{49, 75}

It is intrinsically resistant to ampicillin, amoxicillin and amoxicillin-clavulanic acid due to an inducible chromosomal AmpC beta lactamase.^{47, 85}

Multiple resistance in these organisms is frequent, leading to the development of multidrug and pandrug resistant *Ps.aeruginosa* strains caused by mutations & or production of beta-lactamases ranging from extended spectrum of beta-lactamases to metallo-beta-lactamases.^{31,34,74}

ACINETOBACTER BAUMANII

Acinetobacter are strictly aerobic, gram negative coccobacillary rods, widely distributed in nature and hospital environments^{8 45, 74} They are second most commonly isolated nonfermenters in human specimens next to *Pseudomonas aeruginosa* with a prevalence of 10% of all gram negative isolates.^{74,90} They are generally considered as nonpathogenic but cause serious infections in debilitated patients. The species most frequently isolated is *A.baumanii* It is most often responsible for hospital acquired infections.^{74, 87} They are the most common gram-negative organisms to be isolated from the hands of medical personnel.⁹⁰

A study conducted by CDC has reported *A.baumanii* to be the cause of 1% nosocomial blood stream infections(CDC) A mortality of 17- 46% is associated with nosocomial bacteremia by these organisms.^{28.}

Analysis of data from the NNIS system showed that the proportion of ICU pneumonia episodes range from 4% -7%.⁴⁷

These organisms have high rate of colonization of the trachea. Respiratory tract is the most common site for *A.baumanii* infections in ICU with a mortality rate approaching 70%.^{67.}

Traumatic wounds, burns and postoperative surgical site infections are also common with multidrug resistant strains being observed.⁵

Several reviews have described these organisms in 2-6% of nosocomially acquired urinary tract infections.^{5, 7,8,49}

IDENTIFICATION⁵⁷

Colonies are translucent to opaque, convex and entire with a diameter between 0.5 and 2mm. It produces nonlactose fermenting colonies on MacConkey agar with a pinkish tint. It is oxidase negative, nonmotile, catalase positive, citrate positive and urease negative. It oxidizes glucose and 10% lactose and dextrose in OF media. It does not reduce nitrates to nitrites. It deaminates arginine, acetamide negative, ONPG negative and grows at 44°C.

ANTIBIOTIC SUSCEPTIBILITY^{5,57, 47,87}

They are universally resistant to penicillin, ampicillin and chloramphenicol. They show variable susceptibility to second and third generation cephalosporins. Recently extended spectrum of betalactamases and carbapenamase resistance is reported in nosocomial infections.

STENOTROPHOMONAS MALTOPHILIA

Originally classified as *Pseudomonas maltophilia*, it is an obligate aerobe and an ubiquitous organism^{73,92,104} It is an emerging opportunistic pathogen.⁷³ It is the third most common encountered nonfermenter in clinical laboratory next to *Pseudomonas* and *Acinetobacter*.^{57,92}

It is an important nosocomial pathogen associated with substantial morbidity and mortality especially in immunosuppressed patients.⁹⁶ It is one among the most common causes of wound infections due to trauma. It is frequently isolated from patients with ventilatory support in ICU.⁷⁴ It is an important pathogen in cystic fibrosis patients.¹⁰⁴ It produces proteolytic enzymes, deoxyribonucleases, ribonucleases, hemolysins, hyaluronidase and mucinase etc. which contribute to its severity in immunosuppressed

patients.⁴⁹

The rate of infections caused by *S.maltophilia* is increased in recent years and are being isolated from wound infections, bacteremia, pneumonia, urinary tract infections, meningitis and peritonitis.⁷⁴

IDENTIFICATION^{57,104}

Colonies formed are pale yellow / lavender green with good growth on Blood agar and MacConkey agar. It is oxidase negative, motile, catalase positive, indole negative, citrate variable, urease negative. It oxidizes glucose and maltose, decarboxylates lysine, ONPG positive, with variable nitrate reduction.

ANTIBIOTIC SUSCEPTIBILITY

Therapy for *S.maltophilia* infections is problematic because of the broad antibiotic resistance that typifies this organism.⁹⁰ The most active agents are trimethoprim-sulphamethoxazole, colistin and quinolones. Like other nonfermenters it is intrinsically resistant to many common antibiotics like aminoglycosides, carbapenams and many betalactam agents.^{57,72}

BURKHOLDERIA CEPACIA

It is a motile free living phytopathogen identified as both endemic and epidemic nosocomial pathogen¹⁰⁴. Its detection rates are low, in the range of 1%-16% of clinical samples.^{90, 96} It belongs to rRNA group Ie. It produces virulence factors like proteases, lipases, exopolysaccharides and lipopolysaccharides.^{8, 11}

A few case reports have described serious infections, including severe pneumonia, invasive otitis and sepsis in cystic fibrosis patients⁴⁹ Diabetes mellitus is a potential risk

factor for development of infections by *B.cepacia*.⁵⁷

B.cepacia is also an important pathogen among patients with chronic granulomatous disease.⁸⁰ Like other nonfermenters, it can contaminate disinfectant solutions. The major importance of this organism lies in its role as opportunistic agent of pneumonia in cystic fibrosis patients seeded in sputum samples.⁸¹

The spectrum of infections by these organisms includes wound infections, bacteremia, UTI, pneumonia, meningitis, peritonitis, and endocarditis.⁹⁶

IDENTIFICATION⁵⁷

Colonies are smooth and glistening, forming non-lactose fermenting colonies on MacConkey agar and yellow pigmented colonies on blood agar. It is weakly oxidase positive, catalase positive, motile, oxidizes all sugars, decarboxylates lysine, ONPG negative, acetamide negative and resistant to Polymixin B. Nitrate reduction is variable.

ANTIMICROBIAL SUSCEPTIBILITY

As with other nonfermenters intrinsic antibiotic resistance typifies *B.cepacia* and greatly complicates treatment. Trimethoprim-sulfamethoxazole has historically been the drug of choice. Most active agents are, ceftazidime, meropenam, ciprofloxacin and other quinolones.^{47, 57, 90}

ALCALIGENES FECALIS

It belongs to *Alcaligenaceae* family¹⁰⁴ It exists in soil and water and in the hospital environment. It is isolated from various environmental sources like respirators, hemodialysis systems, intravenous solutions and disinfectants.⁹⁶

They are important causes of hospital acquired infections in patients who have severe underlying diseases. It is found in ulcers of diabetics, urinary tract infections, blood, sputum, CSF and wound burns.⁴⁹

IDENTIFICATION⁵⁷

Colonies are thin, spreading with irregular edges on MacConkey and a greenish discolouration on blood agar. It is catalase positive, oxidase positive, motile, citrate positive, urease negative, reduces nitrite but not nitrate, acetamide positive, ONPG negative, and sensitive to polymixin B.

ANTIMICROBIAL SENSITIVITY⁵⁷

They are resistant to aminoglycosides, chloramphenicol, tetracycline, ciprofloxacin, betalactams and carbapenams.

PSEUDOMONAS FLUROSCENS

P.fluroscens is a psychrophilic organism which favours its presence in blood products.⁴⁹ Outbreaks of bacteremia, respiratory tract infections in cystic fibrosis patients, wound infections, urinary tract infections and rare cases of community acquired pneumonia have been reported.¹⁰⁴ They behave as opportunistic pathogens in immunocompromised patients.⁵⁷

IDENTIFICATION⁵⁷

Colonies are large with spreading edges forming nonlactose fermenting colonies on MacConkey agar and hemolytic colonies on blood agar. It is oxidase positive, catalase positive, motile, oxidizing glucose, deaminating arginine, reducing nitrates to

nitrites, ONPG negative, acetamide negative, sensitive to polymyxin B and do not grow at 42°C

ANTIBIOTIC SUSCEPTIBILITY^{47,57}

It is sensitive to antipseudomonal penicillins like Piperacillin, betalactam agents and carbapenams. It is resistant to penicillins.

WEEKSELLA VIROSA⁵⁷

Flavobacteriaceae comprises indole positive organisms like *Chyseeobacterium*, *Empedobacter*, *Spingobacterium* and *Weeksella*.⁴⁹

Weeksella virosa are associated with urinary tract infections.⁴⁹

IDENTIFICATION⁵⁷

Weeksella virosa form yellow colonies on blood agar. They are oxidase positive and nonmotile, form indole, citrate variable and urease negative, do not oxidizes glucose and maltose. They are nitrate negative. *Weeksella* is sensitive to penicillin and polymyxin B.

ANTIBIOTIC SUSCEPTIBILITY⁵⁷

They are resistant to aminoglycosides, third generation cephalosporins, Imipenam and erythromycin. They are sensitive to ciprofloxacin and betalactamase inhibitors.

MULTIDRUG RESISTANCE IN NONFERMENTING GRAM NEGATIVE BACILLI

Nonfermenting Gram Negative Bacilli pose a particular difficulty for healthcare community because they represent the problem of multidrug resistance to the maximum⁴⁷ They are resistant to three or more drugs and important members of this group are *Ps. aeruginosa*, *A.baumannii.*, *S.maltophilia* and *B.cepacia*²². They use several mechanism of resistance including intrinsic and rapidly acquired resistance. Intrinsic resistance is due to relative impermeability of outer membrane proteins compared to that of other gram negative bacteria (ten fold times lower). Efflux system also contributes to intrinsic resistance Acquired resistance is by mutational changes and acquisition of exogenous genetic material. Lastly resistance may also develop during therapy turning an initially susceptible isolate into a resistant one⁷⁵ *Ps. aeruginosa* exhibits multidrug resistance to 4 antibiotic classes -ceftazidime, imipenam, gentamicin, and a fluroquinolone. The increase in multidrug resistant strains suggests that therapy with compounds like polymyxinB or colistin must be considered.⁴⁷ A report in Germany revealed multidrug resistant profiles in *Acinetobacter* to drugs like cefepime, ciprofloxacin and amikacin⁴⁷ *S.maltophilia* and *B.cepacia* are associated with intrinsic drug resistance.^{40, 92} Multidrug antibiotic resistance negatively affects outcomes of the patients⁴⁷.

