"Comparative evaluation of phenotypic tests for identification of Extended Spectrum Beta Lactamases (ESBL) and Metallo-Beta-Lactamases (MBL) producing *Pseudomonas aeruginosa* from pus samples of hospitalized patients of tertiary care hospital, one year study."

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ENDORSEMENT BY THE HOD, PRINCIPAL/HEAD OF THE INSTITUTION

This is to certify that the dissertation entitled **"Comparative evaluation of phenotypic tests for identification of Extended Spectrum Beta Lactamases (ESBL) and Metallo-Beta-Lactamases (MBL) producing *Pseudomonas aeruginosa* from pus samples of hospitalized patients of tertiary care hospital, one year study."** is a bonafide research work done by REG. NO.BI0110002.

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LIST OF ABBREVIATIONS

ESBL	:	Extended spectrum beta lactamases
MBL	:	Metallo-beta-lactamases
MDRPA	:	Multi drug resistant *P.aeruginosa*
EDTA	:	Ethylene diamine tetra acetic acid
MDR	:	Multidrug resistance
ICU	:	Intensive care unit
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
MRSA	:	Methicillin-sensitive *Staphylococcus aureus*
O/C/O	:	Operated Case of
VAP	:	Ventilator Associated Pneumonia
GNB	:	Gram Negative Bacilli
GPC	:	Gram Positive Cocci
MAT	:	Maternity
DDST	:	Double Disc synergy test
DDT	:	Disc diffusion test
IMP-EDTA CDT	:	Imipenem (IMP)- EDTA combined disc test
IMP-EDTA DDST	:	Imipenem (IMP)- EDTA double disc synergy test
Disc potentiation test	:	EDTA disc potentiation using ceftazidime, ceftizoxime, cefepime and cefotaxime
CSOM	:	Chronic Suppurative Otitis Media

ABSTRACT

Background: *Pseudomonas aeruginosa* is one of the most common pathogens causing nosocomial infection mainly causing wound infections and an opportunistic pathogen with physiologically versatile nature. It flourishes as a saprophyte, with innate resistance to many antibiotics and disinfectants. In addition to its innate resistance, acquired resistance is particularly associated with indiscriminate antibiotic use. ESBL and MBL production are important examples of them. With worldwide increase in occurrence and rate of dissemination of ESBL and MBLs, early detection is essential. This will help in timely implication of strict infection control practices as well as clinical guidance regarding potential risk of therapeutic failure.

Aim of the Study: The present study was undertaken

1. To compare different phenotypic tests for identification of ESBL and MBL producing *P.aeruginosa* from pus samples received at microbiology department.
2. To know the prevalence of ESBL and MBL producing *P.aeruginosa isolated* from pus samples.

Materials and Methods: The present study was undertaken at the Department of Microbiology, J.N.Medical College, Belgaum during the period from Jan 2011 to Dec 2011. All the *P.aeruginosa* isolated from 1200 pus samples in hospitalized patients of K.L.E.'S DR. Prabhakar Kore Hospital and MRC, Belgaum, received at Microbiology department were included in the study. Isolates showing resistance to Ceftazidime were selected for detection of ESBL enzyme by Double Disc Synergy Test and Disc Diffusion Test**.** Isolates showing resistance to Imipenem were selected for detection of MBL enzyme by Imipenem (IMP)- EDTA Combined Disc Test, Imipenem(IMP)-

EDTA double disc Synergy Test, EDTA disc potentiation using Ceftazidime, Ceftizoxime and Cefotaxime and Modified Hodge test **.**

Results: Out of the 90 *P.aeruginosa* isolated from 1200 pus samples, 57(63%) were Cefazidime sensitive and 33(37%) were resistant. Of the 33 *P.aeruginosa* resistant to Ceftazidime, Disc Diffusion Test detected 9(27%) of ESBL producers and Double Disc Synergy Test detected 17(51%).

And out of the 90 *P.aeruginosa* isolated from 1200 pus samples, 66(73.3%) were Imipenem sensitive and 24(26.6%) were resistant. Of the 24 *P.aeruginosa* resistant to Imipenem, IMP-EDTA CDT detected 14(58%) of ESBL producers, IMP-EDTA DDST detected 7(29%), EDTA disc potentiation test detected 11(46%) and Modified Hodge method detected 8(33%).

Conclusion: This study showed that Double Disc Synergy Test is a better method to detect ESBL producers and IMP-EDTA Combined Disc Test for detection of MBL producing *P.aeruginosa*.

Key words: *Pseudomonas aeruginosa*, Extended Spectrum beta lactamases, Metallo beta lactamase, Ceftazidime, Imipenem.

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INTRODUCTION

Pseudomonad literally means 'False Unit' derived from the Greek Pseudo (false) and monas (a single unit). The word "monad" was used in the early history of Microbiology to denote single-celled organisms.

A pseudomonas infection is caused by *Pseudomonas aeruginosa* bacteria and has a distinct feature of "blue pus" symptom. The cause of blue pus is pyocyanin, which is a pigment produced by this pathogen.

Pseudomonas is physiologically versatile and flourishes as a saprophyte in multiple environments, including soil, marshes, and coastal marine habitats. It can survive under conditions that few other organisms can tolerate, it produces a slime layer that resists phagocytosis (engulfment), and it is resistant to most antibiotics. Pseudomonas can multiply in an extraordinary assortment of environments including eye drops, soaps, sinks, anesthesia and resuscitation equipment, fuels, humidifiers and even stored distilled water. It has also been reported in kidney dialysis machines.

Normally, for an infection to occur, some disruption of the physical barriers (skin or mucous membrane), or by-passing of them (invasive devices), and/or an underlying dysfunction of the immune defense mechanisms is necessary. Therefore, *P.aeruginosa* is mostly a nosocomial pathogen.

Emergence of nosocomial bacterial pathogens with acquired resistance to almost all available antimicrobial agents, namely 'Super bugs', has severely threatened therapeutic choices in the last few decades.

Nosocomial infections due to multidrug resistant bacterial pathogens have been associated with increased hospital expenditures and poorer clinical outcomes.

Among the three most common multidrug resistant gram negative bacteria emerged in medical centers such as *P. aeruginosa*, *A.baumannii* and *Klebsiella pneumoniae, P. aeruginosa* is the most frequently isolated troublesome pathogen causing surgical site infections, life threatening respiratory tract infection (ventilator associated pneumonia) and Urinary tract infections in patients from intensive care units.[1]

Pseudomonas aeruginosa is one of the most prevalent opportunistic human pathogen and the most common gram-negative bacteria causing nosocomial infections.

A major challenge has aroused regarding the treatment of infections caused by opportunistic pathogens, predominantly those with high level resistance to all antibiotic classes and extreme ability to acquire resistance, such as *Pseudomonas aeruginosa* and *Acinetobacter baumanni.*[2, 3]

This bacterium makes use of distinctive mechanisms to become resistant to virtually all the available antibiotics.[4, 5] Several mechanisms can contribute to acquired resistance in *P. aeruginosa*, including β lactamase production, the up regulation of efflux systems and decreased outer membrane permeability. In addition to its innate resistance, acquired resistance is particularly associated with discriminated antibiotic use.

Extended-spectrum-β-lactamases (ESBL) and Metallo-β-lactamases (MBL) mediated resistance is important emerging resistance mechanisms in *P.aeruginosa*. Despite of abundant literature, little is known about the prevalence of these mechanisms among clinical strains of *P. aeruginosa.*

At present Clinical and Laboratory Standards Institute (CLSI) guidelines do not describe any method for detection of these enzymes in *P. aeruginosa.*[91]

A 3 years prevalence of *P.aeruginosa* in our hospital (K.L.E.'S DR.Prabhakar Kore Hospital and MRC, Belgaum) is approximately 7% of all the pus samples. Thus this study is designed to know the prevalence of ESBL and MBL producing *P.aeruginosa* and hence will enable us to evaluate antibiotic therapy and take preventive measures to control the outbreaks in future.

Thus, this study was undertaken, to compare different phenotypic tests for identification of ESBL and MBL producing *P.aeruginosa* from pus samples and also to know the proportion of ESBL and MBL producing *P.aeruginosa* isolated from pus samples, in patients at K.L.E.'S DR.Prabhakar Kore Hospital and MRC, Belgaum, received at Microbiology Department.

This possibly will help to prevent the associated morbidity and mortality caused due to this organism by implementing proper control measures and to evaluate antibiotic therapy.

OBJECTIVES

1. To compare different phenotypic tests for identification of ESBL and MBL producing *P.aeruginosa* from pus samples received at Microbiology department.
2. To know the prevalence of ESBL and MBL producing *P.aeruginosa* isolated from pus samples.

REVIEW OF LITERATURE

HISTORY:

Pseudomonas is the most important genus in the order *Pseudomonadales* and the family *Pseudomonaceae*. The genus *Pseudomonas* has more than 60 species. *Pseudomonad* literally means 'False Unit' derived from the Greek Pseudo (false) and monas (a single unit). The word "monad" was used in the early history of Microbiology to denote single-celled organisms.

Gersard first isolated *P.aeruginosa* from surgical wounds. But surgical entity of BLUE PUS was known even before this.[6]

In 1953, Hugh and Leifson made a breakthrough in identification of Nonfermenters by the development of OF-medium.[7]

In 1950s *Staphylococcus aureus* emerged as organisms causing nosocomial infections, followed by Nonfermenters in 1970s and multi drug resistant Enterobacteriaceae in 1960s, with the introduction of new broad spectrum antibiotics.[8]

Emergence of resistance to beta-lactam antibiotics began even before the first beta-lactam penicillin was developed. These enzymes are thought to be evolved from penicillin binding proteins, with which they show some homology. The first plasmid mediated beta-lactamase in gram negatives, TEM-1, was described in *E.coli* from a patient named Temoniera.[9]

Pseudomonas aeruginosa producing MBL was first reported from Japan.[9]

Metallo-beta-lactamases (MBL) can hydrolyze most beta-lactams except for monobactams and confer a broad-spectrum beta-lactam resistance phenotype to the

bacterial host, which is not reversible by conventional therapeutic beta-lactamases inhibitors.

Extended spectrum beta lactamase (ESBL) hydrolyze expanded spectrum Cephalosporins with an oxyimino side chain. These Cephalosporins include Cefotaxime, Ceftriaxone, and Ceftazidime, as well as the oxyimino-monobactam aztreonam. Thus ESBLs confer resistance to these antibiotics and related oxyimino-beta lactams.

ESBL producing bacteria may not be detectable in the routine disc diffusion susceptibility tests leading to inappropriate use of antibiotics and treatment failures.[10]

BACTERIOLOGY OF NONFERMENTERS

The Nonfermenters comprise a heterogeneous group of gram negative aerobic and facultative anaerobic bacilli or coccobacilli that do not ferment glucose. The chaotic nomenclature and classification of many of these organisms have recently been clarified by exhaustive nutritional and genetic studies. Data regarding ecology, pathogenicity and antimicrobial susceptibility of Nonfermenters are gradually becoming available.[18]

TAXONOMY

Unlike Enterobacteriaceae the Non-fermenting Gram Negative Bacilli (NFGNB) do not fit conveniently in single family of well characterized Genera and the correct taxonomic placement of Nonfermenters remains unresolved.

The major genera of Nonfermenters have been classified into five families. The remaining genera of clinically important Nonfermenters are, as yet not assigned

to a family and are grouped under the heading Organisms whose taxonomic position is uncertain.[5]

With this approach medically important Nonfermenters can be grouped as follows:

MEDICALLY IMPORTANT NONFERMENTERS

Motile with polar flagella

Family *Pseudomonadaceae*

rRNA group I

rRNA group II

rRNA group III

rRNA group IV

rRNA group V

Unknown nucleic acid homology

Family *Methylococcaceae*

Genus Methylobacterium

Organisms Whose Taxonomic Position Is Uncertain

Genus Rosemonas

Genus Balneatrix

Motile with peritrichous flagella

Family *Alcaligenaceae*

Genus Alcaligenes

Genus Bordetella

Family *Rhizobiaceae*

Genus Agrobacterium

Organisms Whose Taxonomic Position Is Uncertain

Genus Achromobacter

Ochrobactrum anthropi

Oligella ureolytica

CDC Group IVc-2

Nonmotile, oxidase-positive

Family *Flavobacteriaceae*

Genus Flavobacterium, Chryseobacterium, Empedobacter

Genus Weeksella, Bergeyella

Organisms Whose Taxonomic Position Is Uncertain

Genus Sphingobacterium

Genus Moraxela

Genus urethralis

CDC groups EO-2, Eo-3, and Psychrobacer immobilis

Gilardi rod group 1

Non motile, oxidase-negative

Genus Acinetobacter

CDC group NO-1

Bordetella holmesii (CDC group NO-2)

Family *Pseudomonadaceae*

Consisting of more than 200 species of gram negative bacilli with Genus *Pseudomonas* and some closely related genera. Many of which were formally placed in Genus Pseudomona. [5, 19]

Characteristic features of members of the family *Pseudomonadaceae* are straight or slightly curved, aerobic, gram negative bacilli. Most of the strains are motile by one or more polar flagella, oxidase positive, oxidatively utilize glucose and other sugars.

Pseudomonas aeruginosa

P.aeruginosa, an important species in family *Pseudomonadaceae* is the common *Pseudomonad* recovered from clinical samples.

Morphology

Pseudomonas aeruginosa is a gram negative, uniformly stained, straight or slightly curved rods, measuring 0.5 to 1.0μm by 1.5 to 5.0μm in length. They are aerobic, non-capsulated, non-spore forming, motile by one or more polar flagella. Can have mucoid slime layer, abundant extracellular polysaccharides made up of alginate polymers which forms a loose capsule (glycocalyx) to protect from host defense mechanisms. They are either incapable of utilizing carbohydrates as source of energy or degrade them "oxidatively" rather than fermentative pathway (few species are asaccharolytic) [20] without gas formation.

Culture characteristics

Obligate aerobe, anaerobic growth only in presence of nitrate, grows at wide temperature range 6-42^{O} C, optimum is 37^{O} C.

On nutrient agar, colonies are large, opaque, irregular colonies and pigmented (Pyocyanin/Fluorescence) and on 5% sheep blood agar, they produce β-hemolytic, large flat spreading, mucoid, rough, pigmented colonies with characteristic metallic

sheen (high carbon, low nitrogen content). Many strains may produce a fruity, sweety, musty or grape like odour due to the presence of 2-aminoacetophenone.

On MacConkey agar, they produce Non-lactose fermenting colonies with metallic sheen.

Dense turbidity with surface pellicle in broth.

Alginate, an exopolysaccharide consisting of mannuronic and guluronic acids aids in forming mucoid colonies. [5, 21, 22]

Pigments

Pseudomonas aeruginosa produces number of pigments:

Pyocyanin - Blue phenazine, a water soluble pigment

Fluorescein - Yellow-green pigment also called as Pyoverdin

Pyomelanin - dark brown pigment, needs 1% Tyrosine for its production

Pyorubin - a red pigment

More than half of clinical isolates produce pyocyanin pigment.[23]

Demonstration of pyocyanin pigment is Absolute Confirmatory Test followed by grape like odour of aminoacetophenone. In old cultures pyoverdin may be oxidized to give brown pigment.[6]

P.aeruginosa is sometimes present as a part of normal microbial flora of humans. Prevalence of colonization in healthy persons outside or just at entry to the hospital is, skin 0-2%, nasal mucosa 0-3.3%, throat 0-6.6% and stool 2.6- 24%. With long term stay in hospital, colonization rate may exceed 50% presaging invasive infection.[16]

Biochemical reactions

Biochemically, they are oxidase and catalase positive, motile, grows well at 42˚C, OF-glucose is oxidatively utilized by the organism, reduces nitrate to nitrite with gas production, Methyl red/Voges Proskauer test negative, donot decarboxylate lysine and ornithine, but dihydrolyze arginine, mannitol not fermented, xylose fermented, lactose not fermented, sucrose not fermented, produce alkaline slant/alkaline butt with no gas and no H_2S in TSI, indole negative, utilize citrate, donot hydrolyze urea, donot produce phenyl pyruvic acid, liquefies gelatin, donot hydrolyze aesculin and utilize acetamide , grows on MacConkey and Salmonella-Shigella agar.[20]

Cetrimide agar is a selective and differential medium for the identification of *Pseudomonas aeruginosa* in which cetrimide acts as detergent which inhibits most bacteria and enhances the production of two pigments pyocyanin and pyoverdine. However, about 4% of clinical strains of *P. aeruginosa* do not produce pyocyanin.

Typing methods [6, 16]

1. Most frequently used and least discriminatory are, antibiogram and biochemical tests determined by Analytical Profile Index (API) profiles.
2. Bacteriocin typing (Pyocyanin and Aeruginosin typing)
3. More specific typing method – Serotyping (Immunotyping)
    a) Fisher – Devlin- Ganabasik system – includes seven types.
    b) International Antigenic Typing System (IATS) – includes 20 different types.