EXTENDED SPECTRUM OF BETALACTAMASES^{23, 112,}

ESBL are a group of betalactamases which share the ability to hydrolyse third generation cephalosporins and are inhibited by clavulanic acid. They are plasmid coded. Carbapenams are treatment of choice for serious infections due to ESBL producing organisms. ESBLs in nonfermenters are Ambler class A. These enzymes are SHV type, TEM type, TEM 1 and 2, CTX-M type, OXA- type , PER- type, VEB, BES – types and others. Screening tests for ESBL producers are disk diffusion and dilution susceptibility testing methods. The phenotypic confirmatory tests for ESBL production are:

1. **Cephalosporin / clavulanate combination disks.**²⁰
2. **Broth microdilution tests**²⁰
3. **E tests**^{20, 22}

CARBAPENAMASES AND METALLOBETALACTAMASES^{82,89, 102, 109}

Carbapenamases are betalactamases with versatile hydrolytic capacities. They have the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenams. Bacteria producing these betalactamases may cause serious infections in which the carbapenamases activity renders many betalactams ineffective. They are members of molecular class A, B, and D betalactamases. Class A and D have serine based hydrolytic mechanisms while class B are metallobetalactamases that contain zinc in the active site. Class D carbapenamases consist of OXA type betalactamases frequently detected in *Acinetobacter baumannii*. The metallobetalactamases belong to IMP, VIM, SPM, GIM and SIM families and have been detected primarily in *Pseudomonas aeruginosa*. Nonfermenters especially *P.aeruginosa* and *A.baumannii* have acquired metallobetalactamases through genetic elements (plasmids / transposons) and

can be transmitted to other bacteria. These enzymes confer resistance to all carbapenams (Imipenams, Meropenams, Ertapenams), all betalactams, aminoglycosides and quinolones. The dissemination is thought to be driven by regional consumption of ESBLs. *S.maltophilia* is naturally resistant to imipenam and meropenam because of chromosomally mediated carbapenamase production.⁴²

The families and subgroups of carbapenamases known till now are IMP-1&2, VIM-1&2, SPM-1, GIM-1, and SIM-1.

IMP was first discovered in *Ps.aeruginosa* in Japan.and this has spread to other gram negative bacteria and reports show their detection in *A.baumannii*, *Serratia* and *Klebsiella*. Currently IMP family members number upto 18 in the published literature.

The second dominant group of acquired MBLs is the VIM type enzymes. It was first described in Verona Italy, from *Ps aeruginosa* isolate. This family currently consists of 14 members and seen mostly in *Pseudomonas aeruginosa*. It has dubious distinction of being the most reported metallo-beta-lactamase worldwide. These genes are easily transferred on mobile elements among species. While considered by some to be rare, reports of their occurrence have increased.

DETECTION OF CARBAPENAMASES ⁸⁹

1. Raise in MIC of carbapenams in the range of 8 >128 µgm / ml.
2. Microbiological test with inhibitors:

- a. **Disc approximation test with EDTA** ¹¹³
- b. **Combined disc method: Imipenam with EDTA** ^{62, 63}
- c. **E test strips with Imipenam and Imipenam EDTA combination** ⁸⁸
- d. **Modified Hodge test** ⁶²

Of these tests, studies conducted showed that both combined disc test and E test were more sensitive and equally effective for MBL detection.

MOLECULAR TESTS ^{40, 89, 102, 109}

When the presence of a carbapenamase is suspected, PCR is the fastest way to determine which family of betalactamase is present. Ultimately the identification of the betalactamase gene requires sequencing of the entire coding region.

MATERIALS & METHODS

STUDY PERIOD

This cross sectional study was conducted from May2008-June 2009

PLACE OF STUDY

Institute of Microbiology, Madras Medical College, Chennai-3.

ETHICAL CONSIDERATION

Ethical clearance was obtained from Institute of Ethical committee, Government General Hospital, Madras Medical College, Chennai -3 Informed consent was obtained from the patients before enrolment into the study.

STATISTICAL ANALYSIS

All statistical analyses were carried out using SPSS for Windows. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. *P* values were calculated using the chi-square test. A *P* value of < 0.05 was considered significant.

SAMPLE

A total of 156 nonfermenting bacteria isolated from various clinical specimens like pus, urine, blood, bronchoalveolar lavage, endotracheal aspirations, drain tip and cerebrospinal fluids collected from both outpatients and inpatients of Government General Hospital, Chennai were studied.

SAMPLE PROCESSING

The samples were processed according to standard procedures available. The collected samples were subjected to direct Gram stain and all specimens were inoculated onto nutrient agar, 5% sheep blood agar and MacConkey's agar medium. Urine samples were inoculated onto Cystine Lactose Electrolyte Deficient agar (CLED).

All the catalase positive, oxidase positive and negative, nonlactose fermenting colonies on Mac Conkey agar were provisionally identified by colony morphology and pigment production. They were inoculated in Triple sugar iron (TSI) agar slope. The colonies which failed to acidify the TSI agar were considered as nonfermenters and subjected to the following tests.(annexure) Motility, Indole, Citrate, Urease, Nitrate reduction, growth at 42° c and 44°c, Sensitivity to Polymyxin B and following special biochemical tests and grouped according to P.C.Schreckenberger scheme⁵⁷

SPECIAL BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF NON FERMENTERS

1. HUGH – LEIFSON OXIDATION - FERMENTATION MEDIUM⁵⁷

Two tubes were required for the test, each inoculated with the unknown organism, using a straight needle stabbing the medium three to four times half way to the bottom of the tube. One tube of each pair was covered with a 1cm layer of sterile mineral oil (or) melted paraffin, leaving the other open to the air. Both tubes were incubated at 35⁰C and examined daily for several days.

In case of oxidative metabolism, yellow color appears along the upper one fourth of the medium and in the tube where no oil overlay was done. In case of fermentative organisms yellow color develops in both the tubes.

CONTROL

Glucose fermentation : *Escherichia coli*

Glucose oxidation : *Pseudomonas aeruginosa*

Non saccharolytic: *Alcaligenes species*.

2. DECARBOXYLATION OF LYSINE, ARGININE ORNITHINE ⁵⁷

Decarboxylases are a group of specific enzymes which react with carboxyl portion of aminoacid forming alkaline reacting amines. The reaction is decarboxylation. Each enzyme is specific for Lysine, Arginine and Ornithine.

PROCEDURE

The organism was inoculated in four tubes. One having the basal medium without aminoacid for control. Other three tubes having lysine, arginine and ornithine each. All tubes were overlaid with liquid paraffin. All were incubated at 37⁰C for 24 hours.

The control tube turned yellow indicating that the organism is viable and the test medium turning blue purple indicating positive result.

3. O-NITROPHENYL β - D GALACTOPYRANOSIDE ⁵⁷

A dense suspension of the test organism grown in TSI agar was prepared in saline. About 1 drop of toluene was added to the suspension and 0.2ml of ONPG solution was added to the suspension and incubated at 37⁰C β -galactosidase producing organism show yellow color after 1 hour or 18-24 hours incubation.

4. ACETAMIDE AGAR ¹¹

The slant was inoculated with a portion of isolated colony and incubated at 37°C overnight and was observed for color change from green to blue Tubes with negative result were further incubated for 7 days.

5. GELATIN LIQUEFACTION TEST ⁵⁷

Gelatin breakdown can be demonstrated by incorporating it in a buffered nutrient agar, growing the culture and then flooding the medium with mercuric chloride that differentially precipitates either gelatin or its breakdown products causing opacity in the medium with clear zones around gelatin-liquefying colonies

ANTIBIOTIC SENSITIVITY²⁰

Antibiotic susceptibility pattern was done on Mueller Hinton Agar by Kirby-Bauer disc diffusion method as recommended by Clinical and Laboratory Standards Institute(CLSI).²⁰ Himedia discs were used for disc diffusion testing.

<i>Antibiotic Discs</i>	<i>Contents</i>
Amikacin	30µg
Gentamicin	10µg
Cephotaxime	30µg
Ceftazidime	30µg
Cefepime	30µg
Ciprofloxacin	5µg
Ofloxacin	5µg
Piperacillin	100µg
Piperacillin – Tazobactam	100/10µg
Imipenam	10µg
Meropenam	10µg
Aztreonam	30µg

The control strains used were *E.coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 Overnight broth culture compared to 0.5 McFarland's was used as inoculum. After incubation at 37°C for 16-18 hrs, zone of inhibition was noted. Results were interpreted according to CLSI standard.

Multidrug resistant (MDR) isolates of the nonfermenters were estimated MDR isolate was defined as resistant to three or more drugs of therapeutic relevance.