4. Most reliable typing method is Restriction Endonuclease typing with Pulsed – Field.
5. Electrophoresis (PFGE).
6. Most sensitive and specific epidemiological typing methods are DNA probes.

Epidemiology

P.aeruginosa is cosmopolitan in its distribution, isolated soil, water, plants, and animals including humans. Minimal nutritional requirement is evidenced by growth in distilled water, tolerance to different temperatures, which contributes to its ecological success and ultimately to its role as opportunistic pathogen. Moisture is a critical factor. Human colonization occurs at moist areas like axilla, perineum and ear. Colonization is also seen in hospital environment like respiratory equipment, clean solutions, medicines, disinfections, sinks, mops, food mixers, vegetables. Infections outside hospital are associated with water reservoirs, swimming pools, whirlpools, hot tubs and contact lens solutions.[10]

According to annual surveillance data collected by CDC and National Nosocomial Infections Surveillence (NNIS) system between 1990-1996 *P.aeruginosa* is: [16]

- **Second** most common cause of nosocomial pneumonia (17% of isolates)
- **Third** most common cause of urinary tract infection (11%)
- **Fourth** most common cause of surgical site infection (8%)
- **Fifth** most common isolate (9%) overall from all sites
- **Seventh** most frequently isolated pathogen from the blood stream infections (3%)

Resistance

Not resistant to heat (killed at $55^{o}C$)

- Resistant to commonly used antiseptics, chloroxylenol, hexachlorophene, quaternary ammonium compounds.
- Sensitive to Beta glutaraldehyde, silver salts, phenolic disinfectants.

Virulence factors

P.aeruginosa produces several substances to enhance the colonization and infection of host tissue.

TABLE 1: Virulence factors of P.aeruginosa[5]

Virulence factors	Biologic Activity
Alginate	Capsular polysaccharide that allows infecting bacteria to adhere to lung epithelial cell surfaces and form biofilms which in turn protects the bacteria from antibiotics and the body's immune system.
Pili	Surface appendages that allow adherence of organism to GM-1 ganglioside receptors
Neuraminidase	Removes sialic acid residues from GM-1 ganglioside receptors, facilitating binding of pili.
Lipopolysaccharide	Endotoxin, causes sepsis syndrome: fever, shock, oliguria, leucopenia or leukocytosis, disseminated intravascular coagulation, metabolic abnormalities.
Exotoxin-A	Tissue distruction, inhibition of protein synthesis, interrupt cell activity and macrophage response

Enterotoxin	Interrupts normal gastrointestinal activity, leading to diarrhea.
Exoenzyme-S	Inhibits protein synthesis
Phospholipase-C	Destroys cytoplasmic membrane; destroys pulmonary surfactant; inactivates opsonins.
Elastase	Cleaves immunoglobulins and complement components, disrupts neutophil activity.
Leukocidin	Inhibits neutrophils and lymphocyte function
Pyocyanins	Suppress other bacteria and disrupt respiratory ciliary activity; cause oxidative damage to tissues, particularly oxygenated tissues such as lung

Pathogenesis[5]

P.aeruginosa is an opportunistic pathogen, rarely causing disease in healthy individual's inspite of being common human saprophyte. Disease process beings with some alteration or circumvention of normal host defenses. Some patients have underlying dysfunction of specific immune mechanism, as in neutropenia, hypogammaglobinemia, complement deficiency, iatrogenic immunosuppressive states or AIDS. Number of potential virulence factors and broad spectrum of diseases caused indicates multifactorial etiology. Organism is both invasive and toxigenic.

Any *Pseudomonas* infection may be seen as comprising of three distinct stages.

a. Bacterial adherence and colonization
b. Local invasion
c. Dissemination and systemic disease

Each stage has a prerequisite dependent on previous one but the disease process can stop at any stage. Particular virulence factors appear to mediate each of these steps in pathogenesis and to be responsible for characteristic syndromes.

Metabolism of Nonfermenters[5]

Metabolic processes of Nonfermenters describe the taxonomic niche of bacteria and also determine tests and procedures used in Laboratory identification of Nonfermenters. Basic understanding of metabolism of Nonfermenters is a much when designing or interpreting test procedures.

Different pathways of metabolism are:

a) EMP pathway and Kreb's cycle

b) Entner-doudroff pathway

c) Warburg-Dickens Hexose monophosphate shunt

The acid produced by Nonfermenters like glucouronic acid and citric acid are in smaller quantity and weak when compared to mixed acids resulting from fermentation by other microorganisms (Enterobacteriaceae). This difference in metabolism necessitates alternate practical approach to the identification of oxidative and fermentative bacteria. Hence, test system with more sensitive detector of acid production must be used when studying Nonfermenters. Test systems designed to detect acid production from fermentative bacteria cannot be applied to Nonfermenters that produce insufficient acids to convert pH indicator.

Initial clues that can unknown isolate may be Nonfermenter

1. Lack of evidence of glucose fermentation
2. Cytochrome oxidase positive
3. Failure to grow on MacConkey agar

Common tests used in identification of Nonfermenters:

1. OF-glucose
2. Motility
3. Pigment production
4. Hydrolysis of urea
5. Nitrate reduction
6. Indole production
7. Denitrification of nitrates to nitrites
8. Decarboxylation
9. Esculin hydrolysis
10. Flagellar staining – Leifson, RYU, Wet mount
11. Flagellar morphology

Approach to isolation and identification of Non-fermenters

Levels of service in identification of Non-fermenters

The level to which species level identification of Nonfermenters is performed depends on the size and performance of the laboratory. Reference laboratories and universities need to identify all Nonfermenters to species level. Laboratories primarily providing service to medical community may identify most commonly isolated Nonfermenters to species level and send rest to reference laboratory if needed.

Commercial packaged systems of identification are available but these depend on bacterial growth and formation of biochemical products in conventional media or marginally nutritious media and biochemically more active species only can be identified. These can be easily identified in many laboratories in few biochemical tests.

Accuracy of performance is increased with automated and semi automated identification systems primarily because they rely on automatic instruments readings and recording results, eliminating the subjective bias inherent in the visual interpretation of equivocal endpoints. Identification is faster compared to conventional systems.

Factors contributing to difficulty in identification of Nonfermenters

1. Most species are infrequently encountered.
2. Laboratory personal are not familiar with many of Nonfermenters.
3. Much of conventional media are not suitable for identification.
4. Grow slowly and biochemical reactivity weak, producing equivocal results with difficulty in interpretation.
5. Quality control of media-difficult and outdating becomes a problem because of infrequent use.
6. Package commercial systems have low accuracy, especially with more fastidious Nonfermenters requiring use of additional media.

Different schemes for identification of Nonfermenters using conventional tests[24]

Several schemes are presently being used in laboratories for identifying unknown Nonfermenter. Approach to select a particular scheme is largely one of personal preference, past experience and local availability of culture media.

1. The King and Weaver scheme

One of the first schemes designed for identification of Nonfermenters was at the Center for Disease Control and Prevention by Elizabeth O. King, this has been followed in most diagnostic laboratories throughout the world. Tests used were difficult, possible in only reference laboratories. It was later modified and simplified to certain extent by Weaver.[122]

2. Weaver – Hollis scheme

It is a scheme of identification by using simple tests. Consists of three part guide

a) An identification key for gram negative aerobic Nonfermenters

b) A set of 12 identification tables

c) A numerical code book by which, derived biotype numbers can be linked to species names

3. Gilardi scheme

Has evolved over the past 20 years into an extensive system. System is as complicated as Nonfermenters themselves.

4. The Pickett scheme

Pickett was the first person to bring some order to identification of Nonfermenters. His scheme was designed to identify two most common isolates,

P.aeruginosa and *A.anitratus*. This system still remains a viable approach to identification of Nonfermenters, particularly in laboratories with low volume were it may be difficult to maintain differential test media within quality control standards, heavy inoculums and buffered single substrates are innovations. This system can be used to check automated and semi automated systems

5. P.C.Schreckenberger's system

This is simple system of identification. Conventional biochemical tests are used in identification of more than 95% of Nonfermenters.

6. Oberhofer and associates scheme

Commercial media routinely available in most of the laboratories are used. Nonfermenters are broadly classified into oxidative and non-oxidative group. This is a rational approach to identification.

7. Romeo system

Dichotomous keys are used, for identification of Nonfermenters and other miscellaneous gram negative organisms. 22 media and tests based primarily on formulations at CDC are used to construct 12 dichotomous keys, representing various subgroups of over 100 species.

8. Identification by biochemical and susceptibility tests.[24]

In 2002 Laffineur, Michele Janssens, et al developed tests identification of Nonfermenters. Tests used in the identification are alkaline phosphatase, benzyl-arginine arylamidase, pyrrolidonyl arylamidase, ethylene glycol acidification, susceptibility to desferrioxamine and colistin. Yields highly discriminative results for

most of the tests with 100% or 0% positivity. This is a newly proposed system for identification of Nonfermenters.

COMPUTER AIDED SCHEMES

BIO-BASED (Biobase Inc., Boston MA) is a DOS-based computer programme for computer aided identification of Nonfermenters. This includes data based of Holmes and colleagues that includes 66 taxa of Nonfermenters identification by using 83 phenotypic tests.

COMMERCIAL KITS SYSTEMS

Packaged kit systems have been designed or adapted to the identification of Nonfermenters.

Advantages

Convenient to use, long shelf life, preclude the need for fresh supply of media and reagents, produce standardized techniques with accurate and reproducible results equal to or better than conventional tests.

Disadvantages

1. Organisms exhibiting slow growth or weak enzymatic and delayed biochemical activity are read as negative.
2. Less than optimal design for cultivation of Nonfermenters
3. Many of the differential tests used are ineffective

Different commercial kits available for identification of Nonfermenters are

1. OXI/FERM TUBE
2. API-20E SYSTEM
3. API-NFT system
4. REMEL NFT system
5. THE RAPID NF PLUS system
6. CRYSTAL ENTERIC/NONFERMENTER system
7. THE BIOLOG system

AUTOMATED SYSTEMS

Various automated systems for identification of Nonfermenters are:

1. THE VITEK SYSTEM
2. MICRO SCAN WALKAWAY-96, WALKAWAY-40, AUTOACAN-40
3. SENSITRE AP-80 SYSTEM

Infections and risk factors of *Pseudomonas aeruginosa*:

Among the Pseudomonads, *P. aeruginosa* is the most frequently isolated leading, emerging opportunistic pathogen causing nosocomial infections, including pneumonia (ventilator associated pneumonia), urinary tract infection and bacteremia, secondary meningitis, wound infections and often presenting with ecthyma gangrenosum of the skin.[25]

Pseudomonas aeruginosa can infect almost any external site or organ. *P.aeruginosa* causes infections commonly at any site where moisture tends to

accumulate e.g. tracheostomies, indwelling catheters, burns, the external ear (swimmers or divers ear) and weeping cutaneous wounds.

P.aeruginosa also causes endocarditis, meningitis, brain abscess, infections of bone and joints from hematogenous spread, as it has a propensity to invade vascular walls of blood vessels, which facilitates dissemination.[26]

P.aeruginosa dermatitis and otitis externa outbreaks associated with swimming pool and a necrotizing rash, referred to as Jacuzzi or hot-tub syndrome and infections of nail beds in artificial nail users are also common. *P.aeruginosa* accounts for about 6% of all bacteremias and 75% of nosocomial bacteremias, and is the third most common cause of bacteremia, after *Esherichia coli* and *Klebsiella pneumoniae.*[26]

A study by Toniolo et al, on surgical site infections with highly aggressive multidrug resistant *P. aeruginosa* which accounted for approximately one quarter of all nosocomial infections, reported that the common source of specimens were from surgical site (24.0%), urinary tract (19.0%), and blood stream (10.6%).[27]

In a study by Shashikala et al, 27.6% of carbapenem resistant *P.aeruginosa* isolates were obtained from surgical wounds and 20.7% were from endotracheal aspirate. In this study, the distribution of resistant isolates was seen to be higher among intensive care patients (62%), when compared to those in non-intensive care areas such as general and special wards (38%).[28]

In another study by Shanthi et al, the common sites of *Pseudomonas aeruginosa* isolation was from respiratory tract (41.8%) followed by urinary tract (25.5%), wound (20%) and blood (12.7%).[29]

According to Page et al, *P. aeruginosa* was frequently found in patients with comorbid illness and compromised on in-dwelling catheters, endotracheal tubes and surgery, where mortality rates of more than 60% have been reported.[30]

Multidrug resistant *P.aeruginosa* was also found to induce apoptosis of blood monocytes on experimental animals.[31]

Multidrug resistant *Pseudomonas aeruginosa* can evade the intestinal epithelial barrier, causes lethal sepsis within the intestinal tract of critically ill and immunocompromised patients through mechanisms involving disruption of epithelial barrier function. Infections can be severe in cases of an impaired specific or nonspecific defense, such as that in neutropenic or cancer patients, patients with burn wounds[32], cystic fibrosis, acute leukemia, solid organ transplants, patient on steroid therapy and intravenous drug addiction.[33]

Trauma like gunshot, knife wounds, punctures, surgery, burns wound and foreign body implantation can also predispose through urinary or blood stream catheters. Joints and valve prosthetic devices, corneal implants or contact lenses and also can be by infused fluids like dialysate and saline irrigations. And other factors like diabetes mellitus, heart failure, chronic obstructive pulmonary disease, hepatic dysfunction, renal failure and presence of decubitus ulcers also favours important role.[34]

A study by Shankar EM et al, on bacterial etiology of diabetic foot infections in South India, found that foot infections are the frequent complication of patients with diabetes mellitus, accounting for 20% of diabetes – related hospital admissions.

They found high prevalence (57.6%) of gram negative bacteria over (42.3%) gram positives. About 44% of isolates were MDR *P. aeruginosa.*[35]

A study by Lodise et al, showed that patients on Respiratory tract infections were at greatest risk for multidrug resistance. The prevalence of MDRPA over 351 patients of *P. aeruginosa* were 36%.They also found that increasing frequency of antibiotics exposure led to increased chance of MDR *Pseudomonas aeruginosa* infections.[38]

The prevalence of Multidrug resistant *P. aeruginosa* has increased over the past decade and has become a major concern among hospitalized patients. The United States ICU surveillance study demonstrated a significant risk in MDRPA isolates from 4% in 1993 to 14% in 2002.[39]

Among non-intensive care unit patients, the surveillance network reported an increase in proportion of MDRPA infections from 5.5% to 70% between 1998 and 200137. Prolonged endotracheal intubation, associated with exposure to antimicrobial therapies, frequently leads to colonization of the upper respiratory tract by *P.aeruginosa* and once established, it is impossible to eradicate them and leads to VAP in 10 to 15% of patients.[40]

A study by Jayakumar S et al, on prevalence of MDRPA with respect to ESBL and MBL in a tertiary care hospital, on 245 patients showed 22% (54) and 4% (11) prevalence of Multidrug Resistant *Pseudomonas aeruginosa* (MDRPA) and Pandrug Resistant *Pseudomonas aeruginosa* (PDRPA) respectively.[10]

According to Shahid M et al, among 42 clinical isolates of *P.aeruginosa*, 96% showed multidrug resistance and majority (71.4%) were resistant to 5 or more antibiotics.[41]

A study by Galdstone et al, has documented high prevalence (42.8%) of carbapenem resistance in *P.aeruginosa* isolated from patients with respiratory tract infections in the ICUs.[42]

Poor prognostic factors associated with *P. aeruginosa* bacteremia include septic shock, granulocytopenia, inappropriate antimicrobial therapy and the presence of septic metastatic lesions.

Resistance pattern and various mechanisms:

P. aeruginosa is a classical opportunistic pathogen with innate resistance to many antibiotics.

Despite the advent of potent antibiotics and improvement in supportive care, *P. aeruginosa* bacteremia remains one of the most serious hospital- acquired infections, with a case-specific mortality ranging from 18 – 39%.[42, 43, 44, 45, 46, 47]

This high mortality may be attributable to the inherent virulence of the organism, as well as the fact that it often occurs with immunosuppression and comorbidity conditions.[48, 49]

In addition, *P. aeruginosa* is susceptible to a limited number of antimicrobial agents, which increases the likehood of inappropriate empirical antimicrobial therapy. *P. aeruginosa* exhibits intrinsic resistance to several antimicrobial agents. The anti-pseudomonal β- lactamase (such as Ticarcilin, Piperacillin, Ceftazidime, Cefepime, Aztreonam, and the Carbepenems) represents a major weapon against *Pseudomonas*

infections, either for monotherapy or for combination therapy, for which β-lactams almost invariably represent one of the components.[50]

Therefore, acquired resistance to these agents constitutes a major challenge for anti-Pseudomanads chemotherapy, especially when it is associated with resistance to other classes of drugs, such as Aminoglycosides and Fluroquinolones.[51]

They exhibit alarming levels of resistance to even most recent antibiotics like third generation Cephalosporins, anti-pseudomonad Penicillin (Ticarcillin, Piperacillin), Aminoglycosides and Fluroquinilones[52, 53] from various parts of the world and India.