DETECTION OF EXTENDED SPECTRUM OF β -LACTAMASES^{20,23}

All nonfermenters that were resistant to cefotaxime and or ceftazidime were tested for Extended Spectrum of β -Lactamases by the following methods:

Phenotypic confirmation test with Cephalosporin / clavulanate combination disks.²⁰

This was done as recommended by CLSI. Mueller Hinton Agar plates were swabbed with test organism having the turbidity equivalent to 0.5 Mc Farland's standard. Aseptically cefotaxime disk (30 μ g), cefotaxime – clavulanic acid (30 μ g/10 μ g) ceftazidime (30 μ g) & ceftazidime clavulanic acid (30 μ g/10 μ g) were placed on surface of agar. The plates were incubated at 35°C for 16-18 hours and diameter of zone of inhibition produced was recorded. A 5mm increase in zone diameter for combination disc than that when tested alone confirmed the presence of ESBL production.

ATCC *Escherichia coli* 25922 & *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive control respectively.

DETECTION OF ESBL BY E-TEST

It is a plastic drug – impregnated strips, one end of which contains a gradient of ceftazidime (MIC test range 0.5 to 32 µg/ml) and the other with a gradient of ceftazidime plus a constant concentration of clavulanate (4 µg/ml). Similar strips were used for cefotaxime and cefotaxime / clavulanate. A 0.5 Mc Farland turbidity standard of the organism was inoculated as a lawn culture on Mueller Hinton agar. E strips were placed on the agar surface and plate were incubated at 35°C for 16-18 hours.

INTERPRETATION

A ≥ 8 fold reduction in cephalosporin MICs in the presence of clavulanate is taken as positive for ESBL.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF IMIPENAM AND MEROPENAM BY BROTH MICRODILUTION METHOD

The Minimum Inhibitory Concentration is the least amount of antimicrobial that will inhibit visible growth of an organism after overnight incubation.

MIC was determined by using Mueller Hinton broth as the medium in a microtitre plate. Serial dilutions of Imipenam and Meropenam were prepared in distilled water. The concentrations used were 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 µg/ml. A young peptone water culture of the organisms corresponding to the concentration of 5×10^5 / ml is used as inoculum. A quality control strain of *Pseudomonas aeruginosa* ATCC 27853 was included. The plate was incubated at 35.c for 16-18 hours.

RESULTS AND INTERPRETATION

MIC is expressed as the highest dilution which inhibited the growth as judged by the lack of turbidity in the tube. MIC > 8µg/ml – Carbapenam resistant. MIC < 8µg/ml – carbapenam susceptible. Level of resistance to carbapenams were also noted.

SCREENING FOR METALLOBETALACTAMASE PRODUCTION

Screening for metalloβ-lactamase production was done in isolates of nonfermenters that were resistant to Imipenam and or Meropenam. Due to intrinsic resistance to carbapenams mediated by resident MBL production, isolates of *Stenotrophomonas maltophilia* were not considered eligible for MBL screening.^{40,48} The methods used were:

MODIFIED HODGE TEST ⁶²

The indicator organism, *Escherichia coli* ATCC 25922, at a turbidity of 0.5 McFarland standard was used to inoculate the surface of a Mueller - Hinton agar plate supplemented with zinc sulfate at a concentration of 70µg/ml. and the test strain was heavily streaked from the centre to the plate periphery. After the plate was allowed to stand for 15 min at room temperature, 10 µg Imipenam disk was placed at the center and the plate was incubated overnight. The presence of a distorted inhibition zone was interpreted as a positive result for carbapenam hydrolysis screening.

IMIPENAM – EDTA DOUBLE DISC SYNERGY TEST ⁶²

Test organism was adjusted to the 0.5 Mc Farland standard and used to inoculate Muller Hinton agar plates. 10µg Imipenam disk was placed on the plate and a blank filter paper disk was placed at a distance of 10mm (edge to edge). To the blank disk, 10µl of 0.5M EDTA solution (1,900 µg of disodium salt, dihydrate) was added. After overnight incubation, the presence of even a small synergistic zone was interpreted as positive.

IMIPENAM – EDTA COMBINED DISK TEST ¹¹³

Test organisms of 0.5 Mc Farland standard were inoculated onto plates of Mueller– Hinton agar. A 0.5M EDTA solution was prepared. Two 10µg Imipenam discs and Meropenam discs were placed on the plate. 5µl of EDTA solution was added to one of the disc. The inhibition zones of the Imipenam and Imipenam + EDTA discs were compared after 16-18 hrs of incubation at 37⁰C. An increase in inhibition zone of ≥ 7 mm in combined disc than imipenam disc alone was considered as positive

**MODIFIED HODGE TEST
DDST
DDCT**

**DETERMINATION. OF METALLOBETALACTAMASE GENE BY
MULTIPLEX PCR METHOD ^{59,61,69}**

In this study, multiplex PCR was used to determine the gene for MBL production in *Ps.aeruginosa* isolates that were resistant to carbapenams PCR was done using primers specific for MBL genes.

Cell lysates of the isolates were used as DNA template for colony lysate PCR. Around 5 – 10 colonies were suspended in 100µl of Milli Q water & boiled for 5 minutes. It is then centrifuged at 10,000 rpm for 10 minutes. The supernatant provided templates for PCR reactions.

Forty amplification cycles were performed with an automated thermocycler according to the following format: Initial denaturation for 5 min at 94. C, 30 cycles of DNA denaturation for 30 s at 94.c, annealing for 30 s at 55.c and extension for 1.5 min at 72.c. The final cycle was followed by an additional 5 min at 72.c to complete partial polymerizations. Amplified products were run using horizontal 1.5 % agarose gel electrophoresis. The gel was visualized using a UV transilluminator. The amplified PCR products and 100 base pair DNA molecular markers were seen as bright fluorescent bands.

INTERPRETATION

A 261 bp corresponds to VIM and 587 bp corresponds to IMP gene-specific oligonucleotides.

RESULTS

A total of 156 strains of nonfermenting gram negative bacteria (NFGNB) from different clinical samples were processed and grouped during the study period.

Table-1

AGE DISTRIBUTION OF PATIENTS n= 156

<i>Age</i>	<i>No. of Patients</i>	<i>Percentage</i>
0-20	16	10.3%
21 – 40	80	51.3%
41 – 60	48	30.7%
>60	12	7.7%

Maximum number of isolates were in the age group of 21 – 40 years (51.3%) followed by 41 – 60 years (30.7%).

Table 2

GENDER DISTRIBUTION OF PATIENTS n = 156

Sex	Number	Percentage
Male	110	70.5%
Female	46	29.5%

Males (70.5%) outnumbered females (29.5%)

Male: Female ratio = 2.4 : 1

Table 3

CATEGORY OF CASES n=156

Category	Number	Percentage
Community acquired	14	9%
Hospital acquired	142	91%

Most of the infections associated with nonfermenters were hospital acquired. (91%)

Table 4

SAMPLE DISTRIBUTION n=156

Sample	Number	Percentage
Pus	63	41.1%
Urine	48	30.2%
Tracheal Aspirate	22	14%
Blood	10	6.3%
Sputum	6	3.8%
Bronchoalveolar lavage	5	3.2%
CSF	1	0.7%
Drain	1	0.7%
TOTAL	156	100%

Pus (41.1%) was the predominating clinical sample harboring the nonfermenters followed by urine (30.2%) & tracheal aspirates (14%).

TABLE 5
SOURCE OF SAMPLES n=156

Specialty	No. of Cases	Percentage
Surgery	40	25.5%
Intensive care unit	29	18.6%
Urology	21	13.5%
Medicine	16	10.5%
Orthopedics	14	8.9%
Diabetology	10	6.5%
Thoracic Medicine	9	5.7%
Otorhinolaryngology	7	4.4%
Oncology	5	3.2%
Nephrology	4	2.6%
Neurosurgery	1	0.6%

Maximum number of the clinical samples infected by NFGNB were from Surgery wards (25.5%) followed by Intensive Care Unit (18.6%) & Urology (13.5%)

TABLE 6**RISK FACTORS ASSOCIATED WITH INFECTIONS BY NONFERMENTERS****n=156**

Risk Factors	Number	Percentage
Surgery/Trauma	31	23.8%
Catheter & Instrumentation	27	20.7%
Diabetes Mellitus	24	18.5%
ICU stay	22	16.9%
Prolonged antibiotic therapy	16	12.3%
Malignancy	5	3.9%
Transplantation	3	2.3%
Dialysis	2	1.6%
TOTAL	130	83%

Risk factor was present in 83% of the infections by NFGNB ($p < 0.05$) and the commonest risk factors associated with these infections were surgery (23.8%), catheters & Instrumentation procedures (20.7%), Chronic illness like diabetes mellitus (18.5%) and prolonged stay in ICU (16.9%).