The definition of Multidrug Resistant *Pseudomonas aeruginosa* (MDRPA) was established as isolates Intermediate or Resistant to at least three drugs in the following classes: β-lactams, Carbapenems, Aminoglycosides and Fluroquinolones.[54]

The Pandrug resistant strains are those that are treatable with a single agent, colistin.[55]

Study by Marilee et al, has reported MDRPA of 27-72% in patients with initially susceptible *P. aeruginosa* isolates. As nosocomial infections caused by *P.aeruginosa* are frequently life threatening and often challenging to treat, the emergence of MDR microorganisms presents a critical problem for patients undergoing surgery.[56]

The acidic pH, which is produced by Oxidized Regenerated Cellulose (ORC), a broad spectrum physiological determinant for the survival of microorganisms is known to cause surgical infections.[57]

The drug resistance in *P. aeruginosa* is acquired, either via mutation of endogenous genes or via acquisition of exogenous resistance genes *P. aeruginosa* displays an elevated intrinsic drug resistance that can be further increased through chromosomal mutations against β-lactams.[58]

The most frequently encountered mutations lead to derepression of the chromosomal Amp β-lactams (hydrolyzing most β-lactams except carbapenem), over expression of multidrug efflux pumps (MeXAB-OprM, MeXCD-OprJ and MexXY-OprM system) excluding fluroquinolone, β-lactams (except Imipenem), Aminoglycosides and Tetracyclines or reduced expression of porin pathways (oprD, limiting the permeation of most carbapenem).[59, 60, 61, 62]

Mutation can occur in antimicrobial targets such as, type II tropisomerases (reducing interaction with Fluroquinolones).[51]

Beta lactamases plays very important role in widespread betalactam resistance. These β-lactamases hydrolyze the amide bond of the β-lactam ring, rendering the antimicrobials ineffective. These enzymes may be chromosomal or plasmid mediated and these are thought to have evolved from Penicillin binding proteins.[63]

Most strains of *P.aeruginosa* which are resistant to third-generation cephalosporins produce a chromosomally mediated molecular class C beta- lactamase, the AmpC enzyme. An increasing complexity of aminoglycoside resistance mechanisms is being observed in *P. aeruginosa*, including drug impermeability, multidrug active efflux systems and enzymatic modification of the amino or hydroxyl groups of aminoglycosides. Resistance to aminoglycosides may involve aminoglycoside or 16S rRNA- modifying enzymes, as well as the MexXY-OprM

efflux pump. Among these enzymes, aminoglycoside 3'-phosphotransferases [APH(3')s], is widely represented in *P.aeruginosa*. In particular, the chromosomal gene *aph(3')-IIb* which was believed to be responsible for the "uniform resistance" of *P. aeruginosa* to kanamycin (in operon downstream of the hpaA gene). Similarly, fluroquinolone resistance may be due to mutations in target genes (*gyrA, gyrB, parC* and *parE)* or due to drug efflux systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM).[64]

Carbapenems are the most potent β-lactams against *P. aeruginosa* because it's strong affinity to Penicillin binding proteins, stability against most serine β-lactamases and high permeability across the outer membrane.[59, 65]

However intensive use of carbapenems facilitated the emergence of carbapenem-resistant *P.aeruginosa*.

A study by Taneja et al, showed 42% resistance to Imipenem in urinary isolates. Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin-binding proteins and carbapenem hydrolyzing enzymes-carbapenemase.[66]

Perhaps the most menacing resistance efflux pumps. These pumps have the ability to extrude multiple classes of antibiotics from the periplasmic space as well as the cytoplasm. Constitutive expression of efflux pumps in 'wild-type' cells plays an important role in the relatively decreased susceptibility of *P. aeruginosa* to antibiotics. However, the greatest therapeutic problems occur when these pumps are over expressed in mutants and high-level multi-drug resistance develops. Even though efflux usually confers a moderate level of resistance, its impact in the clinics could be

important, because it may make antibiotics inefficient in infected sites, where antibiotic concentrations are less than optimal, and it confers cross-resistance to unrelated antibiotic classes. About 12 potential efflux systems have been identified in the *P. aeruginosa* genome, four of them (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) are well characterized as antibiotic transporters.[54]

Although the development of infections with extremely resistant strains of *P.aeruginosa* can prevent serious therapeutic challenges, the most troublesome threat associated with the chromosomally-encoded resistance mechanisms is the potential for high-level resistance to emerge during the course of therapy. Unfortunately the emergency of resistance mechanisms is not a rare event with *P. aeruginosa.* It was also found that the standard combination of an aminoglycoside and beta-lactam has shown to be ineffective in preventing the emergence of some resistance problems.[54]

Resistance to all antibiotics except polymyxins is now a reality in many medical centers. *P. aeruginosa* develop resistance to polymyxin by lipid-A modifications with l-Ara4N controlled by PmrA/PmrB.[67]

Panresistance typically is the result of the convergence of multiple resistance mechanisms. Deplano et al, described an outbreak of pan resistant *P. aeruginosa* in an intensive care unit in Belgium.[68]

The isolates over expressed the chromosomal β-lactams Amp and had decreased expression of the porin OprD. Additionally, up-regulation of the MexXY efflux system was present. Lolans et al described an outbreak of Panresistance in *P.aeruginosa* in an ICU in Chicago. The isolates produced Metallo-β-lactamase enzyme (VIM-2), the chromosomally encoded AmpC β- lactamase and the genes

encoding 2 AMEs (aacA7 and aacC-A5). The isolates were variably resistant to Aztreonam, Metallo-enzymes do not hydrolyze Aztreonam.[69]

In order to successfully combat multi-resistant pathogens, it is necessary to clearly define the molecular basis of the general resistance mechanisms associated with the expression of active efflux pumps, which strongly restrict the intracellular concentration of antimicrobial drugs. The activity of some efflux systems requires ATP hydrolysis for transport while others require a sodium or proton antiport.

The enzymatic modification of aminoglycosides results in high-level resistance in numerous bacterial species [Aminoglycoside modifying enzymes (AMES)]. Efflux system in association with distinct mechanisms such as the porin down-regulation, AmpC overproduction and secondary betalactamases also play an important role in the MDR phenotype among *P.aeruginosa* clinical isolates.

According to Cardoso O et al, MDR was recognized in 100 isolates (9.1%), 20 out of these 100 were pandrug resistant (20%) and all these were from nosocomial infections from the respiratory sources (70%).[70]

Acquired resistance to betalactamase can lead to therapeutic failure, especially when it is associated with resistance to other classes of drugs such as Aminoglycoside and Fluroquinolones. In 2003, the National Nosocomial Infections Surveillance System reported that resistance rates of *P.aeruginosa* to Imipenem, Quinolone, and broad-spectrum Cephalosporins were 21.1, 29.5 and 31.9% respectively. Compared to rates in the period between 1998 and 2002, these rates were increased by 15, 9, and 20% respectively.[68]

Study done by Alifkan et al, with patients on Intensive care unit and in-patients clinics between 2003 to 2006 from various clinical samples with *P.aeruginosa* showed, the most effective antibiotics in 2003 were Piperacillin-Tazobactam (84%), Ciprofloxacin (79%), Imipenem (77.5%) and Meropenem (69%) and in 2006 decreasing sensitivity was observed to Imipenem(51%), Meropenem(45%), Cefepime (51%), Piperacillin (38%), Ciprofloxacin (72%), Cefoperazone-Sulbactam (44%) and Piperacillin-Tazobactam (67%) for *Pseudomonas.*[71]

According to a study by Dong et al, regarding analysis of resistant genes of beta-lactam antibiotics from *P.aeruginosa* in pediatric patients, over 146 *P.aeruginosa* strains showed 41.1% Imipenem and 35.6% Meropenem resistance.[72]

In another study by Fadeyi et al, on antibiotic sensitivity pattern of *P. aeruginosa*, showed increasing resistance to Gentamicin and Ofloxacin(77.6%), Cefuroxime(76.6%), Ciprofloxacin(75.3%), Perfloxacin(66%), Streptomycin(64%) and Ceftazidime(50.7%).[73]

A study by Yapar et al, on antibiotic resistance patterns of *P.aeruginosa* isolated from ICU between 2000-2002 and 2003-2006. Seventy nine *P.aeruginosa* isolated during January 2000- December 2002 and 66 *P.aeruginosa* isolated during January 2003- December 2006 were included. All the isolates were from deep tracheal aspirate or bronchoalveolar lavage specimens. Study showed resistance rate for ceftazidime 84%, cefepime 83%, carbapenemes 73%, ciprofloxacin 87% and piperacillin-tazobactam 88%. The study concluded that there was no decrease in the antibiotic resistance rates after.[74]

According to Regina et al, towards the impact of empirical antibiotic therapy and outcomes in patients with *P.aeruginosa* bacteremia, between January 2001 and June 2005 showed, resistance pattern of, Piperacillin-Tazobactam 17.4%, Imipenem 15.5%, Cefepime 9.3%, Gatifloxacin 35.6%, Ciprofloxacin 31%, Amikacin 14.3%, Gentamicin 16.2%, Ticarcillin-Clavulanate 30.1% and Polymyxin-B 0%.[75]

A surveillance study carried out by Olga Cardoso et al, from Portugal between April 2003 to April 2006 to assist in the determination of guidelines for empirical regimen, with 414 communities acquired *Pseudomonas aeruginosa* isolates and 685 isolates from nosocomial infections. A total of 1099 *Pseudomonas aeruginosa* isolates from nosocomial and community-acquired infection showed overall susceptibility for Meropenem, 90%; Piperacillin plus Tazobactam, 89%; Piperacillin, 86.2%; Amikacin, 85.6%; Ceftazidime, 85%; Imipenem, 82.4%; Aztreonam, 81.7%; Ciprofloxacin, 69.2% and Gentamicin, 65.9%. Among hospital isolates, 85.1% were susceptible to Piperacillin plus Tazobactam, followed by Meropenem 84.8%, Piperacillin 81.5%, Ceftazidime 79.4% and Aztreonam 75% and Imipenem 74.7%.[76]

According to Gur et al, on resistance to newer beta-lactams and related ESBL types in gram negative nosocomial isolates in Turkish hospital, done with 1196 gram negative isolates between June 2004- January 2005, showed 28.9% of *P.aeruginosa* were resistant to Imipenem, 27.9% resistant to Cefoprazone/Sulbactam and 22.7% resistant to Piperacillin/Tazobactam.[77]

A study by Yakupogullari Y et al, on MDRPA from Turkey showed MIC of Imipenem and Meropenem were 128mg/L, and those of Ceftazidime, Cefepime, Aztreonam and Piperacillin/Tazobactam were >256mg/L. MIC of Chloramphenicol,

Tetracycline, Gentamicin, Amikacin, Sulphonamides and Rifampicin were more than 32mg/L.[78]

A study by Fernando et al, showed MIC of Ticarcillin(1,024µg/ml), Piperacillin(MIC>1,024µg/ml), Piperacillin-Tazobactam(MIC-512µg/ml), Cefotaxime (MIC-512µg/ml), Ceftazidime(MIC-256µg/ml), Cefepime(MIC-64 µg/ml), Aztreonam (MIC-32 µg/ml), Imipenem(MIC-512 µg/ml), Meropenem(MIC-128 µg/ml), Amikacin(MIC-256 µg/ml), Gentamicin(MIC >512 µg/ml), and Ciprofloxacin (MIC-32 µg/ml), but Colistin showed MIC of 0.5 µg/ml.[79]

Study by Shashikala et al, showed that twenty nine out of 266 (10.9%) isolates of *P.aeruginosa* were resistant to Meropenem and Imipenem. The MIC of Meropenem for the resistant strains ranged from 8 µg/ml to 64 µg/ml and 20% of the resistant strains were sensitive to Amikacin, 13% and 10% each to Piperacillin and Cefepime respectively and 3% to Ciprofloxacin24. A multicentric study conducted by Prashanth et al., 2010, with 29 stains of Imipenem resistant *P.aeruginosa* isolated from three different hospitals showed, showed high Imipenem MIC levels ranging from 32 to 128 µg/ml.[80]

Among various mechanisms of resistance, ß-lactamase production is the most important, resistance mechanism among *P.aeruginosa*. Under these ß-lactamases, Extended spectrum ß-lactamase and Metalo-betalactamanse producing *P. aeruginosa* continues to pose a challenge to infection control worldwide.

Extended spectrum beta lactamases (ESBLs):

ESBLs are beta-lactamases that hydrolyze extended-spectrum Cephalosporins with an oxyimino side chain. These Cephalosporins include Cefotaxime, Ceftriaxone,

and Ceftazidime, as well as the oxyimino-monobactam Aztreonam. Thus ESBLs confer resistance to these antibiotics and related Oxyimino-beta lactams.

The so-called Clavulanic acid-inhibitory extended-spectrum ß-lactamases (ESBLs) belong mostly to class A of the Ambler classification scheme and group 2b according to the Bush-Jacoby-Medeiros functional scheme.[80]

Table 2: Classification of β-lactamases according to Amber molecular scheme.[134, 135, 136]

	Class	β - lactamases	Examples
Serine β - lactamases	A	Broad spectrum β - lactamases	TEM-1, TEM-2, SHV-1
		ESBL TEM – type	TEM-3
		ESBL SHV – type	SHV-5
		ESBL CTX-M – type	CTX-M1, CTX-M9
		Carbapenemases	KPC
	C	AmpC cephamycinases (chromosomal encode)	AmpC
		AmpC cephamycinases (plasmid encode)	CMY, DHA
	D	Broad spectrum β - lactamases	OXA-1, OXA-9
		ESBL OXA – type	OXA-2, OXA-10
		Carbapenemases	OXA-48, OXA-23
Metallo β - lactamases	B	Metallo β - lactamases	VIM, IMP

In Amber scheme, ESBLs are classified into 4 classes, A to D, by its homology of amino acid at the active site. At the beginning, there were 2 classes, serine-β-lactamase (class A) contained serine at the active site and Metallo β-lactamase (class B) which needed bivalent cation, usually Zn2+, for their activity. Due to the discovery of new types of serine β-lactamases, this class was separated to class C and class D

Table 3: Classification of β-lactamases according to Bush-Jacoby-Medieros system.[134, 135, 136]

Group		Type	Examples
1		Cephalosporinases	AmpCs, CMY-2
2		All clavulanic acid susceptible	
	2a	Penicillinases	PC-1 from *S.aureus*
	2b	Broad-spectrum penicillinases	TEM-1, SHV-1
	2be	ESBLs	SHV-2, TEM-10, CTX-Ms
	2br	Inhibitor resistant	TEM, IRT
	2c	Carbenicillin hydrolyzing	PSE-1
	2d	Oxacillin hydrolyzing	OXA-10, OXA-1
	2e	Cephalosporinase inhibited by clavulanate	FEC-1
	2f	Carbapenemases	KPC-1, SME-1
3		Metallo-beta-lactamases	IMP-1, VIM-1
4		Miscellaneous	

In 1995, Bush – Jacoby – Medeiros proposed the new system to categorize ESBLs using enzymatic activity. They classified ESBLs into 4 groups (1-4) and 5 subgroups (a-f)

Group 1 involves cephalosporinases that resist to clavulanic acid, comparable with class C in Amber scheme.

Group 2 consists of penicillinases and cephalosporinases that can be inhibited by clavulanic acid, comparable with class A and D in Amber classification. The TEM and SHV were originally classified in this group.

Due to the increase in number of TEM- and SHV- derived **β**-lactamase, this group was separated into 2 subclasses, 2a and 2b.

The member of **subclass 2a** is only penicillinases, while **2b** is composed of a broad spectrum **β**-lactamases which can inactivate penicillins and cephalosporins at the

same rate. Moreover, there are sub-subgroups, 2be and 2br, separated from subgroup 2b.

Sub-subgroup 2be (e = extended spectrum of activity) is composed of ESBL that can hydrolyze the 3rd generation cephalosporins (cefotaxime, ceftazidime, and cefodoxime) and monobactams (aztreonam).

Sub-subgroup 2br (r = reduced binding to clavulanic acid and sulbactam) contains enzymes that are resistant to clavulanic acid and sulbactam. This group is called inhibitor-resistant TEM derivative enzymes. However, this group is still sensitive to tazobactam.

Subgroup 2c was separated from subgroup 2b later because the members of this group could inactivate carbenicillin more efficiently than benzylpenicillin and had slight effect on cloxacillin.