TABLE 7

GROUPING OF NON FERMENTING GRAM NEGATIVE BACILLI

Group	Number	Percentage
Oxidase positive and Motile	101	64.1%
Oxidase positive and Nonmotile	2	1.9%
Oxidase negative and Motile	7	4.5%
Oxidase negative and Nonmotile	46	29.5%
TOTAL	156	100%

64.1% of the isolates were oxidase positive and motile

TABLE 8
SPECIATION OF NFGNB ISOLATED n=156

Clinical Isolates	Number	Percentage
<i>Pseudomonas aeruginosa</i>	92	58.9%
<i>Acinetobacter.baumannii</i>	46	29.5%
<i>Stenotrophomonas.maltophilia</i>	7	4.5%
<i>Pseudomonas fluorescens</i>	4	2.6%
<i>Alcaligenes fecalis</i>	3	1.9%
<i>Weeksella virosa</i>	2	1.3%
<i>Burkholderia cepacia</i>	2	1.3%
TOTAL	156	100%

Pseudomonas.aeruginosa (58.9%) was the predominant isolate among the nonfermenters, followed by *Acinetobacter.baumannii* (29.5%) *S. maltophilia* (4.5%), *Ps.fluorescens* (2.6%), *Alcaligenes fecalis* (1.9%), *B.cepacia* (1.3%), and *Weeksella virosa* (1.3%)

TABLE 11
MULTI DRUG RESISTANT NONFERMENTERS n=156

Organism	Total Number	MDR	Percentage
<i>Ps.aeruginosa</i>	92	71	77%
<i>A.baumannii</i>	46	36	78%
<i>S.maltophilia</i>	7	7	100%
<i>B.cepacia</i>	2	2	100%
<i>Ps.fluorescens</i>	4	1	25%
<i>A.fecalis</i>	3	1	33%
<i>W.virosa</i>	2	-	-
Total	156	118	75.6%

75.6% of NFGNB were multidrug resistant.

TABLE 12
DRUG SUSCEPTIBILITY OF MDR STRAINS

DRUGS	SUSCEPTIBLE	RESISTANT	P value
GENTAMICIN	5.1%	94.9%	< 0.01
AMIKACIN	41.8%	58.2%	< 0.01
CIPROFLOXACIN	38.8%	61.2%	< 0.01
OFLOXACIN	33.7%	66.2%	< 0.01
CEFOTAXIME	4.1%	95.9%	< 0.01
CEFTAZIDIME	57.1%	42.9%	< 0.01
PIPERACILLIN	12.2%	87.8%	< 0.01
PIP-TAZO	65.3%	34.7%	< 0.01
CEFIPIME	16.3%	83.7%	< 0.01
AZTREONAM	23.8%	76.2%	< 0.01
IMIPENAM	82.7%	17.3%	< 0.05
MEROPENAM	82.7%	17.3%	< 0.05

Multidrug resistant isolates were susceptible to carbapenams, ceftazidime and piperacillin-tazobactam. Maximum resistance was seen with cefotaxime and gentamicin.

TABLE 13
ESBL PRODUCTION IN NONFERMENTERS n=156

Organism	Total No.	ESBL Producers	Percentage
<i>Ps.aeruginosa</i>	92	63	68.4%
<i>A.baumannii</i>	46	32	69.5%
<i>S.maltophilia</i>	7	7	100%-
<i>P.fluorescens</i>	4	1	25%
<i>B.cepacia</i>	2	2	100%
<i>A.fecalis</i>	3	1	50%
<i>W.virosa</i>	2	-	-
Total	156	106	67.9%

67.9% of NFGNB were ESBL producers.

TABLE 14
ESBL PRODUCTION IN SAMPLES n=156

Samples	Total No.	ESBL	Percentage
Pus	63	47	74%
Urine	48	33	70%
Trachea	22	16	72%
Blood	10	4	40%
Sputum	6	3	50%
BAL	5	3	60%
CSF	1	--	-
Drain	1	-	-
Total	156	106	67.9%

Majority of samples with ESBL isolates were Pus (74%), tracheal aspirates (72%). and Urine (70%),

TABLE 15
DRUG SUSCEPTIBILITY OF ESBL ISOLATES

DRUGS	SUSCEPTIBLE	RESISTANT	P value
GENTAMICIN	6.7%	93.7%	< 0.01
AMIKACIN	44%	56%	< 0.01
CIPROFLOXACIN	34.7%	64.3%	< 0.01
OFLOXACIN	30.7%	69.3%	< 0.01
CEFOTAXIME	5.3%	94.7%	< 0.01
CEFTAZIDIME	41.3%	58.7%	< 0.01
PIPERACILLIN	10.7%	89.3%	< 0.01
PIP-TAZO	60%	40%	< 0.01
CEFIPIME	17.3%	82.7%	< 0.01
AZTREONAM	25.2%	74.8%	< 0.01
IMIPENAM	82.7%	17.3%	< 0.05
MEROPENAM	82.7%	17.3%	< 0.05

ESBL producing isolates were susceptible to carbapenams, amikacin and piperacillin-tazobactam. Maximum resistance was seen with cefotaxime and gentamicin.

TABLE 16

DISTRIBUTION OF ACQUIRED CARBAPENAM RESISTANT ORGANISMS

Organisms	Total No.	Carbapenam resistant isolates	Percentage
<i>Ps.aeruginosa</i>	92	9	9.7%
<i>A.baumannii</i>	46	3	6.5%
<i>S.maltophilia</i>	7	-	-
<i>P.fluorescens</i>	4	-	
<i>B.cepcia</i>	2		
<i>A.fecalis</i>	3	-	
<i>W.virosa</i>	2	-	
Total	156	12	7.7%

7.7% of strains were acquired carbapenam resistant organisms. Predominant organisms were *Ps.aeruginosa* (9.7%) & *A.baumannii* (6.5%).

TABLE 17
PATTERN OF CARBAPENAM RESISTANCE n=12

RESISTANCE	MIC µgm/ml	NO.	PERCENTAGE
High - level	>32	7	58%
Low - level	8 - 32	5	42%

High level resistance to carbapenams was found in 7 isolates. Low level resistance was found in 5 isolates.

TABLE 18
ACQUIRED MBL PRODUCTION IN NONFERMENTERS

ORGANISM	TOTAL NO	CARBAPENAM RESISTANT	MBL
<i>P.aeruginosa</i>	92	9	5(5.43%)
<i>A.baumannii</i>	46	3	2(4.34%)
<i>S.maltophilia</i>	7	-	-
<i>P.fluorescens</i>	4	-	-
<i>A.fecalis</i>	3	-	-
<i>B.cepacia</i>	2	-	-
<i>W.virosa</i>	2	-	-
TOTAL	156	12(7.7%)	7(4.48%)

4.48% of isolates were MBL producers.

TABLE 19

RESULTS OF PCR IN IMEPENAM RESISTANT *PSEUDOMONAS* n=9

MBL GENE	TOTAL NO OF CASES	PERCENTAGE
Positive	5	55%
Negative	4	45%

The gene responsible for metalloβ-lactamase production was observed in 5 isolates (55%)

TABLE 20

PATTERN OF GENE OBSERVED IN MBL PRODUCING

***Ps. aeruginosa* n=5**

TYPE OF MBL GENE	TOTAL NO OF CASES	PERCENTAGE
IMP	2	40%
VIM	3	60%

VIM type isolates (60%) were predominant than IMP type isolates.

TABLE 21

COMPARISON OF MBL DETECTION BY DIFFERENT METHODS

Organism	No	Modified Hodge test		Double disc synergy test		Double disc combined test		PCR	
		+ve	_ve	+ve	_ve	+ve	_ve	+ve	_ve
<i>P.aeruginosa</i>	9	3	6	4	5	5	4	5	4
<i>A.baumannii</i>	3	2	1	2	1	2	1	Not done	Not done
Total	12	5	7	6	6	7	5	5	4

Sensitivity and specificity of Modified Hodge test for *P.aeruginosa* were 60% and 93.4% respectively and for the EDTA – disk synergy test sensitivity was 80% and specificity 95.3% whereas for the EDTA- combined disc test both were 100% The differences in sensitivity and specificity between these tests were statistically significant($p < 0.01$ by chi-square test)

All the three tests were equally sensitive and specific for *A.baumannii*

DISCUSSION

Non fermenting Gram Negative bacilli (NFGNB) are being isolated with increasing frequency from clinical specimens and clinical problems posed by their multi drug resistance in recent years has led to the interest in the present study.

One hundred and fifty six isolates of non fermenting gram negative bacilli were taken from various clinical samples like pus, urine, endotracheal aspirates, blood, sputum, cerebrospinal fluids and drain tube and evaluated for their role in infections in hospitalised patients including the characteristics of their drug resistance.

Analyzing the age group, higher incidence of infection by NFGNB was seen in the age group of 21-40 years (51.3%) (Table 1). This was comparable with the study by Meharwal et al from Chandigarh (53%)⁷⁰ It can be attributed to the increased activity of the age group and higher chance of exposure to infections.

The incidence of infections by nonfermenters is very high in males (70.5%) as compared to females (29.5%) (Table 2). This correlated with the study by Chacko.B et al who observed 66.6% of males to be affected as compared to (33.3%) of females¹⁷. The increase in male to female ratio (2.4:1) could be due to the involvement of outdoor activities by males and therefore increased chance of exposure to infections.

The present study shows a higher rate of hospital acquired infections (91%) by nonfermenters than community acquired infections (9%) (Table 3). Studies like

Meharwal et al (88%), from India and Marcus.et al (92%) from Israel support this finding^{70, 68}.

In the current study, community acquired infections were most commonly caused by *Pseudomonas* (7.05%) followed by *A.baumannii* (1.92%) The infections were otitis externa , chronic suppurative otitis media and urinary tract infections.Urinary tract infection was the commonest community acquired infection by nonfermenters. This finding is comparable with the studies by Paraskaki et al (6.5%), Marcus.et al (8%) , Gilad J et al, (8% & 2%) and Badura et al(7%).^{78, 68, 41, 10}

Among the clinical samples, nonfermenters were predominantly observed in pus samples (41.1%) followed by urine (30.2%) and tracheal aspirates (14.1%)in the present study(Table 4). Kharangate et al from India reported 50% incidence in pus samples.⁵⁴ Vijaya et al from India reported a lower incidence of 32.4% in pus samples¹⁰⁷.Veenu et al reported 29% incidence in urinary tract infections.¹⁰⁶ Gladstone et al from CMC Vellore showed a higher incidence in endotracheal aspirates (42.4%) due to large number of samples ⁴².