Subgroup 2d is able to hydrolyze cloxacillin more than benzylpenicillin and has similar activity on carbenicillin. This group is slightly inhibited by clavulanic acid.

In addition, **subgroup 2f**, which consist of serine-based carbapenamases, was designated later. This group is different from zinc-based carbapenamases in group 3.

Group 3 consists of zinc-based or metallo-β-lactamases comparable with Amber class B. This group is the onlygroup that requires metal ion, usually Zn2+, for their activity. These enzymes can hydrolyze penicillins, cephalosporins, and carbapenams. Therefore, carbapenams can be inhibited by either group 2f (serine-based mechanism) or group 3 (zinc-based mechanism).

Group 4 is composed of penicillinases that resist to clavulanic acid and non-comparable with any groups in Amber classification. ESBLs are classified into class

A and D in Amber molecular scheme and in group 2be in Bush-Jacoby-Medieros functional classification system. These enzymes can hydrolyze oxyimino **β**-lactams at least 10% more than benzylpenicillin and are inhibited by clavulanic acid.

The 2 be designation consists of '2b' denoting that the enzyme is derived from 2b enzyme (e.g., SHV-1, TEM-1, and TEM-2) and 'e' representing the ' extended spectrum of activity. They have been extensively reported in members of the family Enterobacteriaceae since the early 1980s, whereas they have been described in *P.aeruginosa* very recently. These enzymes are either of the TEM and SHV types, which were also well known in the Enterobacteriaceae. ESBL producing gram negatives are typically resistant to Penicillin, first and second generation Cephalosporins and also third generation oxyimino Cephalosporins and Monobactams. The ESBLs in *P.aeruginosa* includes the, SHV, TEM, PER, VEB and IBS/GES types.[81]

PER type, mostly originated from Turkish isolates; and VEB type from Southeast Asia; and finally GES and IBC types, which have been reported from France, Greece, and South Africa.[82, 83, 37, 84, 85]

These five types of enzymes are distantly related from a genetic point of view, although they share similar hydrolytic profiles. Recent studies indicates that dissemination of the genes for these β-lactamases may play an important role in the spread of antibiotic resistance and may limit future choices of antibiotic regimens for the treatment of life-threatening infections due to ESBL-producing *P.aeruginosa.*[86]

Most of these are plasmid mediated, initially confirmed to one group of bacteria and spreads to other groups subsequently. It is likely that the genes for the

TEM- and SHV-type ESBLs in *P.aeruginosa* originated in Enterobacteriaceae, from which the genes were transferred by gene transfer. This has been shown for the sequence of TEM-24[87] and the downstream-located DNA sequences of the chromosome of *P.aeruginosa* RP-1, which produces SHV-2a, which were found to be identical to those reported to be plasmid encoded in a *Klebsiella pneumoniae* isolate.[88, 89]

Differences in the replication origins of plasmids from Enterobacteriaceae and *P.aeruginosa* may, however, limit such intergeneric transfers.

Additionally, the difficulty of detectionof TEM- and SHV-type ESBLs in the clinical laboratory may underestimatetheir true prevalence in *P.aeruginosa*.

Table 4: Ambler class A extended-spectrum ß-lactamases reported in *Pseudomonas aeruginosa* are, [90]

ß-lactamase	Genetic support[a]	Country of first isolation	Yr of first isolation	Other countries of isolation
VEB-1	C,P,I	France	1998	Thailand, China,[b]India,[b]
VEB-1a	C,I	Kuwait	1999	
VEB-1b	C,I	Kuwait	1999	
VEB-2	C,I	Thailand	1999	
PER-1	C	France	1991	Turkey, Itlay, Belgium
SHV-2a	C,P	France	1995	Thailand, poland[b]
SHV-5	P	Thailand	1994-1996	Greece[b]
SHV-12	C	Thailand	1994-1996	
TEM-4	P,C	France	1996	
TEM-21	C	France	1997	

TEM-24	P	France	1998	
TEM-42	P	France	1992	
GES-1	C,I	France	1999	
GES-2	P,I	South Africa	2000	
IBC-2	C,I	Greece	1998	

[a] C, chromosomal location; P, plasmid borne; I, integron-borne.

[b] P. Nordmann, personal data.

Table 5: Percent amino acid identity between representatives of each type of Ambler class A ESBL identified in *P. aeruginosa*

ESBL	% Amino acid identity			
	TEM-4	SHV-2a	VEB-1	PER-1
SHV-2a	63			
VEB-1	19	21		
PER-1	18	20	38	
GES-1	31	30	19	23

A study done by Aggarwal et al, on detection of Extended-spectrum beta-lactamase in *P.aeruginosa* with 148 isolates, 30 (20.27%) were ESBL positive. And the maximum ESBL production was found in isolates of sputum and tracheostomy swabs (28.57%), followed by pus (24.13%), urine (19.04%), cerebrospinal fluid (CSF) and other sterile body fluids (15.38%) and blood (7.14%).[14]

In one of the study by Upadhyay et al, out of 202 isolates, 3.3% ESBL production among MDRPA got documented.[91]

The sensitivity of screening for ESBLs can be increased by use of more than one third generation Cephalosporin for screening. Cefpodoxime and Ceftazidime show the highest sensitivity for ESBL detection.

Types of ESBL genes in *Pseudomonas aeruginosa*

Oxacillinases:

Unlike TEM and SHV enzymes, Oxacillinases belongs to the molecular class D of Ambler's scheme and 2d of functional group under Bush-Jacoby-Medeiros classification.[103] They are so named because of their Oxacillin hydrolyzing abilities. These OXA β-lactamases are characterized by hydrolysis rate for Cloxacillin and Oxacillin greater than 50% that for benzylpenicillin.[92]

OXA type β-lactamases are predominant in *P.aeruginosa* and other gram negative bacteria. OXA type-A β-lactams confer resistance to Ampicillin and Cephalothin and are characterized by their high hydrolytic activity against Oxacillin and Cloxacillin and this fact that they are poorly inhibited by Clavulanic acid. OXA-10 weakly hydrolyzes Cefotaxime, Ceftriaxone and Aztreonam leading to reduced susceptility to these antibiotics. OXA-10 and its derivatives like OXA- 11, 14, 15,16, 17, 19, 18, 28, 31, 35 and 45, differs by their amino acid sequences.[93]

OXA-14 differs from OXA-10 by only one amino acid residue, OXA-11 and OXA- 16 differ by 2 amino acid and OXA-13 and OXA-19 differs by nine amino acids. OXA-10 and its derivatives have one of two amino acid substitutions, an asparagine for serine at position 73 or an aspartate for glycine at position 157, in particular, the Gly 157 Asp substitution may be necessary for high level resistance to ceftazidime, thus either of these mutations may be required to confer ESBL

phenotype on the OXA hypervariant. In contrast to other OXA type, ESBLs which confer resistance to Ceftazidime, the OXA 17 beta-lactamase confers resistance to Cefotaxime and Ceftrioxone but provides marginal protection against Ceftazidime. OXA type ESBLs are not inhibited by clavulanic acid, but OXA 18 & 45 beta-lactamase was reported to be inhibited by this. OXA type ESBLs in *Escherichia coli* provide weak resistance to oxyimino cephalosporins but in *P.aeruginosa* it provides high level of resistance (transconjugate). Several oxacillinases (OXA-2 and OXA-10 derivatives and OXA-18) that have extended substrate profiles, including extended spectrum Cephalosporins, have been reported in *P. aeruginosa.*[94]

***PER* gene:**

The ß-lactams PER-1(*Pseudomonas* extended spectrum betalactamase) was the first ESBL (class A) identified and fully characterized in *P.aeruginosa*, which occurred in 1993[4]. It shares only 18 to 20% amino acid identity with the TEM- and SHV-type ESBLs . It was identified for the first time in a *P.aeruginosa* isolate from a Turkish patient hospitalized in the Paris, France, in 1991.[54]

A subsequent study on the distribution of the *bla*PER-1 gene revealed that it is widespread in Turkey, with PER-1 being identified in up to 46% of *Acinetobacter* strains and 11% of *P.aeruginosa* isolates analyzed in a nation-based survey performed over a 3-month period in1999. PER-1 was identified in up to 38% of Ceftazidime-resistant *P.aeruginosa* isolates, with ribotyping results indicating. The spread of different clones. Since screening for the *bla*PER-1gene has not been performed in *P.aeruginosa* isolates originating from countries neighboring Turkey, such as Syria, Iran, and Iraq, no current data exist on the true prevalence of PER-1in the Middle East. PER occurring less frequently has clinical importance by conferring resistance

to Oxyimino betalactams. Poor outcome as a result of infections caused by PER-1 producers has been reported. It is possible that the spread of PER-1 in Western Europe may be mostly related to the immigration of Turkish nationals. Interestingly, although it has been reported in several members of Enterobacteriaceae including community-acquired pathogens such as *Salmonella* spp., the PER-1 ß-lactamase mostly seems to be expressed by *P. aeruginosa* and *Acinetobacter* sp. isolates in Turkey.[94]

PER-1 was more prevalent in almost all the countries like Italy, Belgium, France, Spain, Romania, Hungary, Serbia, Korea, Japan, China, Europe, etc,. The blaPER-1 gene is found mostly in *P.aeruginosa*, where it resides within a specific transposon, named Tn*1213* by Poirel et al, and Tn*4176* by Mantengoli and Rossolini.[93, 95]

The expression of PER-1 confers clear resistance to oxyimino-β- lactams, especially Ceftazidime, Ceftibuten, and Aztreonam. PER-1 β-lactamase efficiently hydrolyses Penicillins and Cephalosporins and is susceptible to Clavulanic acid inhibition. PER-2 shares 86% homology to PER-1 and has been detected in *Salmonella enterica serovar Typhimurium, E.coli, K.pneumonia, Proteus mirabilis* and *Vibrio cholera 01 Eltor*. It has been found to occur in ~10% of nosocomial *P.aeruginosa* isolates in Turkish hospitals. Many studies evidenced that nosocomial outbreaks of infection with multi-drug resistant strains of *P.aeruginosa* producing PER-1 carries poor therapeutic outcomes. PER-1 shows a broad substrate profile, in that it hydrolyzes Benzylpenicillin, Amoxicillin, Ticarcillin, Cephalothin, Cefoperazone, Cefuroxime, Ceftriaxone, Ceftazidime , and moderately Aztreonam but not Oxacillin, Imipenem or Cephamycins.

A study by Pagani et al, on multifocal detection of MDRPA producing the PER-1 ESBL in Northern Italy reported that the MDRPA clones producing the PER-1 ESBL are endemic in areas of northern Italy.[96]

Another study by Vahaboglu et al, on widespread detection of PER-1 type ESBL among nosocomial *Acinetobacter* and *P. aeruginosa* isolates in Turkey, with a total of 367 *P.aeruginosa* isolates and reported PER-1 type beta-lactamase in 11% (40/367). They found that PER-1 type producers were highly resistant to Ceftazidime and Gentamicin, intermediately resistant to Amikacin, and susceptible or moderately susceptible to Imipenem and Meropenem.[94]

A study by Shacheraghi et al, from Iran, which included 120 strains of *P.aeruginosa* from a burns unit for a period of one year, showed 41 strains of ESBL producers phenotypically. They found blaPER-1 (927bp) in 68.3%(28) of isolates.[97]

Mirsalehian et al, reported the prevalence of ESBLs and antimicrobial susceptibilities of *P. aeruginosa* isolated from burn patients in Tehran, Iran and found that 50(74.62%), 33(49.25%) and 21 (31.34%) strains among 67 ESBL-producing strains amplified blaOXA-10, blaPER-1 and blaVEB-1 respectively.[98]

***VEB-1* gene**

Another unrelated ESBL from *P.aeruginosa*, i.e. the ß-lactamase VEB-1, was originally identified in *Escherichia coli* and *Klebsiella* isolates from a 4-month-old Vietnamese child transferred from Vietnam and hospitalized in France, it was also subsequently found in *P.aeruginosa* strains from Thailand, Kuwait and China. It is a variety of β-lactamases which are plasmid –mediated or integron –associated class A enzymes, recently discovered also confers resistance to non β lactam antibiotics.[54]

It was distantly related to other class A ESBLs . Subsequent isolation of VEB-1 from *P.aeruginosa* strains from two patients hospitalized in France transferred from Thailand was documented.[99]

A study conducted in a university hospital in Thailand revealed that *bla* VEB-like genes were present in up to 93% of the Ceftazidime -resistant isolates, whereas Ceftazidime resistance occurred in 24% of *P.aeruginosa* isolates. The latest development in the analysis of *bla*VEB- 1-likegenes was the isolation of *P.aeruginosa* strains from an intensive care unit of a Kuwait hospital harboring *bla*VEB-like genes, *bla*VEB-1a and *bla*VEB-1b, that differed from the *bla*VEB-1 gene by nucleotide substitutions in the DNA sequence encoding theleader peptide. Unpublished data have also identified VEB-1 in *P.aeruginosa* strains in India and China.[100] It is likely that VEB-type enzymes may be isolated mostly from patients coming from or hospitalized in Asia. VEB -1 has greatest homology with PER1 and PER-2 (38%).[101]

It confers high level resistance to Ceftazidime, Cefotaxime and Aztreonam which is reversed by Clavulanic acid.[102]

SHV gene

A study by Laurent Poirel et al, represented the first description of the bla SHV-5 gene in the Pseudomonas species and it adds to the list of ESBLs identified in *P.aeruginosa.*[103]

The SHV- type of ESBL was found to be more frequent among clinical isolates of Enterobacteriaceae and it refers to sulfhydryl variable and hence named as SHV. This type of ESBL efficiently hydrolyzes Cefotaxime and to a lesser extent Ceftazidime and was identified as SHV-2. SHV-2 differs from SHV-1 by replacement

of glycine by serine at 238 position by point mutation.[103] Few outbreaks of SHV producing Pseudomonas aeruginosa have been reported (SHV-2a) in France and (SHV-12) in Thailand. SHV-5 also has Lysine at 240 instead of Glycine, which is crucial for Cefotaxime and Ceftazidime hydrolytic activity. SHV acts against penicillin and narrow spectrum cephalosporins such as cephalothin and cephaloridine. The ß-lactamase SHV-5 confers a high level of resistance to ceftazidime and to monobactams. While this work was in progress, an outbreak of 11 *P .aeruginosa* isolates producing SHV-5 was reported in Heraklion, by Neonakis in 2003 which is in the vicinity of Athens.

TEM gene:

The TEM type ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was reported in 1965 from an Escherichia coli isolate from a patient in Athens, Greece named Temoneira hence named as TEM. TEM-1 hydrolyzes Ampicillin at a greater rate than Carbencillin, Oxacillin or Cephalothin and has negligible activity against extended spectrum cephalosporins and they are inhibited by Clavulanic acid.[91]

TEM-2 has same hydrolytic profile as TEM-1, but differs from TEM-1 by having single amino acid substitutions. Over 150 TEM type beta lactamaces have been described, of which the majority are ESBLs and others are inhibitor resistant enzymes.

The TEM-type enzymes described in *P.aeruginosa*, namely, TEM-4, TEM-21, TEM-24, and TEM-42, have been reported in rare isolates from France. A French survey indicated that only 10% of ticarcillin-resistant *P.aeruginosa* isolates (1.9% of *P.aeruginosa* isolates) produce a TEM-type ß lactamase, whereas other narrow-spectrum ß-lactamases (OXA and CARB) are more frequently encountered in that

species.[91] GES the other example of non-TEM, non-SHV ESBLs. GES is not closely related to any other plasmid mediated β lactmases.

According to Poriel et al, the single documented outbreak involving GES-2 producing *P.aeruginosa* strains, a total mortality rate of 62.5% (5 of 8 patients) was reported.[103]

A study by Xavier et al, on efflux pumps expression and its association with porin down-regulation and β-lactamase production among *P.aeruginosa* causing bloodstream infection in Brazil, showed highest susceptibility to Aztreonam of 64.4%, whereas for Imipenem and Meropenem susceptibility was 47.5%. The MexXY-OprM and MexAB-OprM efflux systems were overexpressed in 50.8% and 27.1% of isolates respectively. Overexpression of the MexEF-OprN and MexCD-OprJ system are not observed. AmpC beta lactamase was overexpressed in 11.9% of *P.aeruginosa*. In addition, decreased OprD expression was also observed in 69.5% and in 87.1% of the Imipenem non-susceptible *P.aeruginosa* clinical isolates. MBL encoding genes *bla*SPM-1 and *blaI*MP-1 were detected in 23.7% and 1.7% of isolates and *bla* GES-1 was detected in 5.1% and, GES-5 and *bla* CTX-M-2were observed in 1.7%.[104]

Metallo- β-lactamases (MBL):

The Metallo-β-lactamase (MBL) is an enzyme which requires zinc for their catalytic activity. The MBL activity is inhibited by metal chelators, such as EDTA and THIOL compounds. MBL hydrolyze all beta-lactam antibiotics including, Penicillins, Cephalosporins and Carbapenems, with the exception of Aztreonam Monobactam).[12]

The emergence of multidrug resistance among gram-negative bacteria is a notable threat. Clinically relevant species of gram negative bacilli are often resistant to β-lactam antibiotics, including extended spectrum Cephalosporins, but rarely to carbapenems. Carbapenems are often used as last resort antibiotics for treating infections caused by multidrug-resistant gram-negative bacilli, as they are stable and respond only to extended spectrum and AmpC β-lactamases.

However, emergence of acquired carbapenemases, particularly Ambler class B metallo-β-lactamases (MBLs), IMP and VIM, have been increasingly reported in Asia, Europe, Canada and in many geographical locations. Another type (SPM-1) has been reported in South America. Five enzymes have been identified (IMP, VIM, SPM, GIM and SIM types) in various host organisms, the most common ones being found in *Pseudomonas* spp. and *Acinetobacter* spp.

Table 6: Classification of Carbepenems(Metallo-β-Lactamases —MBLs)

Ambler class	Enzyme	Function	Known organisms
A	KPC[1]	Hydrolyzes all β-lactam antibiotics; inhibited by clavulanate	*K pneumoniae,* Enterobacteriaceae
B	MBLs[2] (NDM, IMP, VIM, GIM, SPM)	Hydrolyze all β-lactams except aztreonam; may be inhibited by clavulanate; require zinc for enzymatic activity; inhibited by EDTA	*P aeruginosa, Acinetobacter* spp, Enterobacteriaceae
D	OXA	Oxacillin hydrolyzing; less able to hydrolyze carbapenems	*P aeruginosa, A baumannii,* Enterobacteriaceae

1KPC—Klebsiella pneumoniae carbapenem-resistant

2MBL—Metallo- β-lactamases (MBLs) (e.g., NDM-1, IPM, VIM, and so forth)

Table 7: Classification of class B metallo beta lactamases.[141]

Functional subgroup	Spectrum	Subclass	Examples	Protein binding ligands		Effect of Zn binding
				Zn 1 site	Zn 2 site	
Group 3a	Broad spectrum	B1	Bc II, IMP- I, Ccr A, VIM, GIM, SPM-1	3 Histidine	Asparagine-Cysteine-Histidine	Binding of 2 Zn atoms for optimal hydrolysis
Group 3b	High specifi city for hydrolyzing carbapenems	B2	Cph A, Sfh-1	2 Histidine, 1 asparagine	Asparagine-Cysteine-Histidine	Binding of second Zn atom is inhibitory
Group 3c	High specifi city for hydrolyzing cephalosporins	B3	LI, FEZ-1, Gob-1, CAU-1	3 Histidine	Asparagine-Histidine-Histidine	Binding of 2 Zn atoms for optimal hydrolysis

Gram-negative bacilli can develop resistance to beta-lactam antibiotics by three mechanisms:

1. Alteration of the antimicrobial target receptor molecule in the bacteria.
2. Decreasing the accessibility of the antimicrobial to the target by altering the entry of the antimicrobial into the cell or increasing the removal of the antimicrobial from the cell.
3. Destruction or inactivation of the antimicrobial. The ability of bacteria to produce beta-lactamase enzymes is the major cause of resistance to beta-lactam antibiotics.

These enzymes hydrolyze four-membered beta-lactam rings, converting them into beta-amino acids, that unlike active members within this class of antibiotics, no longer interfere with bacterial cell wall synthesis and growth.

The β-lactamase enzymes fall into four classes on the basis of their sequence homology, or on the basis of their substrate spectrum and responses to inhibitors into a larger number of functional groups. In the Ambler classification, class A, C and D enzymes employ serine as the reactive site to attack the β-lactam bond of penicillins, cephalosporins and carbapenems. These enzymes cleave the amide bond of the β-lactam ring thus inactivating the antibiotic, while class B (metallo-β-lactamases) requiring zinc ions for their activity. Metallo-β-lactamases (MBLs) catalyze the identical (as serine enzymes) chemical reaction, using one or two divalent cations (Zn^{2+}) coordinated to two water molecules as the reactive nucleophiles (Figure 2).

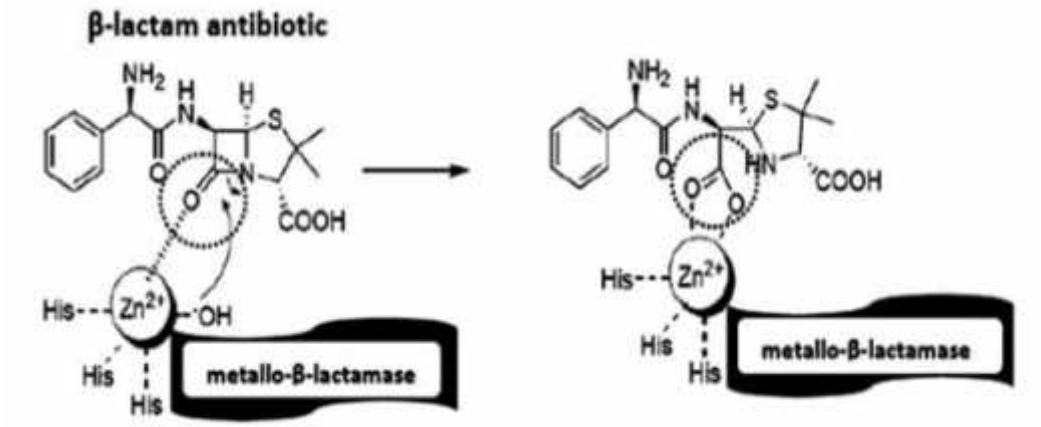


Figure 1: Mechanism of the hydrolysis of β-lactam antibiotics through metallo-β-lactamases[98]

MBLs are produced by bacteria as extracellular or periplasmatic enzymes. All known representatives' posses conserved metal binding sites and require zinc ions as enzymatic cofactors. These enzymes can degrade all of β-lactam antibiotics except

monobactams and are special constant and efficient carbapenemases activity. Moreover, metallo-β-lactamases are not susceptible to therapeutic β-lactamase inhibitors. Their increasing emergence in pathogenic bacterial strains (particularly *P. aeruginosa* and Enterobacteriaceae) due to a rapid dissemination by horizontal gene transfer induced a growing interest in this enzyme family because of the lack of efficient therapies to treat infected patients [11].

Characteristics of Metallo –β-lactamase (MBL):

1. Metallo –β-lactamase requires zinc for their catalytic activity.
2. Their activity is inhibited by Metal chelators, such as EDTA and THIOL compounds.
3. Metallo –β-lactamase hydrolyze all beta-lactam antibiotics including carbapenems, with the exception of aztreonam (monobactam).
4. MBL producing strains are not susceptible to serine beta lactamase inhibitors,(e.g. clavulanate).[137, 138, 139]

Acquired drug resistance is frequent in nosocomial isolates of *P. aeruginosa* and often involves more than one antimicrobial class. Acquired metallo-β lactamases (MBL) have recently emerged as one of the most worrisome resistance mechanisms owing to their capacity to hydrolyze with the exception of aztreonam, all β-lactams including carbapenems and also because their genes are carried on highly mobile elements, allowing easy dissemination. Such strains are not susceptible to therapeutic serine β lactamase inhibitors (such as Clavulanate and Sulfones). [5, 12, 13].

MBL producing isolates are also associated with a higher morbidity and mortality. Moreover, given that MBLs will hydrolyze virtually all classes of β-lactams

and that we are several years away from the development of a safe therapeutic inhibitor; their continued spread would be a clinical disaster.

Metallo-β-lactamases belong to class B, which requires divalent cations of zinc as cofactors for enzyme activity and are therefore characterized by inhibition of metal chelators.[105] They have potent hydrolyzing activity not only against carbapenem but also against other β-lactam antibiotics. Carbapenems, mainly Imipenem, Meropenem and Panipenem (available only in Japan) are potent agent for the treatment of infections due to multidrug resistant *P.aeruginosa*. However the prevalence of carbapenem resistant *P.aeruginosa* has been increasing very recently. The IMP and VIM genes responsible for MBL production are horizontally transferable via plasmids and can rapidly spread to other bacteria. Thus, MBL-producing *P.aeruginosa* strains have been reported to be important causes of nosocomial infections associated with clonal spread.

The level of resistance may be low-level to carbapenems (MIC 8-32mg/L) in case of *P.aeruginosa* and it may also be high- level (MIC >32mg/L). Low level resistance to Imipenem is mostly due to reduced uptake of the drug as a result of down-regulation of the outer membrane protein (OprD), which serves as the primary route of entry for carbapenems or as a result of loss of the porin OprD 66(i.e) lack of 'carbapenem specific porin'. High level resistance to carbapenems (MIC> 32µg/ml) can be due to the presence of metallo- β-lactamase, which exhibit a broad-spectrum β-lactam resistant genes as mobile genetic cassettes with other resistance determinants inserted into class1 or 3 integrons[106] and this mechanism is still uncommon towards *P.aeruginosa.*Resistance to Meropenem but not Imipenem may also arise via over expression of the MexA-MexB-OprM efflux Pump.[107]

Carbapenemases are class B metallo-β-lactamases (MBLs; IMP, VIM) or class D oxacillinases (OXA 23 to OXA 27) (carbapenem-hydrolyzing class D β-lactamases (CHDLs) or class A clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC). Class A β-lactamases with activity against carbapenems are uncommon and divided into 5 groups (GES, IMI, KPC, NMC-A and SME). GES and KPC enzymes have been described in *Pseudomonas aeruginosa* due to loss of OprD outer membrane porin. Genuinely hydrolytic resistance has been confined to infrequent pathogens like *Stenotrophomonas maltophilia, Aeromonas spp., Flavobacterium spp., Leginella gormanii and Bacillus cereus*, which have chromosomal zinc (molecular class B) enzymes and to a small sub-group of *Bacteroides fragilis* isolates that possess the Ccr A zinc β-lactamases.[108]

ESBLs and carbapenemases are typically encoded by plasmid or transposon-borne genes, often on integron, which are genetic elements capable of capturing and subsequently mobilizing resistance genes, although some β-lactamase genes are associated with novel mobile insertion sequences termed ISCR elements. Acquired MBLs includes the VIM and IMP enzymes, of which there are numerous variants of the original VIM-1 and IMP-1 MBLs as well as the SPM-1, GIM-1, NDM-1, AIM-1 and SIM-1 enzymes. The VIM and IMP enzymes are by far the most common MBLs found in carbapenem-resistant bacteria, including carbapenem-resistant *P.aeruginosa*. The predominance of VIM vs IMP in *P.aeruginosa* appears to be geographical, with IMP-type MBLs predominating in Asia where it was first discovered and VIM-type enzymes predominating in Europe though both enzymes are now disseminated globally, with VIM-2 well established. IMP and VIM possess the broadest substrate of hydrolysis range among *Pseudomonas aeruginosa* β- lactamases, including

Penicillins, Cephalosporins, Cephamycin, Oxacephamycins and Carbapenems, but not Monobactams. With the exception of SPM-1, all the clinically relevant MBLs are encoded by gene cassettes, as part of integrons, which inturn are often found as part of complex transposons.

According to study by Zavascki AP et al, infections by MBL-carrying *Pseudomonas aeruginosa* (MBL-PA) resulted higher in-hospital mortality, than those by non-MBL *Pseudomonas aeruginosa* (51.2% versus 32.1%, respectively. And their higher mortality rates 17.3 per 1000 versus 11.8 per 1000 patient-days, respectively.[108]

Currently, no standardized method for MBL detection has been proposed. Several nonmolecular techniques have been studied, all taking advantage of the zinc dependence of the enzymes, by using chelating agents such as EDTA or 2-mercaptopropionic acid to inhibit their activities.

Types of MBLs:

IMP gene:

IMP (Imipenemase) was the first mobile MBL discovered from *P.aeruginosa* strain GN17203 in Japan during 1988, with a high MIC on Imipenem of 50µg/ml and also resistant to extended spectrum Cephalosporins with Imipenem MIC of 50µg/ml. The resistance allele was found on a transferable conjugative plasmid that could be readily mobilized to other *Pseudomonas* strains. Following that, three years later, an identical gene was found in *Serratia marcescens* Tn9106 from an UTI patient at Aichi hospital in Okazaki, Japan. And it was found within a class 3 integron adjacent to a aac(6')Ib-like gene on a plasmid (120kb).[109]

13 IMP variants differing in sequence by 0.4-22.2% were studied and their genetic and kinetic analysis showed that substitution of glycine for serine at position 196 caused a reduction in activity against Penicillin. Discovery of IMP-10 was a result of study of IMP-producing isolates of *Pseudomonas aeruginosa* and *Alcaligenes* spp. collected from 1995 to 2001. The position of the changed amino acid found in both IMP-3 and IMP-6 corresponds to the same amino acid (glycine) in other innate MBLs such as BCII, leading to the hypothesis that IMP-3 may actually be the progenitor of IMP-1 rather than just being a variant of IMP-1.

VIM* gene*:

Second dominant group of acquired MBL is VIM (Verona Integron encoded Metallo β-lactamases) (Veronese Imipenemase) type enzyme, which was first described in Verona, Italy from a *Pseudomonas aeruginosa* isolate in 1997 which was resistant to Piperacillin, Ceftazidime, Imipenem and Aztreonam, with MIC of Imipenem >128μg/ml. VIM-1 is distantly related to other Metallo enzymes and also found that, it was closely related to BCII from *Bacillus cereus* sharing about 39% amino acid identity.The *bla*VIM-1 gene was integrated as a gene cassette into a class 1 integron. This integron also carries an integrase gene typical of class1 integrons in addition to the *bla*VIM-1 gene cassette; it has an *aac* A4 gene cassette which encodes resistance to aminoglycosides. In *P.aeruginosa*, this integron of *bla*VIM-1 was probably located on the chromosome. [110]

Similarly VIM-1 was also detected from *Pseudomonas putida* from Italy, Greece and France recently. Among several types of MBL enzymes identified, VIM type enzymes appears to be most prevalent.[111] The blaVIM-2 was first identified in southern France from a *P.aeruginosa* isolated from blood culture of a neutropenic

patients and the isolate was found to be resistant to most of the β-lactams, including Ceftazidime, Cefepime and Imipenem but remained susceptible to Aztreonam. Similarly, VIM-2 producing *P.aeruginosa* was also isolated from Italy and Greece during the same period. The most common MBL identified worldwide is VIM-2.[112]

VIM-2 has got 90% amino acid identity with VIM-1 and was encoded by a gene cassette, the only resistance gene identified in the blaVIM-2 is positive class 1 integron.[113]

More recently, a novel variant of the VIM series, VIM-3 has been also identified in *P.aeruginosa* in Taiwan. VIM-2 has been reported from nearly 23 countries and the first characterization of an MBL (blaVIM-2) from India was reported from Chennai in 2003 from a bronchoalveolar lavage fluid of a 60yrs old man with VAP studied by Shanthi et al, and this strain showed genetic structure similar to those of integrons from the United States and Russia.

Study by Wang et al, from China, with 93 *Pseudomonas aeruginosa* strains showed that only 14 strains produced carbapnemases, 2 strains produced MBL by 2-mercaptopropanoic acid inhibition assays, 13 strains produced AmpC and 2 strains were AmpC and ESBL positive. The 2 MBLs were amplified by PCR with VIM 2 specifiC. [114]

A Study on molecular characterization of Imipenem resistant *P. aeruginosa* in Hiroshima, Japan from six hospitals by Dhara et al, with 1,058 strains of *P. aeruginosa* strains showed, 100 strains resistant to Imipenem (9.5%). Of the 100 strains, 14 (14%) were MBL positive by double disc synergy test using Sodium mercaptoacetic acid disk and 18 (18%) were bla (IMP-1) or bla(VIM-2) allele positive

by PCR and 32(32%) were multidrug resistant strains. Of 33, 13 were positive for MBL gene. 51 (51%) among 100 Imipenem resistant strains had elevated RND efflux pump activity against Levofloxacin. [60]

According to Dong et al, among 146 strains of Multi drug resistant *Pseudomonas aeruginosa*, 46(31.5%) were positive for the MBL genotypes; 38 (82.6%) carried the blaIMP gene, 8(17.4%) carried the blaVIM gene, and 114(78.1%) were oprD2 negative 74. A study done by Joseph *et al.* from Puducherry, India, on Ventilator Associated Pneumonia and the role of MDR pathogen, showed 37 (78.7%) of the 47 VAP pathogen were multidrug resistant. Among these, 20% were MBL producing *Pseudomonas aeruginosa.*[36]

A study done by Pitout et al, on detection of *Pseudomonas aeruginosa* producing metallo-betalactamases in a large centralized laboratory from Canada using 241 clinical strains of imipenemnonsuseptible *Pseudomonas aeruginosa* between 2002 to 2004, 110/241 (46%) were MBL positive using phenotypic methods. Among 241 non-susceptible *Pseudomonas aeruginosa* strains, 110/241 (46%) were PCR positive for MBL genes, 103/241 (43%) for *bla*VIM and 4/241(2%) for *bla*IMP.[115]

And using EDTA disk screen test for meropenem showed 100% sensitivity and 97% specificity for detecting MBLs in control and clinical strains.