In the present study, majority of the isolates of nonfermenters were from General Surgery wards (25.5%).(Table 5) A study by Anupurba et al from India reported higher prevalence of 29% isolations in surgery wards⁶. Another study by Ezeltahawy from Saudi Arabia also observed 25% isolations from General surgical wards²⁹.This might be due to prolonged stay in hospital following an operation resulting in colonization and

subsequent infection The second highest source observed in the present study was intensive care unit (18.6%) followed by urology units (13.5%). These data clearly state the importance of the infections caused by nonfermenters in the intensive care settings. Several studies also report a higher incidence in these units. (Trautmann et al -50% , Aikaterini Mastoraki et al- 15%, Gladstone et al -12.2%)^{105, 3, 42}

Risk factors for development of infections by nonfermenters were present in 83% cases ($p < 0.05$) in this study while Meharwal et al also reported presence of risk factors in 87.2% of infections by these organisms⁷⁰ The significant risk factors in the present study were surgery/trauma (23.8%) catheters/instrumentation (20.7%) and diabetes mellitus (18.5%). and stay in the intensive care unit (16.9%). (Table 6) .The identification of ICU as a strong risk factor is not unexpected. Studies by .Joshi et al (30.8%) & Ezeltahawy et al. (34%) have identified a longer stay in intensive care unit as a risk factor^{51, 29} . In addition, in the present study, variables related to hospitalization and invasive procedures like intubation, prolonged therapy with antibiotics, catheter lines, dialysis, malignancy and transplantation were identified as risk factors for infections caused by NFGNB. as observed in the studies by. Falagas et al, Saad Nseir et al, Nicholas et al^{32, 94, 75}

Grouping of NFGNB (Table 7) showed 64.1% of the isolates belonging to oxidase positive and motile group in the present study as compared to Veenu et al.¹⁰⁶ *Pseudomonas aeruginosa* was the predominant isolate (58.9%) (Table 8) among the

nonfermenting gram negative bacilli observed in the present study followed by *Acinetobacter. baumannii* (29.5%), *Stenotrophomonas. maltophilia* (4.5%), *Pseudomonas fluorescens* (2.6%) *Alcaligenes faecalis* (1.9%), *Burkholderia cepacia* (1.3%), and *Weeksella virosa* (1.3%), Kharangate et al from India reported 56% isolation of *Ps.aeruginosa*⁵⁴ Taneja et al, from Chandigarh, India found *Ps aeruginosa* (51%) as the predominant isolate followed by *A.baumannii* (31%), and *A faecalis* (7%)¹⁰⁰. Similar to the present study, a study from Saudi.Arabia by Ezeltahawy et al. identified *Ps. aeruginosa* as the most common isolate (56%) followed by *A.baumannii* (34%) & *S.maltophilia* (.6%)²⁹. Wang H et al from China reported most predominant pathogens as *Ps.aeruginosa* (46.9%), *Acinetobacter* species (31%) & *S.maltophilia* (9.2%).¹¹⁰

PSEUDOMENAS AERUGINOSA (Table 9)

In the present study, out of the 92 isolates of *Ps aeruginosa* 41 isolates (44.6%) were from pus samples comprising wound swabs, ulcers and ear swabs This is similar to the study by Basu et al who reported 40% isolates from pus samples¹². Other Indian reports by Veenu et al & Behera et al showed higher isolation rate of 53.4% & 66.7% respectively.^{106,13} A report by Kiani et al from Pakistan also showed *Ps.aeruginosa* as commonest isolate from pus samples (55%).⁵⁵ However reports by Frank et al from USA and Olayinka from Africa were less at 12% and 33%.^{35,77} Higher rate of infections found here compared to western studies necessitates the implementation of proper infection control measures.

Ps. aeruginosa was next commonly present in urine samples – 24 isolates among 92 isolates (26%). It is an opportunistic pathogen in urinary tract infections accounting upto 40% of infections in the hospital ⁴⁴. The rate in the present study was higher than that reported in Taiwan (11%).⁸⁴ but comparable to the Indian study by Veenu et al (29%).¹⁰⁶ Similar to the studies in literature (Meharwal et al - 45.5% Dedeic et al -16%, Milan et al -10% , Aaron et al -30% & Tabibian et al - 38%) *Ps.aeruginosa* infection were associated with catheter related, cytосcopy related & other urinary tract procedures (20%)in the present study.^{70, 24, 72, 1, 99}

Investigations have shown that *Ps.aeruginosa* were significant pathogens from endotracheal aspirates especially in ICU⁴². This study by Gladstone et al from Vellore showed an incidence of 42.8% of drug resistant *Ps.aeruginosa* from endotracheal aspirates. The present study shows 10 / 92 isolates (11%) from endotracheal aspirates which is lower than the previous study due to lesser number of samples but correlates with the study by Chien Liang Wu et al (10%)¹⁹ A study by Jonaidi et al from Iran showed 32% isolations and *Ps aeruginosa* as the most common isolate from tracheal specimens.⁴⁸ Culture of endotracheal aspirates is a valuable tool in the diagnosis of ventilator associated pneumonia where NFGNB especially *Ps. aeruginosa* is a significant pathogen (Furtado et al & Aikaterini Mastorati et al).^{37, 3}

In the present study, 8.8% of *Ps aeruginosa* isolates were from blood cultures similar to the Indian study by Vijaya et al (8%)¹⁰⁷ SENTRY survey of blood stream

infections due to gram negative bacilli collected in USA, Canada and Latin America showed an incidence of 10.6% of *Ps.aeruginosa* bacteremia²⁵ A report by Mayo clinic in 2008 showed an incidence (10.2%) comparable to the present study⁶⁵

Four out of 92 (4.4%) isolates of *Ps.aeruginosa* were from sputum samples and 3/92 (3.3%) from bronchoalveolar lavage samples. The predisposing factors in the respiratory tract observed in the present study were COPD, chronic bronchitis and underlying chronic diseases. Various studies by Po-Ren Hseuh et al, Italy, Aikaterini Mastoraki et al (6%), Veenu et al (9 %) , Ferrara et al (5%), Arora et al (12%). show that *Ps. aeruginosa* is frequently isolated from respiratory tract.^{85, 3, 106, 33, 9,}

Ps.aeruginosa is an unusual cause of bacterial meningitis associated with immunosuppressed patients. In the present study one isolate each from CSF(1.08%) and drain tube (1.08%) was observed comparable to the study by Chi et al. who showed *Ps.aeruginosa* as the most common isolate from shunt infections.¹⁸ A study by Damsbrauskiene et al found 3 isolates of *P.aeruginosa* in CSF.²¹

In the present study, 88% of *Ps. aeruginosa* isolates were susceptible to Imepenum & Meropenam,(Table 10) which was similar to Indian studies by Prakesh et al 86% & Anupurba et al 84%^{86, 6} An Italian study also reported 81% susceptibility similar to present study³⁰ Susceptibility to Piperacillin – Tazobactam in the present study was 82.6% comparable to the study in Canada conducted in the year 2008 (80.5%)¹⁰⁸. A study in India by Basu et al in the year 2009 also reported high sensitivity

to Piperacillin – Tarobactam¹² The susceptibility rate of *P. aeruginosa* isolates to amikacin (61.9%), ceftazidime (65.2%), and ciprofloxacin (42.3%) in the present study were relatively low compared to the sensitivity pattern to these drugs reported world wide. In US more than 90% of the isolates were susceptible to amikacin, 80-90% of isolates were susceptible to ceftazidime while 70-80% of isolates were susceptible to ciprofloxacin⁵³ In Trinidad and Tobago 80 % isolates were susceptible to ceftazidime³⁴. Reports from France have shown *P. aeruginosa* susceptibility rates of 78.5% and 61.7% to ceftazidime and ciprofloxacin, respectively¹⁵. Studies in China & India gives results similar to the present study - 68% and 63% to ceftazidime and amikacin^{111, 100} In the current study, high rate of resistance was seen with Gentamicin (71.8%) and cefotaxime (87%). similar to reports from India by Veenu et al (69% & 75%) and, Anupurba et al (75% & 82%)^{106, 6}. This is much higher compared to reports from USA (30%) and Trinidad (12 %) ^{53, 31}. This shows the selective pressure from the use of these agents in empirical therapy in India as the main determinant for the emergence of drug resistant strains.

ACINETOBACTER BAUMANII (Table 9)

The next predominant isolate was *A.baumannii* in the present study. A total of 46 out of 156 NFGNB were isolated accounting for 29.5% Fourteen from pus, 19 from urine, 2 from blood, 8 from tracheal aspirates, 2 from bronchoalveolar lavage. , and 1 from sputum

A. baumannii as major species of *Acinetobacter*, isolated from this hospital is reportedly a major pathogen responsible for nosocomial infections in other parts of the world also ⁸³.