A study by Quinines-Falconi et al, on Emergence of *Pseudomonas aeruginosa* strains producing metallo-beta-lactamases of VIM-2 types, from 86 carbapenem non-susceptible *P.aeruginosa* isolates collected in the National Institute of Respiratory Disease of Mexico City, showed presence of VIM-2 in two clonally related isolates.[116]

SPM-1

A novel gene *bla SPM-1* was isolated from Sao Paulo, Brazil in1997.[142] On comparison with the sequence of other MBLs, maximum identity was seen with IMP1 enzyme.[142] The genetic context of bla-SPM-1 is unique in that it is immediately associated with common region elements and not with transposons or integrons.[143] Like IMP-1 and VIM-1, SPM-1does not hydrolyze clavulanic acid and aztreonam.[144]

GIM-1

In 2002, five *P. areuginosa* isolates were recovered from different patients in Germany, which were shown to possess a novel class B beta lactamase designated, as GIM-1.[145] The isolates were susceptible to polymyxin only. The amino acid sequence of GIM-1 displayed maximum identity with IMP-6, IMP-1 and IMP-4 isolates. Like majority of *MBL* genes, bla GIM-1 was also found on class 1 integron that is carried on a small plasmid of 45 kb. [146]

SIM-1

In a tertiary care hospital in Seoul (Korea), 17% of the carbapenem resistant *P. aeruginosa* and *Acinetobacter* spp. were found positive for metallo beta lactamases. Out of these 96% had either the bla IMP-1 or bla VIM-2 allele. Seven *Acinetobacter baumanii* isolates were found to have a novel *MBL* gene, which was designated as bla SIM-1.[147] This belongs to subclass B1 and exhibits 64 – 69% identity with the IMP type MBL's. All SIM-1 producing isolates exhibited low imipenem and meropenem MICs and had a multi drug resistant phenotype. Expression of the cloned gene revealed that the enzyme is capable of hydrolyzing a broad range of beta lactams including penicillins, narrow to expanded spectrum cephalosporins and carbapenems.[148] The gene for bla SIM-1 is carried on a gene cassette inserted into a

class 1 integron. The pulse field gel electrophoresis of SmaI digested genomic DNA showed that the strains belonged to two different clonal lineages, indicating horizontal transfer of this gene and suggesting the possibility of further spread of resistance in the future.[148]

AIM-1

AIM-1 constitutes the first member of a new subgroup of MBLs. This has been reported from a male original immunocompromised patient having *P. aeruginosa* infection from Australia. The *MBL* structural gene was 915 bp long encoding a protein of 305 AA's. The protein displayed a typical MBL active site HXHXD and most identity to B3 lineage MBL's and a very low identity to the other clinically relevant MBLs i.e., IMP, VIM, SPM-1, and GIM-1. The prescence of ISCR 10 adjacent to bla AIM-1 indicates that like bla SPM-1, ISCR elements are implicated in its mobility.[149]

NDM-1

Metallo-beta-lactamase-1 (NDM-1)[150] is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics. These include the antibiotics of the carbapenem family, which are a mainstay for the treatment of antibiotic-resistant bacterial infections. The gene for NDM-1 is one member of a large gene family that encodes beta-lactamase enzymes called carbapenemases.

NDM-1 was first detected in a *Klebsiella pneumoniae* isolate from a Swedish patient of Indian origin in 2008. It was later detected in bacteria in India, Pakistan, the United Kingdom, the United States, Canada, Japan and Brazil.[150]

Gene for NDM-1 can spread from one strain of bacteria to another by horizontal gene transfer. *bla*$_{\text{NDM-1}}$ gene produces NDM-1, which is a carbapenemase beta-lactamase - an enzyme that hydrolyzes and inactivates these carbapenem antibiotics. The NDM-1 enzyme is one of the class B metallo-beta-lactamase; other types of carbapenemase are class A or class D beta-lactamases.[155]

The NDM-1 enzyme was named after New Delhi, the capital city of India, as it was first described by Yong et al. in December 2009 in a Swedish national who fell ill with an antibiotic-resistant bacterial infection that he acquired in India.[156] The infection was unsuccessfully treated in a New Delhi hospital, and, after the patient's repatriation to Sweden, a carbapenem-resistant *Klebsiella pneumoniae* strain bearing the novel gene was identified. The authors concluded that the new resistance mechanism "clearly arose in India, but there are few data arising from India to suggest how widespread it is".[156] Its exact geographical origin, however, has not been conclusively verified. In March 2010, a study in a hospital in Mumbai found that most carbapenem-resistant bacteria isolated from patients carried the *bla*$_{\text{NDM-1}}$ gene.[157]

In May 2010, a case of infection with *E. coli* expressing NDM-1 was reported in Coventry in the United Kingdom.[158] The patient was a man of Indian origin who had visited India 18 months previously, where he had undergone dialysis. In initial assays the bacterium was fully resistant to all antibiotics tested, while later tests found that it was susceptible to tigecycline and colistin. The authors warned that international travel and patients' use of multiple countries' healthcare systems could lead to the "rapid spread of NDM-1 with potentially serious consequences".

In July 2010, a team in New Delhi reported a cluster of three cases of *Acinetobacter baumannii* bearing $bla_{\text{NDM-1}}$ that were found in the intensive care unit of a hospital in Chennai, India, in April 2010.[159]

A study by a multi-national team was published in the August 2010 issue of the journal *The Lancet Infectious Diseases*. This examined the emergence and spread of bacteria carrying the $bla_{\text{NDM-1}}$ gene. This reported on 37 cases in the United Kingdom, 44 isolates with NDM-1in Chennai, 26 in Haryana, and 73 in various other sites in Pakistan and India.[150] The authors' analysis of the strains showed that many carried $bla_{\text{NDM-1}}$ on plasmids, which will allow the gene to be readily transferred between different strains of bacteria by horizontal gene transfer. All the isolates were resistant to multiple different classes of antibiotics, including beta-lactam antibiotics, fluoroquinolones, and aminoglycosides, but most were still susceptible to the polymyxin antibiotic colistin.

On 21 August 2010, Ontario, Canada, had its first confirmed case of the "superbug" in Brampton. There were other confirmed cases in British Columbia and Alberta.[162]

In August 2010, a chemical compound GSK 299423 was found to significantly fight against antibiotic-resistant bacteria by making such bacteria unable to reproduce, citing a likely treatment to the NDM-1 strain. [162, 163]

On 6th September 2010, Japan detected its first ever case of the NDM-1 enzyme. In May 2009, a Japanese man in his 50s who had recently returned from vacation in India was struck with a fever and hospitalized, later making a full

recovery. Hospital officials confirmed that tests carried out after the patient's recoveries were positive for the NDM-1 enzyme.[163]

An environmental point prevalence study conducted between 26 September and 10 October 2010 found bacteria with the NDM-1 gene in drinking water and seepage samples in New Delhi. 50 tap water samples and 171 seepage samples were collected from sites within 12 km of central New Delhi. Of these samples, 20 strains of bacteria were found to contain NDM-1 gene in 51 out of 171 seepage samples and 2 out of 50 tap water samples.[166]

On 18 May 2012, the presence of NDM was found in a patient who died at Royal Alexandra Hospital in Edmonton, Alberta. The patient was also found to be carrying a strain of bacteria known as acinetobacter. The patient had surgery on the Indian subcontinent, traveled to Canada and was admitted to hospital with an infection.

In August 2010, the first reported death due to bacteria expressing the NDM-1 enzyme was recorded after a Belgian man, who had become infected while being treated in a hospital in Pakistan, died despite being administered colistin. A doctor involved in his treatment said: "He was involved in a car accident during a trip to Pakistan. He was hospitalised with a major leg injury and then repatriated to Belgium, but he was already infected".[166]

World scenario of ESBL producing *P.aeruginosa*:

There are a number of studies across the world done to know the prevalence of ESBL and MBL producing *P.aeruginosa*. National surveys have indicated the

presence of ESBLs to be 5-8% in Japan, Korea, Malaysia and Singapore and 12-24% from Thailand, Philippines and Indonesia.[179] Countries which showed high prevalence of *P.aeruginosa* producing ESBL were United states [177], Turkey [176], Portugal [175], Africa [186], China [178], France [189] and Nepal [172] with 75%, 58%,34%, 30.1%, 25-40%, 25%, 24.27% respectively. Incidence of ESBL producing *P.aeruginosa* in other countries were, South-West Nigeria [180],Hongkong [190],Thaiwan [173],Iran [182, 186],America [185],Kuwait [187],Brazil [169, 171], United Kingdom [184] with 22.2%, 12%, 8.5%, 8.1%-9.2%, 7.7%, 6.5%, 4%-21% and 4.2% respectively. Some countries showed the lowest prevalence like Malaysia [183],Sweden [175],Belgium [181] and Netherland [188] with 3.7%, 3%, 2.74% and <1% respectively.

The prevalence varies in different countries may be due to the population size or difference in sample size studied or due to their differences in hygienic practices.

Indian scenario of ESBL producing *P.aeruginosa*:

In India a number of studies have been done in various places to know the prevalence of *P.aeruginosa* producing ESBL. The overall prevalence rate in different institutions varies from 28 to 84%.[191] The places which showed the highest prevalence were New Delhi [194, 195, 196, 206],Nagpur [193], Coimbatore [192],Chandigard [200] ,Mysore [123],West Bengal [125],Davangere [207,]Haryana [14],Tamil Nadu [128],Pondicherry [197] and Chennai [204] with 30%-68.78 %, 50%, 40%, 29.7%, 27.2%, 20.5%,20.27%, 20.27%,20% and 20% respectively. Places showing lower prevalence included like Hyderabad [205], Kanchipurum [202], Eastern Indian states [201], Mumbai [203] and Varanasi [199] with 19.8%, 16%, 15%, 9%, 5.9% and 3.3% respectively.

World scenario of MBL producing *P.aeruginosa*:

There are a number of studies across the world done to know the prevalence of MBL and MBL producing *P.aeruginosa*. Different countries showed different prevalence like Brazil [169, 171, 210], Tehran [153], Beijing, Iran [211], Canada [208], Bangladesh [212] and America [151] with 37-79%, 94.2%, 66.1%, 53%, 46%, 43% and 40% respectively. Some countries showed low prevalence like Italy [213], Japan [214], Africa [219], Scotland [209], Korea and China [154] with 20%, 20%, 13.7%, 13%, 11.4% and 9.1% respectively.

The prevalence varies in different countries may be due to the population size or difference in sample size studied or due to their differences in hygienic practices.

Indian scenario of MBL producing *P.aeruginosa*:

In India a number of studies have been done in various places to know the prevalence of *P.aeruginosa* producing MBL. The prevalence of *P. aeruginosa* which are MBL producers in different places included Loni [174], Varanasi [199], New Delhi [217], Vellore [129], Manipal [117], Mumbai [3, 130], Pondicherry [167, 121] with 66.7%, 46.6%, 36%, 32%, 30%, 20-32%,19%-74.5% respectively. Some places in India showed low prevalence like Chennai [165], Bangalore [112, 128], Kashmir [216], Puducherry [28, 164], Ahmedabad [218], Nagpur [161, 171] and Davangere[207] with 14%, 12-27.7%, 11.66%, 10.9%, 9.09%, 8% and 4.8% respectively.

The prevalence differs in different places due to the difference in population size or difference in sample size studied or due to their differences in hygienic practices.

Risk factors for acquring ESBL and/ MBL producing organisms. [219]

There are a number of facters which attribute for the spread of ESBL and / MBL producing organisms. Important of them are as follows:

• Recent patient in ICU / NICU (ET tubes, central lines)

• Immunocompromised

• Post transplant

• Premature babies

• Frequent / long term antibiotic therapy

• Indwelling urinary catheters

• Surgical procedures

Steps to be taken to prevent of ESBL and or MBL in hospitals: [219]

1) Handwashing: This is the single most important way to help prevent the spread of germs. Healthcare workers washing their hands with soap and water or use an alcohol-based hand cleanser before and after treating each patient will help to prevent spread of ESBL and / MBL by a large percentage.

2) Protective clothing: Wearing of gloves and gown when entering the room of a patient with ESBL by healthcare workers and visitors.

3) Private rooms: Patients with ESBL infection are to placed in private rooms or in a room with another patient who also has ESBL.

4) Personal care items: Patients with ESBL must have their own patient care items, such as thermometers and stethoscopes. If these items are shared, they should be fully cleaned and disinfected before reuse.

METHODOLOGY

The present study was conducted at the Department of Microbiology, J. N. Medical College, Belgaum.

Source of data:

Pus samples from hospitalized patients of K.L.E.'S DR. Prabhakar Kore Hospital and MRC, Belgaum, received at Microbiology department.

Sample Size:

The prevalence of *P.aeruginosa* in our hospital (K.L.E.'S DR. Prabhakar Kore Hospital and MRC, Belgaum) in 3 years is 7% of all pus samples.

Formula: $n = 4pq / d^2$

Where, n = sample size

p = prevalence = 7%

q = 100-p = 100-7 = 93

d = error = 1.5

After substitution of the above values, n = 1157~ 1200.

So, 1200 pus samples has to be screened.

Inclusion Criteria:

All the *P.aeruginosa* isolated from pus samples in hospitalized patients of K.L.E.'S DR. Prabhakar Kore Hospital and MRC, Belgaum, received at Microbiology department.

Exclusion Criteria:

All the organisms other than *P.aeruginosa* isolated from pus samples in hospitalized patients of K.L.E.'S DR. Prabhakar Kore Hospital and MRC, Belgaum, received at Microbiology department.

Criteria for deciding an organism as ESBL producers:

1. Zone diameter of various third generation cephalosporin,

   Cefotaxime 30mcg = <27mm or
   Ceftazidime 30mcg = <22mm or

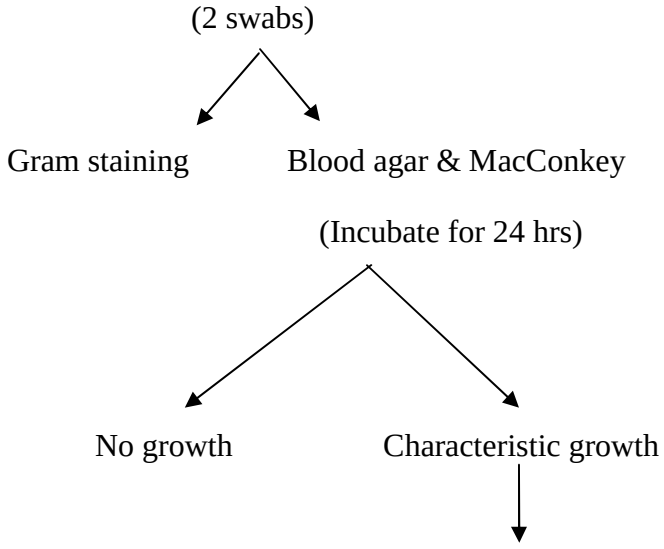
   Ceftriazone 30mcg = <25mm

   Increase in zone size with addition of an inhibitor (ESBL) by >5mm.

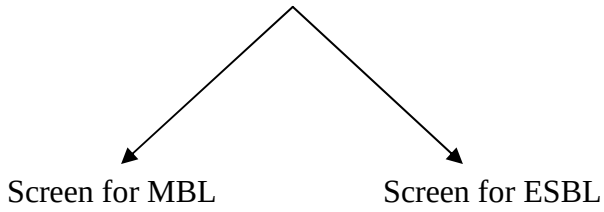
Criteria for deciding an organism as MBL producers:

1. Resistance to Imipenem or Meropenem. or
2. Resistance to all beta-lactam drugs including third generation Cephalosporins and Azetreonam.

Pus samples of hospitalized patients received at microbiology laboratory under aseptic precaution



Identification of *P. aeruginosa* by biochemical reactions and antibiotic sensitivity



Quality controls

For MBL tests[117]:

- Positive control-*P.aeruginosa* ATCC 68549
- Negative control-*P.aeruginosa* ATCC 27853

For ESBL tests[118]:

- Positive control- *Klebsiella pneumoniae* ATCC 700603.
- Negative control- *E.coli* ATCC 25922

Identification:

Isolates of *Pseudomonas aeruginosa* from pus samples were identified on the basis of colony characters on 5% sheep blood agar, MacConkey agar.