Urinary samples (41.3%) were the predominant among the clinical specimens showing infection with *A baumannii*. This is in accordance with Indian reports by Meharwal et al (39%) & Taneja et al (41%).^{70, 100}

Isolates in pus in this study was 30.4% comparable to Indian study by Joshi et al (27.5%) and a study from Pakistan reporting *Acinetobacter* spp as one of the commonest isolates from wound infections.^{51, 55}

The present study shows *A.baumannii* as a major pathogen in Intensive care units as well with an isolation rate of 17.3% in endotracheal aspirates.similar to the reports in Italy (Ferrara et al- 16%) and .Gladstone et al (19%) from CMC Vellore India.^{33, 42} Literature gives a morality rate going upto 70% associated with ventilator associated pneumonia by *A.baumannii*.^{74, 66} No mortality was observed in the present study in endotracheal aspirates..

Epidemiological studies on blood stream infections show *A.baumannii* as the tenth most common etiologic agent at 1.3% of these infections⁴⁷. The present study shows 4.3% of *A.baumani*. isolates from blood culture samples comparable to a report from India by Arora et al (5.4%) but less than the reports from USA, France, Belgium and Germany ranging from 7-9% (Lahiri et al) ^{8, 60} Invasive procedures like intravenous

catheterization was a major risk factor in the study by Joshi et al and it was observed in this study as well.⁵¹

Isolation rate from bronchoalveolar lavage and sputum samples were 4.4% & 2.1%.comparable to the studies by Patwardhan et al, at 7% and 6 %.respectively⁷⁹

There have been isolations from CSF and other body fluids ⁷⁴ but no such isolate is reported in the current study.

A.baumannii is an organism which exhibits high resistance to antibiotics in hospital environment. In the present study 93.4% of isolates were sensitive to Imipenam & Meropenam,(Table 10), 86.9% to Piperacillin-Tazobactum, 69.5% to Amikacin, 48.6% to ceftazidime, while cefotaxine & gentamicin showed maximum resistance at 76% and 85%. This finding is nearly comparable with Aroma et al, Ferrara et al from Italy and Jones et al ^{7, 33, 50}

S.MALTOPHILIA (Table 9)

S.maltophilia is an emerging nococomial pathogen in hospital settings. The significance of this organism lies in its intrinsic multidrug resistance. ⁹². Seven out of 156 (4.5%) nonfermenting gram negative bacilli were observed in this study. This is less compared to the reports in China (9.2%)¹¹¹. However Indian reports found 3.35% (Vijaya et al) & 1% (Meharwal, Taneja, & Veenu et al) isolations among NFGNB from clinical specimens. ^{107, 70, 100, 106}

Four isolates were obtained from endotracheal aspirates in this study. A study by Chien Liang Wu et al had reported *S.maltophilia* as the third most common organism (2 isolates) isolated from endotracheal aspirates.¹⁹ Kollef et al reported that isolation of this high risk pathogen is an important predictor of mortality in Ventilator Associated Pneumonia.⁶⁶

In the present study 3 isolates were obtained from pus (,2 from diabetic foot ulcer &1 from postoperative wound swab). similar to the studies by Veenu et al. ¹⁰⁶.Studies have shown that *S.maltophilia* is a frequent isolate from wounds and other skin lesions ⁷³

S.maltophilia is highly resistant to many of the antibiotics commonly used in hospital settings⁷³ In the present study,(Table10) these isolates were sensitive to quinolones (57%), and cotrimoxatole (100%), Resistance was observed with betalactam agents – ceftazidime (82%), and cefotaxime (100%).as observed by Robert et al⁹²

***P.FLUORESCENS*(Table 9)**

P.fluorescens is also a common isolate in various clinical specimens mentioned in different literature studies ^{49,74}

In the present study 4/156 (2.6%) strains were obtained. All of them were isolated from pus. A study by Veenu et al reported 6 isolates from pus¹⁰⁶. Sheretz et al reported 2.38% of isolates.⁹⁵ Although isolates from blood culture have been reported, the present study did not give any blood isolate.

These organisms (Table 10) were susceptible to carbapenams (100%) Amikacin (100%), Ceftazidime (100%), and Piperacillin – Tazobactam (100%) Studies in literature show sensitivity of these organisms to betalactam agents & carbapenams ⁷⁴

BURKHOLDERIA CEPACIA (Table 9)

Burkholderia cepacia has emerged as a significant respiratory pathogen ⁹². Study done in Chandigarh show majority of these isolates from blood.³⁹ In the present study one each were isolated from pus and sputum. The isolation of this organism from diabetic foot ulcer was significant in this study.

B.cepacia is an important pathogen isolated from sputum of cystic fibrosis patients ⁹⁰ In the present study one isolated was obtained from sputum

These organisms (Table 10) were sensitive to carbapenams (100%), quinolones (50%) and ceftazidime (50%). A report by V.Gautam et al found a similar susceptibility profile with resistance to aminoglycosides as observed in the current study.³⁹

ALCALIGENES FECALIS (Table 9)

Alcaligenes fecalis is a pathogen in hospital acquired infections especially in persons with underlying diseases⁷⁴. In the present study 3/156 strains were isolated from urinary tract infections. Vijaya et al and Veenu et al isolated 3 and 1 isolates from urine respectively.^{106, 107}

In the present study (Table 8) these isolates were sensitive to Amikacin (100%), ceftazidime (100%) and carbapenams (100%). Resistance was observed to quinolones (100%) and gentamicin (50%) comparable to the study by Meharwal et al.⁷⁰

WEEKSELLA VIROSA (Table 9)

Flavobacteriaceae have been recovered from hospital environment causing nosocomial outbreaks.⁵⁷

In the present study 2 *Weeksella virosa* isolates were obtained from urine. Meharwal et al isolated one strain of *W.virosa* from urine.⁷⁰

In the present study these organisms (Table 10) were susceptible to carbapenam (100%), ceftazidime (100%) Amikacin (100%). Reports in literature showed sensitivity to piperacillin-tazobactam & ciprofloxacin. similar to the present study⁷⁴

MULTIDRUG RESISTANT NONFERMENTERS (Table 11)

Multidrug resistance is a major problem with non fermenting gram negative bacilli and so the infections caused by them are very difficult to be treated. In the present study 75.6% of NFGNB were resistant to 2 or more drugs of relevance. and *Ps aeruginosa* (77%), *A. baumannii* (78%) & *S.maltophilia* (100%) were the most common multidrug resistant strains. This is higher than the studies in India reported previously in the year 1998 (Vijaya et al) and 2000.(Veenu et al) at 31% & 62% respectively and comparable to the study .by Patwardhan et el in 2008 who showed >68% of

Pseudomonas isolates and >90% *Acinetobacter* isolates were multidrug resistant.^{107,106,79}

While *S.maltophilia* is intrinsically resistant to antibiotics, *Ps aeruginosa* & *A.baumannii* acquire resistance by many different mechanism like Extended spectrum of betalactamases (ESBL) and Metallobetalactamases(MBL). This is of concern as efficacious antimicrobial options are limited.⁴⁷ The present study showing *Acinetobacter* as more multidrug resistant than *Pseudomonas* correlated with the studies by Tanya et al (53% and 49%) and Homer et al (62% and 58%)^{101, 46} Multidrug resistant organisms were susceptible to Piperacillin – tazobactam (62.3%), and carbapenams (82.7%) Maximum resistance was seen with gentamicin(94.9%) and cefotaxime.(95.9%) (Table12)

Extended spectrum of betalactamase (Table13)

Extended spectrum of betalactamase production was observed in 67.9% of NFGNB. *S.maltophilia*(100% , *B.cepacia*(100%),*Ps.aeruginosa* (68.4%) & *A.baumannii* (69.7%) were the predominant ESBL producers. comparable with the report by SridharRao(61.7%)⁹⁸. While *S.maltophilia* and *B.cepacia* show intrinsic resistance , ESBL production by *Ps.aeruginosa* and *A.baumannii* is significant⁹² All the ESBL producers were multidrug resistant. Maximum sensitivity among the ESBL producers was seen with Imepenam (82%) followed by piperacillin – Tazobactam (60%) (Table14) as seen in the studies conducted by Brauers et al and Freshteh et al^{16,36}

Maximum ESBL production was found in pus (74%), tracheal aspirates(72%) and urine samples(70%), (Table15).similar to the reports by RituSinghal et al ⁹¹These data shows an increase in the prevalence of ESBL producing organisms in India.

Pan drug resistant NFGNB

Pan drug resistant isolates in the present study was seen in 7.7% (12/156) strains. *Ps.aeruginosa* & *A.baumannii* were the most common isolates. Various studies in the literature show similar reports (Obritsch et al -9%, Aikaterini Mastoraki et al -12%, Falgas et al -9%)^{76, 3, 31}. Antibiotics to which these strains were resistant were carbapenams, third generation cephalosporins, Piperacillin, Fluoroquinolones and aminoglycosides.

Carbapenam resistant NFGNB (Table 16)

Acquired carbapenam resistance was observed in 7.7% strains (12/156 strains) *Stenotrophomonas* possesses intrinsic carbapenamase resistance and hence *Ps aeruginosa* (9.7%) and *A.baumannii* (6.5%) were the most common acquired carbapenam resistant organisms.^{40,48} The incidence is lesser compared to study by Taneja et al (36.4%) but comparable to the report by Berges et al (7.2%), Prakash (8.1%), Kanungo et al from Pondicherry (10%) and,Gladstone et al (12.2%)^{14,100,86, 52, 42}.These data show an emerging resistance to carbapenams in this tertiary hospital. All the carbapenam resistant isolates were multidrug resistant. They were susceptible to colistin & polymyxin.B. Studies show that potentially toxic drugs like polymyxin B & colistin remained the only option

among the drugs for the treatment of PDR isolates. (Michaelopoulos et al, Koomanachi et al, Po-Ren Hseuh et al, Hachem et al)^{71,56, 83, 43}

In the present study (Table 17)high level of resistance >32 MIC ugm/ml was observed in 7 isolates and low level 8 - 32 MIC ugm/ml found in 5 isolates. compared to the study by Jones et al and Massaru et al who shoerd High level resistance can be caused by MBL enzymes and low level resistance is associated with reduced uptake of drug as a result of loss of porin Opr D ^{50, 69}

METALLOBETALACTAMASE PRODUCING NFGNB (Table 18)

The present study shows 4.48% of total NFGNB as MBL producers.Of these 5 isolates of *Pseudomonas aeruginosa* (5.43%) were confirmed to be positive by PCR and 2 isolates were *A.baumannii* (4.34%) Various studies across the world, has reported MBL production ranging from 2%-60% ¹⁰⁹. Indian reports by Navneeth et al show 12% of MBL production and Agarwal et al in 2008 reported an incidence of 8% *Ps. aeruginosa* strains to produce MBL^{42,2} A study in Korea showed MBL production in 10% of *Ps. aeruginosa* isolates and a study in Japan showed 1.9% of MBL production in *A.baumannii* isolates ^{26, 40}

In the present study MBL production was observed in tracheal aspirates(13%), followed by blood samples(20%) and diabetic foot ulcer (3%)in the present study compared to that by Gian Marria (13%,17%,10%) respectively.⁴⁰ Organisms with MBL production are associated with high mortality rate ⁸² In the present study, one mortality

was observed in a renal transplant patient from whom MBL producing *A.baumannii* was isolated from blood culture sample. All the MBL isolates were sensitive to colistin and polymyxin B similar to the studies by Yoo Chul et al, and Hachem et al ^{114, 43}

Molecular analysis (Table 19 and 20) by Multiplex PCR performed on the carbapenam resistant *Pseudomonas* isolates revealed 2 types of MBL genes circulating in this hospital –bla_{VIM} and bla_{IMP}. bla_{VIM} were more prevalent (3 isolates) similar to the study by Luzzare et al (4 isolates) than bla_{IMP} (2 isolates) similar to the study by Masaru et al (2 isolates) and Gian et al ^{64, 69, 40}. This data suggests the emerging resistance by carbapenamase production in NFGNB in this tertiary care hospital. Rapid dissemination of MBL producer is a danger as we are left with the option of using potentially toxic drugs like Colistin & Polymyxin B for treatment of infections with MBL producers apart from the high mortality associated with them. This necessitates not only the implementation of surveillance studies but also proper and judicious selection of antibiotics in a tertiary care hospital.

Of the various methods used to detect MBL production (Table 21) Double Disc Combined. Test with imipenam & imepenam EDTA showed 7 isolates as MBL producers. Double Disc Synergy Test showed 6 isolates as MBL producers. Modified Hodge Test showed 5 isolates as MBL producers. The present study observed DDCT to have sensitivity and specificity of 100% in *Pseudomonas* isolates compared to the PCR as gold standard. This study showed DDCT as the simple, inexpensive and reproducible functional screening test for MBL production in *Ps.aeruginosa*. Various studies by Ting-

ting Qu et al, Pitout et al, Dongeun Yong et al, Behera et al , Lee et al, and Berges et al have shown Double Disc combined and Synergy Tests as better methods / methods of choice to detect the MBL production in the absence of PCR. ^{103, 88, 26, 13, 62, 14}

MANAGEMENT OF INFECTIONS BY NFGNB

The prevalence and sensitivity of nonfermenters often varies between communities, hospitals in the same community and among different patient populations in the same hospital. Faced with these variations, the physician in clinical practice has the responsibility of making clinical judgments and should have access to recent data on the prevalence and antimicrobial resistance pattern of commonly encountered pathogens. It is therefore important to institute a system for the surveillance of antimicrobial resistance that will involve the collection and collation of both clinical and microbiological data. The present study observed highest resistance of NFGNB against cefotaxime & gentamicin antibiotics which are commonly used drugs. This necessitates the judicious use of these antibiotics in empirical therapy. Maximum sensitivity was observed with newer agents like carbapenams and piperacillin-tazobactam. Moderate sensitivity was seen with aminoglycosides and fluoroquinolones. Major risk of using monotherapy is the emergence of antibiotic resistant bacteria as observed in the present study which showed high rate of multidrug resistance and ESBL producers. Carbapenamase resistance, though not high was still observed as an emerging drug resistant mechanisms in the NFGNB from this hospital. Antibiotic therapy either empiric or documented is based upon antibiotic combination supplemented by knowledge of

local epidemiology of resistance and susceptibilities in choosing a suitable combination. Therefore combination antibiotic therapy like piperacillin-tazobactam, quinolones-amikacin, imipenam-amikacin would be an ideal choice of therapy on the basis of antibiotic susceptibility testing.as observed in this study along with an adequate infection control measures especially in the surgical and ICU units ^{6,15,28,34,48,106}

SUMMARY

1. A total of 156 strains of NFGNB from various clinical samples were studied for their role in hospital infections.
2. Higher incidence of nonfermentative isolates was observed in the age group of 21-40 years (51.3%) with a preponderance in males.(70.5%)
3. Hospital acquired infections were more compared to community acquired infections (91%) and Pus samples (41.1%) were the predominant among the various clinical samples harboring nonfermenters.
4. Predominant source of these infections were from surgical wards.(25.5%)
5. Infections by NFGNB were associated with a higher number of risk factors.(83%) ($p < 0.005$) and Intensive Care Units formed a significant source of infections by these organisms(16.9%)
6. *Pseudomonas aeruginosa* was the predominant isolate (58.9%) among the nonfermenting gram negative bacilli, followed by *A. baumannii* (29.5%) *S.maltophilia* (4.5%), *Pseudomonas fluorescens* (2.6%) *Alcaligenes fecalis* (1.9%), *Burkholderia cepacia* (1.3%), *Weeksella virosa* (1.3%),
7. *Pseudomonas aeruginosa* was predominantly isolated from pus (44.6%), *A.baumannii* from urine (41.3%), *S. maltophilia* from tracheal aspirates (57.2%) ,*Weeksella virosa* from urine, *Pseudomonas fluorescens* from pus,

Alcaligenes fecalis from urine *Burkholderia cepacia* from pus and sputum

8. The antibiotic susceptibility pattern of the isolated nonfermenters revealed 87.8% sensitivity to Imipenam and Meropenam, followed by Piperacillin-Tazobactum (83%), ceftazidime (59.6%), amikacin (62.8%) and quinolones (53.2%). Resistance was seen against cefotaxime (80.8%), and gentamicin (72.5%),
9. Incidence of multidrug resistant nonfermenters was 75.6%
10. 7.7% of nonfermenters were pandrug resistant showing resistance to all commonly used antibiotics.
11. Extended spectrum of betalactamase production was observed in 67.9% of the strains. and majority of these isolates were from pus samples (74%) followed by tracheal aspirates (72%) and urine (70%)
12. Acquired Metallobetalactamase production was seen in 4.48% of NFGNB and 58.3% of carbapenam resistant isolates. *Pseudomonas aeruginosa* (5.43%) and *A.baumannii* (4.54%) were the predominant MBL producers. Blood sample (20%) was the predominant sample harboring MBL producers.
13. Double disc combined test method was a better method of detection of metallobetalactamase production phenotypically with a sensitivity and specificity of 100%

14. Molecular characterization of MBL production revealed prevalence of VIM like isolates (60%) of *Ps aeruginosa* than IMP like isolates (40%)
15. Combination therapy of piperacillin-tazobactam, quinolones-aminoglycosides, and imipenam-aminoglycosides was recommended for treatment of infections by nonfermenters.
16. Potentially toxic agents like Colistin and Polymixin B were the only option available for treating MBL isolates

CONCLUSION

Observations from the present study showed the aerobic NFGNB which are usually considered as contaminants are now emerging as important nosocomial pathogens. The various clinical specimens from which they were isolated proved their existence in all sites leading to a range of diseases. Different sensitivity pattern and multidrug resistance exhibited by nonfermenters pose a great problem in treating these infections. ESBL and MBL production by these organisms lead to high morbidity and mortality as we are left with the only option of treating them by potentially toxic agents like Colistin and Polymyxin B. Awareness of their entry into a hospital environment is the first step that clinical microbiologists can take to address this problem. Care in detection, evaluation of effective antibiotic options, judicious use of antibiotics by instituting antibiotic policy of combination therapy and rigorous infection control measures will help to fight against these multidrug resistant nonfermenters in the effective management of patients

APPENDIX

1. *oxidase test*⁵⁷

This test is mainly done to differentiate organisms lacking cytochrome oxidase enzyme, mainly the members of enterobacteriaceae. This enzyme helps in the transfer of electrons to oxygen, with formation of water.

The dye tetramethyl paraphenylene diamine dihydrochloride is substituted for oxygen as artificial electron acceptor. In the reduced state, it is colorless and in the presence of cytochrome oxidase and oxygen it forms indophenol, which is purple.

Strips impregnated with dried reagents were used. The colony was taken on a wooden stick and smeared onto the strip. The appearance of purple color within 10 sec. was taken as positive.