Further identification were done by standard biochemical reactions like,

1. Oxidase,
2. Catalase,
3. Motility,
4. Growth at 42˚C,
5. Oxidative/Fermentative medium (Glucose, Maltose, Lactose),
6. Nitrate reduction test,

7. MR/VP,
8. Arginine dihydrolase,
9. Mannitol Motility Medium,
10. Triple Sugar Iron agar,
11. Indole production,
12. Urea hydrolysis,
13. Citrate utilization,

Common initial steps:

1. 4-5 colonies of the test strain were touched with a straight wire and transferred to 1ml of normal saline to match turbidity to 0.5 McFarland standard.
2. Using this inoculum, lawn culture was made on cation balanced Muller Hinton Agar (MHA) plate with a sterile cotton swab.
3. Excess broth was expressed by rotating the swab against the inner side of the suspension tube.

EDTA solution preparation:

A 0.5 M Ethylenediamine tetraacetic acid (EDTA) solution was prepared by dissolving 186.1 g of disodium EDTA.$2H_2O$ in 1000 ml of distilled water and adjusted it to pH of 8.0 by Sodium hydroxide (NaOH). The mixture was sterilized by autoclaving. 10µl of 0.5 M EDTA solution was used each time. Stored in refrigerator at 4^0C or -20^0C in airtight vials without significant loss of activity for atleast 16 weeks.[120]

ESBL screening tests

1. Disc diffusion test:[10]

This test requires use of Ceftazidime (30mcg) alone and in combination with lCavulanic acid (10mcg). Ceftazidime & with Ceftazidime/Clavulanic acid (30mcg/10mcg) discs were placed with distance between the two discs 10mm edge to edge on MHA plate inoculated with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture and was incubated overnight at 37^{o}C.

An increase in the zone diameter by > 5mm of Ceftazidime versus its zone when tested in combination with Clavulanic acid is considered as an ESBL producer.

2. Double Disc synergy test:[14]

30mcg disc of each third generation cephalosporin antibiotics: Cefotaxime, Ceftriazone and Ceftazidime, are placed on MHA plate inoculated with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture at distance of 15mm center to center from Augmentin disc (Amoxicillin/Clavulanic acid-20mcg/10mcg) and was incubated overnight at 37^{o}C.

Increase in the inhibition zone of any one of the three third generation antibiotic disc towards augment disc is considered as an ESBL producer.

MBL screening tests

1. Imipenem (IMP) - EDTA combined disc test:[13]

MHA plate is prepared by inoculating with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture. Two 10mcg Imipenem discs are placed on the plate. 10µl of 0.5M EDTA solution is added to one of the Imipenem discs to obtain a concentration of 750mcg and was incubated overnight at 37^{o}C.

If increase in inhibition zone with IMP and EDTA disc was >7mm than IMP disc alone, it is considered as MBL producer.

2. Imipenem (IMP) - EDTA double disc synergy test:[13]

MHA plate is prepared by inoculating with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture. An IMP (10mcg) disc is placed 20mm center to center (10mm edge to edge) from a blank disc(6mm diameter, Whatmann No.2) containing 10mcl of 0.5 M EDTA(750mcg) and was incubated overnight at 37^{o}C.

Enhancement of zone of inhibition in area between IMP and EDTA disc in comparision with zone of inhibition on far side of the drug was considered as MBL producer.

3. EDTA disc potentiation using ceftazidime, ceftizoxime and cefotaxime:[13]

MHA plate is prepared by inoculating with standard inoculum (0.5 McFarland) of the test organism to form a lawn culture. A filter paper (6mm diameter, Whatmann No.2) was placed and the above four antibiotic discs were placed 25mm center to center from blank disc.10mcl of 0.5 M EDTA (750mcg) solution was added to the blank disc and was incubated overnight at 37^{o}C.

Enhancement of zone of inhibition in area between EDTA disc and any one of the four cephalosporin disc in comparison with zone of inhibition on far side of the drug was interpreted as an MBL producer.

4. Modified Hodge test.[121]

MHA plate is prepared by inoculating with standard inoculum (0.5 McFarland) of the E.coli ATCC 25922 to form a lawn culture. The test organism is

then streaked across the plate to form a plus sign and IMP disc (10mcg) is placed at the center of the plate and was incubated overnight at 37^{o}C.

Cloverleaf shaped inhibition is considered as a carbapenemases producer.

PHOTOGRAPHS

Photo 1: Hucker's modification of Gram staining of *P.aeruginosa*

Gram negative, uniformly stained, straight or slightly curved rods, measuring 0.5 to 1.0µm by 1.5 to 5.0µm in length, non-capsulated, non-spore forming.

Photo 2: Blood agar plate showing irregular grayish white colonies of *P.aeruginosa*

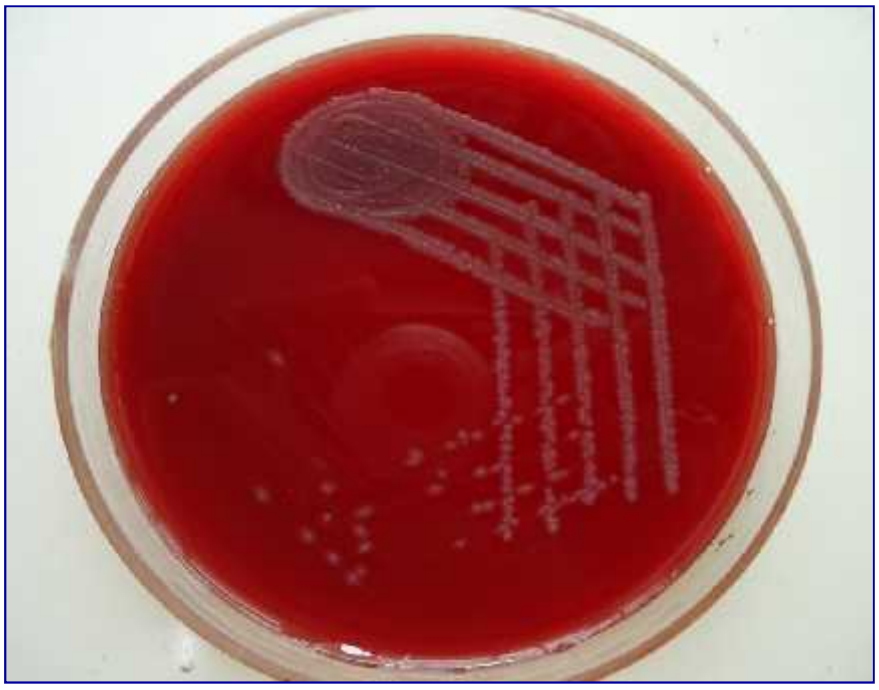
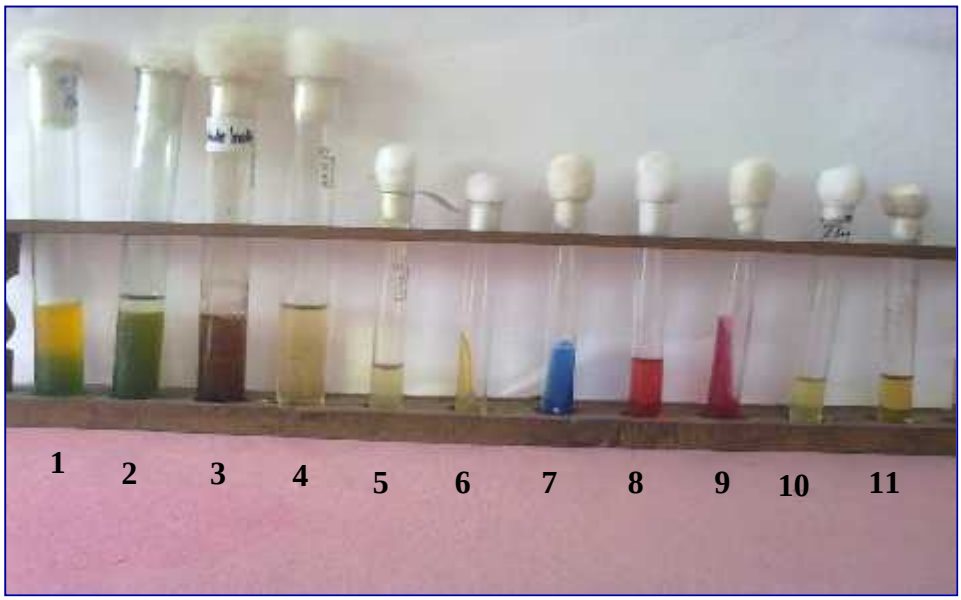


Photo 3: MacConkey agar plate showing NLF colonies with metallic sheen of *P.aeruginosa*.

Photo 4: Nutrient agar plate showing green pigment production by *P.aeruginosa*

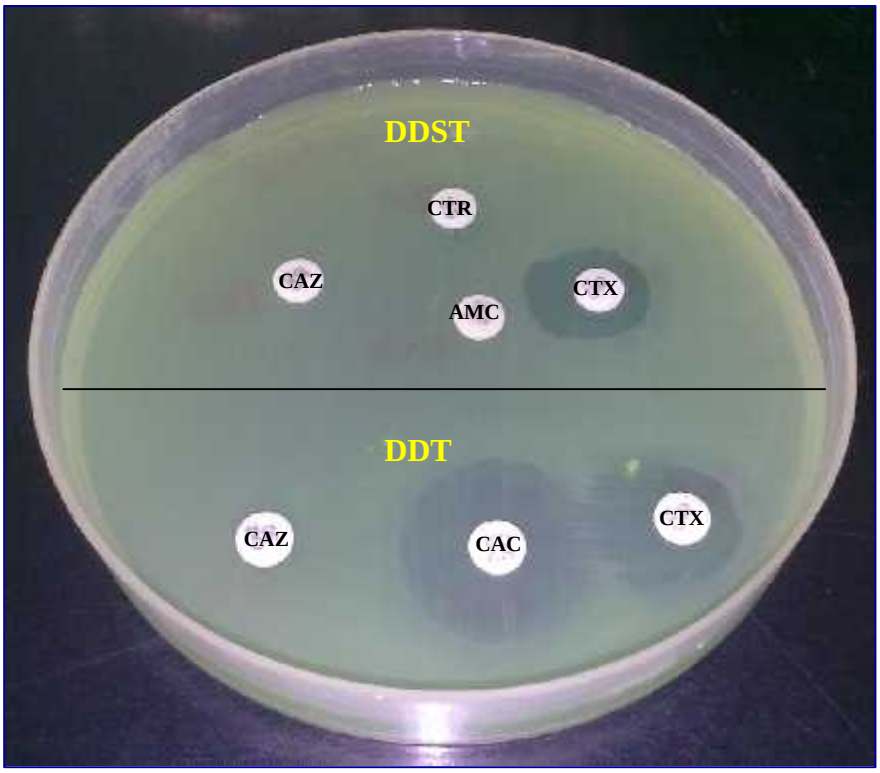
Photo 5: Biochemical tests for the identification of *P.aeruginosa*



1.OF-Glu-oxidative
2.OF-Glu- not fermented
3.Nitrate reduced to nitrites
4.Glucose not fermented
5.Peptone water with Indole and H2S paper-both not produced
6.Urease not produced
7.Citrate utilized
8.Mannitol not fermented and motile
9.TSI-K/NC
10.MR negative
11.VP negative

1. LD not decarboxylated
2. OD not decarboxylated
3. AD dehydrolysed
4. D-Base

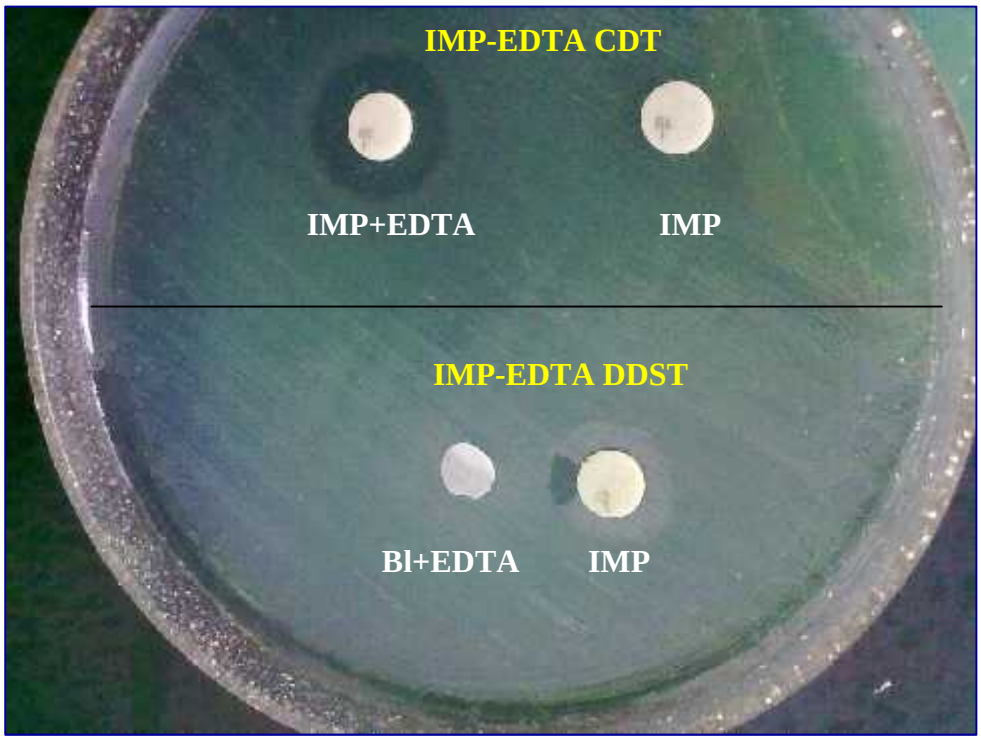
Photo 6: Extended-spectrum-β-lactamase (ESBL) producing *P.aeruginosa* detection by Double disk synergy test (DDST)& Disc diffusion test (DDT):



Interpretation of DDST : Increase in inhibitory zone around CTX antibiotic disc towards Augmentin disc **.**

Interpretation of DDT: Increase in zone diameter of Ceftazidime by >3mm.

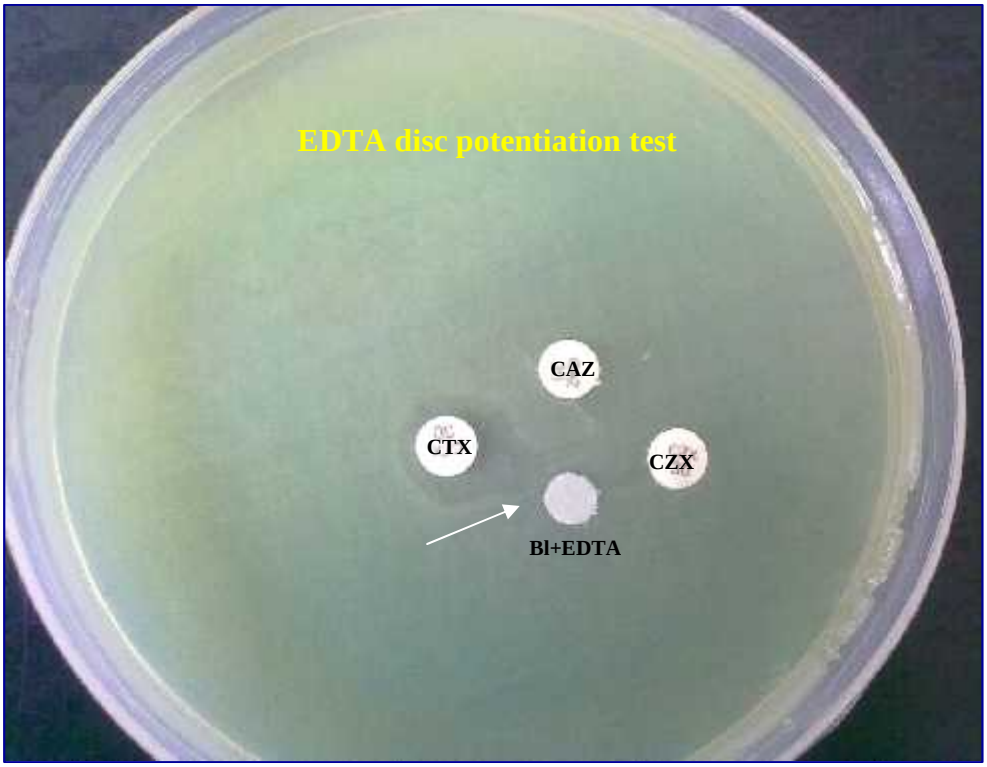
Photo 7: Metallo-β-lactamase (MBL) producing *P.aeruginosa* detection by Imipenem (IMP)-EDTA combined disc test (IMP-EDTA CDT) and Imipenem(IMP)-EDTA double disc synergy test (DDST).