2. **CATALASE TEST**⁵⁷

Organisms' possessing the enzyme catalase splits hydrogen peroxide into water and oxygen. The evolution of oxygen appears as bubbles.

Tube catalase test was performed 3ml of 3% Hydrogen peroxide was taken in a test tube. The colony of the organism to be tested was taken on a glass rod and introduced into the tube. Appearance of brisk effervescence was taken as positive.

3. **MOTILITY**⁵⁷

Motility was done by hanging drop method. A drop of saline with the test organism was placed on the coverslip. The hanging drop slide is inverted over the coverslip where wax had been applied on the corners. The slide is turned quickly so that drop is in centre of the concavity. The edge of the drop is focused on the low power and high power objectives. Motility was observed.

4. INDOLE TEST⁵⁷

Indole is a benzyl pyrrole which is a metabolic product of tryptophan. Bacteria which possess tryptophanase hydrolyse tryptophan to indole.

The organism was inoculated in peptone water medium, incubated at 37°C for 18-24 hours, 1ml of xylene was added and Erlich's reagent (Para dimethyl amino benzaldehyde) was added drop by drop. Formation of fuchsia red ring was positive. It is a red complex of indole and paradimethylaminobenzaldehyde.

5. TRIPLE SUGAR IRON MEDIUM⁵⁷

Triple sugar iron medium was taken and the organism was stabbed to the butt as well streaked on the surface. It was incubated for 18-24 hours at 37°C, and then looked for the presence of growth and change in pH. Growth with no change in pH (slant and butt) indicated the organism to be nonfermenting.

6. CITRATE UTILIZATION TEST ⁵⁷

Sodium citrate is a salt of citric acid seen in metabolism in Krebs cycle. Some bacteria utilize citrate as the sole source of carbon and it is detected by the production of alkaline byproducts Christensen's citrate media was used.

The organisms were streaked on the surface of the slant and incubated at 37⁰C for 18-24hrs. Development of deep blue color of the medium with growth was taken as positive.

8. UREASE TEST ⁵⁷

Urea is a diamide carbonic acid which when hydrolyzed releases ammonia and carbon dioxide. Urease is an enzyme when present hydrolyses urea and release ammonia changing the medium to alkaline, PH.

The organism was streaked on the surface of the slant and incubated at 37⁰C for 18-24 hours. Development of magenta pink of the medium along with growth was taken as positive.

9. NITRATE REDUCTION TEST ⁵⁷

The capacity of an organism to reduce nitrates to nitrites is shown by this test. The presence of nitrate in the medium is detected by addition of α naphthalamine and sulphanilic acid.

The organism was inoculated in nitrate broth and incubated at 37°C for 18-24 hours and observed for gas production by Durham's tube. One ml each of reagents α naphthalamine & sulphanilic acid were added simultaneously and looked for the development of red color.

Development of red color was taken as positive and when it was negative zinc dust was added. When the red color developed after adding zinc the test was taken as negative because it indicated the presence of residual nitrates.

10. Growth at 42°C⁵⁷

The organism was plated in nutrient agar plate and incubated at 42°C for 18-24 hours and looked for growth. Presence of uniform growth indicated positive results.

11. Growth at 44°C⁵⁷

The organism was plated in nutrient agar plate and incubated at 44°C for 18-24 hours and looked for growth. Presence of uniform growth indicated positive results.

12. Polymyxin B Sensitivity⁵⁷

The organism was plated on nutrient agar plate and a polymyxin B disc of 300U was kept. It was incubated at 37°C for 18-24 hrs and looked for zone of inhibition

SPECIAL BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF NON

FERMENTERS

1. HUGH – LEIFSON OXIDATION - FERMENTATION MEDIUM⁵⁷

Peptone	:	2g
Sodium Chloride	:	5g
D-Glucose	:	10g
Bromothymol blue	:	0.03g
Agar	:	3.0g
Dipotassium Phosphate	:	0.3g
Distilled water	:	1 litre
pH	:	7%

Medium was sterilized by autoclaving. After cooling the medium to 45⁰C, filter sterilized carbohydrate solution was added to get a final concentration of 1%. Carbohydrates used for the study were glucose, maltose, xylose, fructose and mannitol. Of medium was poured as a butt and without a slant into tubes. Two tubes were required for the test, each inoculated with the unknown organism, using a straight needle stabbing the medium three to four times half way to the bottom of the tube. One tube of each pair was covered with a 1cm layer of sterile mineral oil (or) melted paraffin, leaving the other open to the air. Both tubes were incubated at 35⁰C and examined daily for several days.

In case of oxidative metabolism, yellow color appears along the upper one fourth of the medium and in the tube where no oil overlay was done. In case of fermentative organisms yellow color develops in both the tubes.

CONTROL

Glucose fermentation : *Escherichia coli*

Glucose oxidation : *Pseudomonas aeruginosa*

Non saccharolytic: *Alcaligenes species*.

2. *DECARBOXYLATION OF LYSINE, ARGININE & ORNITHINE*⁵⁷

Decarboxylases are a group of specific enzymes which react with carboxyl portion of aminoacid forming alkaline reacting amines. The reaction is decarboxylation. Each enzyme is specific for Lysine, Arginine and Ornithine.

INGREDIENTS

Yeast extract	:	5g
Peptone	:	5g
Glucose	:	0.5g
Pyridoxal	:	5mg
Bromocresol Purple	:	5ml
Cresol Red	:	2.5ml
Distilled water	:	1 ltr.

All aminoacids were added individually to a final concentration of 1% pH adjusted at 6.0 & autoclaved at 15lbs for 15 minutes.

PROCEDURE

The organism was inoculated in four tubes. One having the basal medium without aminoacid for control. Other three tubes having lysine, arginine and ornithine each. All tubes were overlaid with liquid paraffin. All were incubated at 37⁰C for 24 hours.

The control tube turned yellow indicating that the organism is viable and the test medium turning blue purple indicating positive result.

3. O-NITROPHENYL β- D GALACTOPYRANOSIDE⁵⁷

Reagent

Sodium Phosphate buffer 1M pH 7.0 – 5ml

O – Nitrophenyl β - D – galactopyranoside – 80mg

Distilled water – 15ml.

A dense suspension of the test organism grown in TSI agar was prepared in saline. About 1 drop of toluene was added to the suspension and 0.2ml of ONPG solution was added to the suspension and incubated at 37⁰C β-galactosidase producing organism show yellow color after 1 hour or 18-24 hours incubation.

CONTROL

Positive Control – *Escherichia coli*

Negative control – *Proteus species*.

4. ACETAMIDE AGAR¹¹

Ingredients

Magnesium Sulphate	:	0.2g
Ammonium dihydrogen Phosphate	:	1g
Pottasium monohydrogen phosphate	:	1g
Sodium Chloride	:	5g
Acetamide	:	10g
Bromothymol blue solution	:	6.4ml
Agar	:	15g
Final pH	:	6.9
Distilled water	:	1 litre

The ingredients are mixed and pH adjusted to 6.9, dispensed into screw cap tubes and sterilized at 121⁰C for 15 min. The medium was allowed to cool in a slant. The slant was inoculated with a portion of isolated colony and incubated at 37⁰C overnight and was observed for color change. Tubes with negative result were further incubated for 7 days.

Control

Positive control	:	<i>Pseudomonas aeruginosa</i>
Negative Control	:	<i>Stenotrophomonas maltophilia</i>

5. GELATIN LIQUEFACTION TEST ⁵⁷

Gelatin breakdown can be demonstrated by incorporating it in a buffered nutrient agar, growing the culture and then flooding the medium with mercuric chloride that differentially precipitates either gelatin or its breakdown products causing opacity in the medium with clear zones around gelatin-liquefying colonies

CONTROL

Positive control : *Pseudomonas aeruginosa*

Negative Control : *Stenotrophomonas maltophilia*

PCR Primers and procedure ⁶⁹

Two sets of primers were used for multiplex PCR. The primers used are given below

IMP FORWARD – 5' CTA CCG CCG CAG CAG AGT CTT TG -3'

REVERSE 5'-AAC CAG TTT TGC CTT ACC AT-3'

VIM FORWARD 5'- AGT GGT GAG TAT CCG ACA G -3'

REVERSE 5'-ATG AAA GTG CGT GGA GAC -3

PCR was conducted with 1µl of boiled bacterial suspensions, 0.5µl of each primer, 2µl 5mM of dNTP, 2.µl 10mM Tris-HCL (pH 8.3), 0.75 µl 50mM MgCl₂ and 2U of Taq DNA polymerase in a total volume of 20µl.

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PROFORMA

Name: I.P.No:

Department:

Age: Sex:

Occupation:

Present Complaints and duration:

Present History:

Past History:

Personal History:

Family History:

Clinical Findings:

Investigations:

Management:

Follow-up:

ABBREVIATIONS

NFGNB	-	Nonfermenting gram negative bacilli
Ps.aeruginosa	-	Pseudomonas aeruginosa
A.baumannii	-	Acinetobacter baumannii
Ps.fluorescens	-	Pseudomonas fluorescens
S.maltophilia	-	Stenotrophomonas maltophilia
B.cepacia	-	Burkholderia cepacia
Mac plate	-	MacConkey plate
MDR	-	Multidrug resistant
ESBL	-	Extended Spectrum Of Betalactamases
MBL	-	Metallobetalactamases
ONPG	-	O–Nitrophenyl β - D Galactopyranoside