Interpretation of IMP-EDTA DDST: Increase in inhibition zone with IMP-EDTA combined disc >7mm than IMP disc alone.

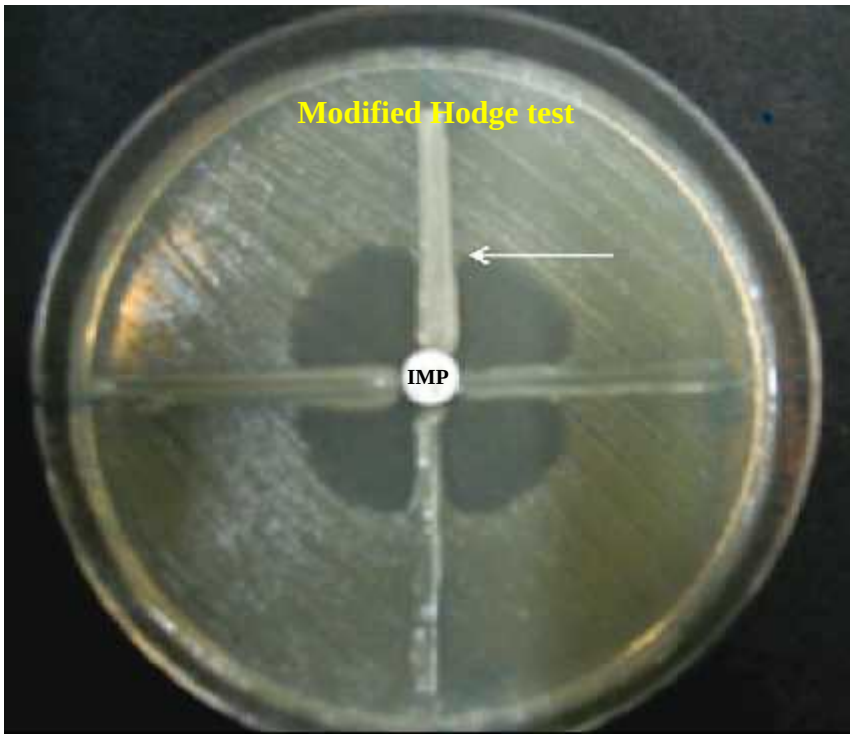
Interpretation of IMP-EDTA CDT: Enhancement of zone of inhibition of IMP in area between IMP and blank disc+EDTA disc in comparison with zone of inhibition on far side of IMP drug.

Photo 8: Metallo-β-lactamase (MBL) producing *P.aeruginosa* detection by EDTA disc potentiation using Ceftazidime, Ceftizoxime, and Cefotaxime.



Interpretation of EDTA disc potentiation test : Enhancement of zone of inhibition in area between blank disc+EDTA disc and CTX in comparison with zone of inhibition on far side of drug.

Photo 9: Metallo-β-lactamase (ESBL) producing *P.aeruginosa* detection by Modified Hodge test.



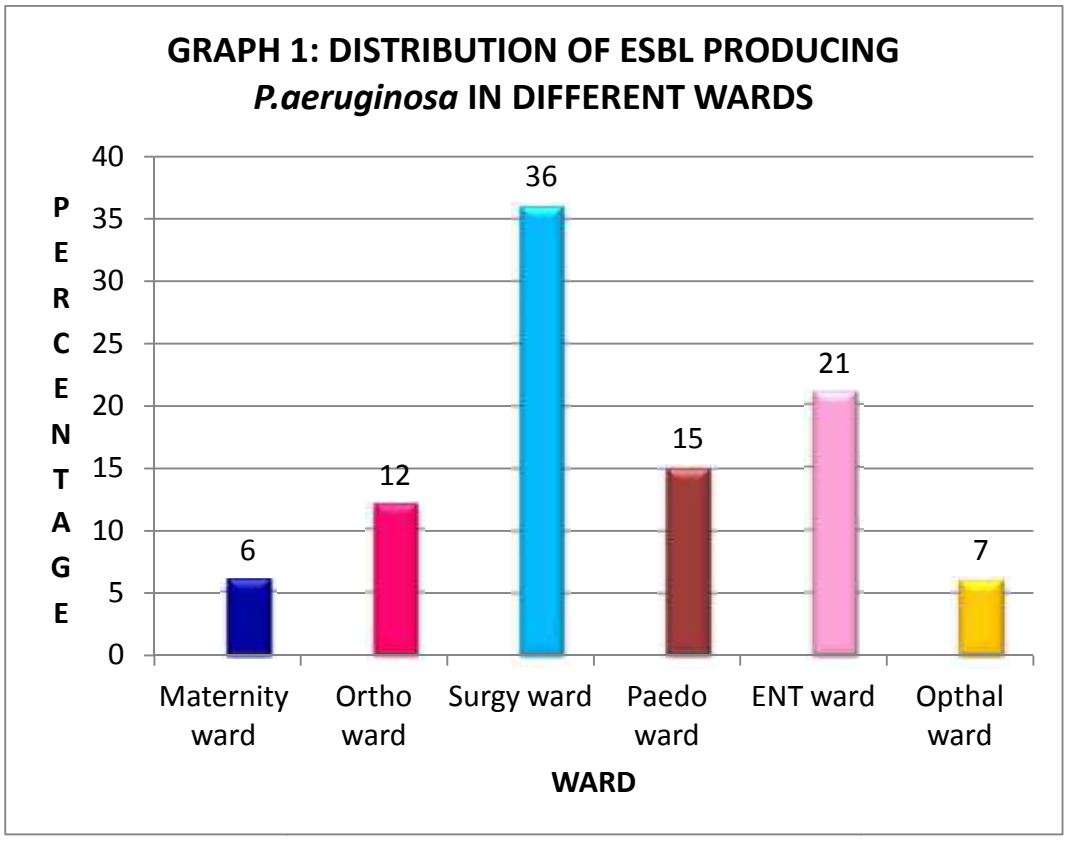
Interpretation of Modified Hodge Test : Presence of cloverleaf shaped zone of inhibition around the disc

RESULTS

A total of 90 *P.aeruginosa* isolated from 1200 pus samples received at Microbiology laboratory from January 2011 - December 2011were include in the study.

TABLE 8: DISTRIBUTION OF ESBL PRODUCING *P.aeruginosa* IN DIFFERENT WARDS:

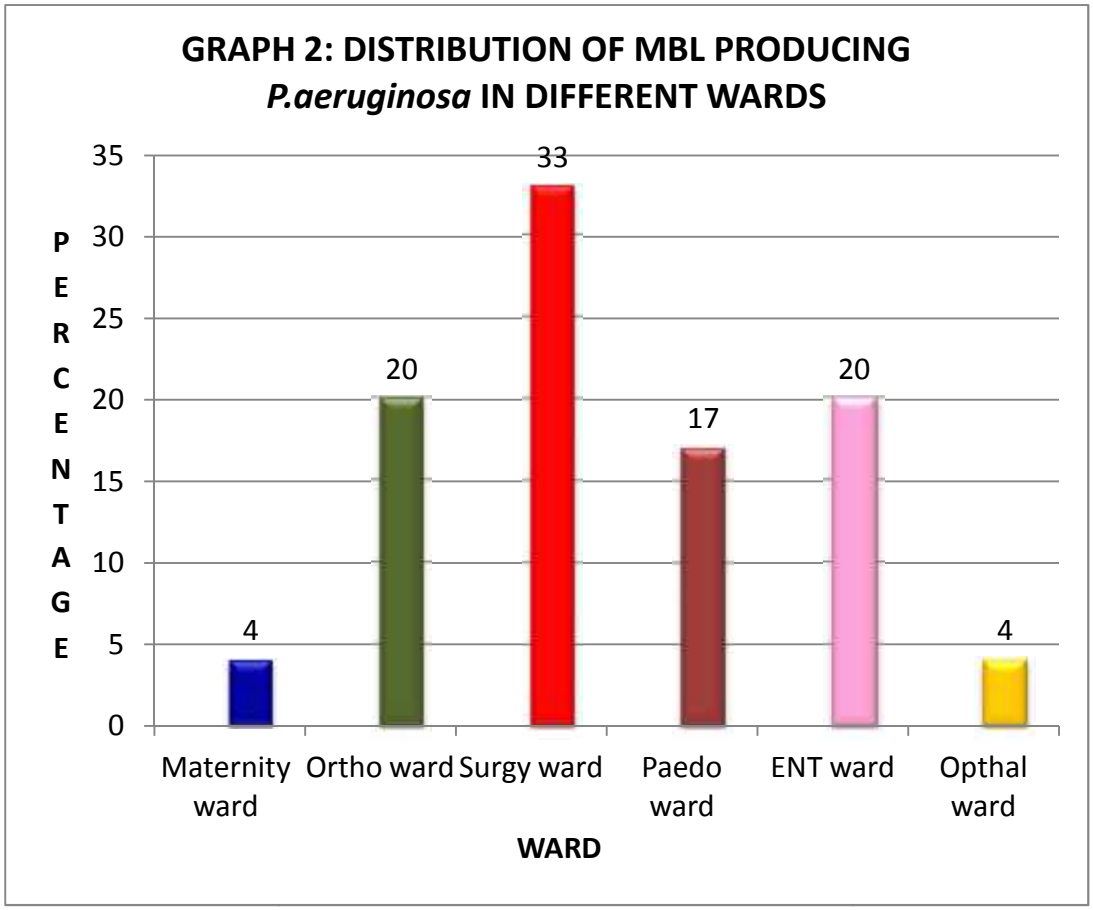
Total ESBL producers	Maternity ward	Ortho ward	Surgy ward	Paedo ward	ENT ward	Opthal ward
33	2(6%)	4(13%)	12(36%)	5(15%)	7(21%)	3(7%)



Of the 33 Ceftazimide resistant *P.aeruginosa*, maximum number of isolates were from Surgery ward with 36% of cases.

TABLE 9: DISTRIBUTION OF MBL PRODUCING *P.aeruginosa* IN DIFFERENT WARDS:

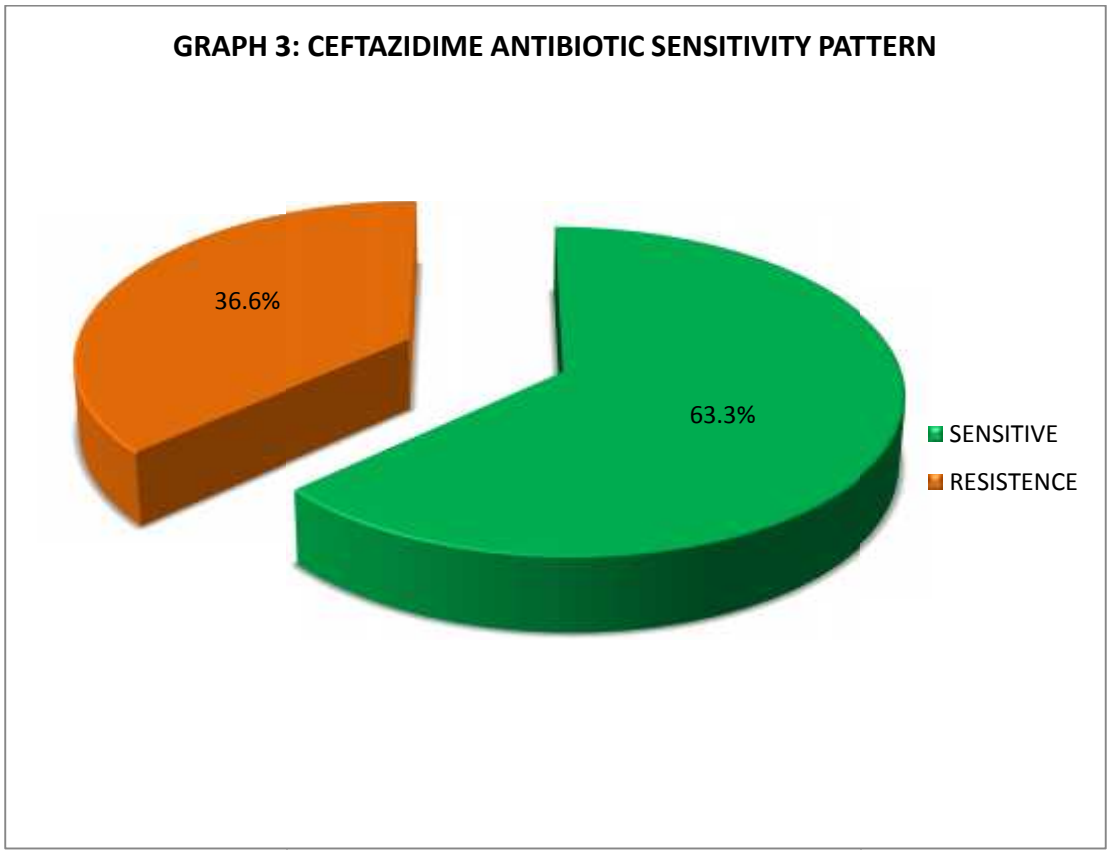
Total MBL producers	Maternity ward	Ortho ward	Surgy ward	Paedo ward	ENT ward	Opthal ward
24	1(4%)	5(20%)	8(33%)	4(16%)	5(20%)	1(4%)



Of the 24 Imipenem resistant *P.aeruginosa*, maximum number of isolates were from Surgery ward with 33% of cases.

TABLE 10: CEFTAZIDIME ANTIBIOTIC SENSITIVITY PATTERN OF *P.aeruginosa.*

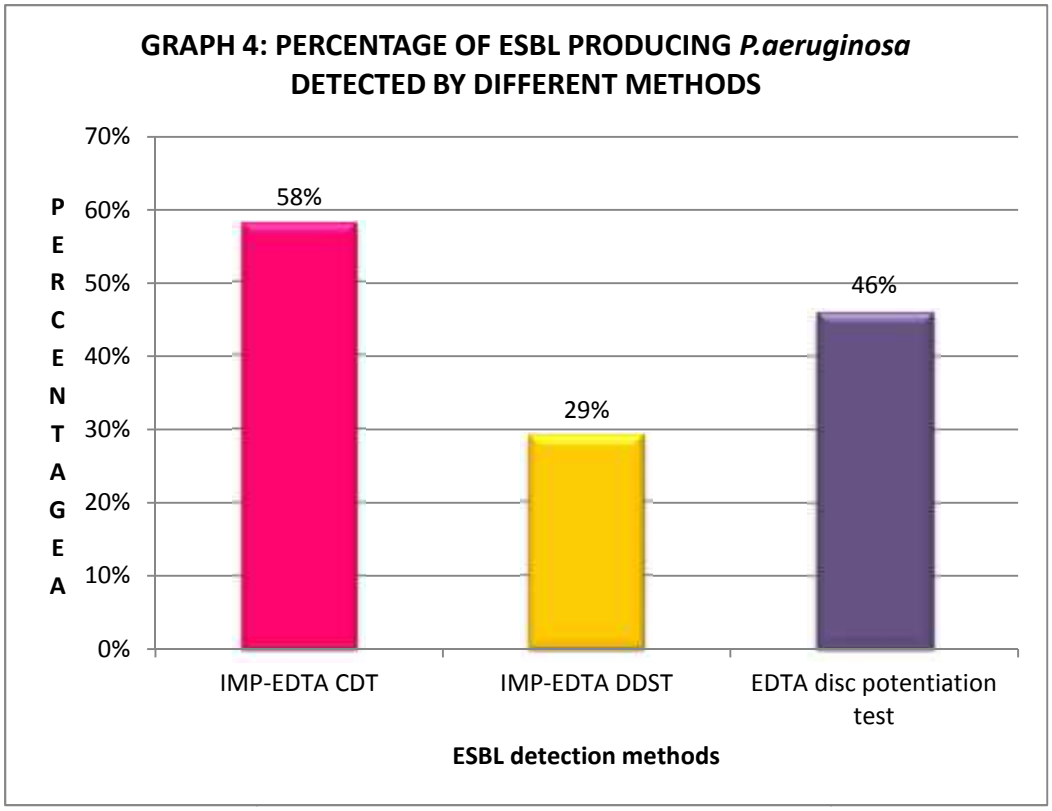
Total number of *Pseudomonas aeruginosa* isolates from pus sample	Ceftazidime resistant	Ceftazidime sensitive
90	33(36.6%)	57 (63.3%)



Out of the 90 *P.aeruginosa* isolated from pus samples, 57(63%) were Cefazidime sensitive and 33(37%) were resistant.

TABLE 11: PERCENTAGE OF ESBL PRODUCING *P.aeruginosa* DETECTED BY DIFFERENT METHODS:

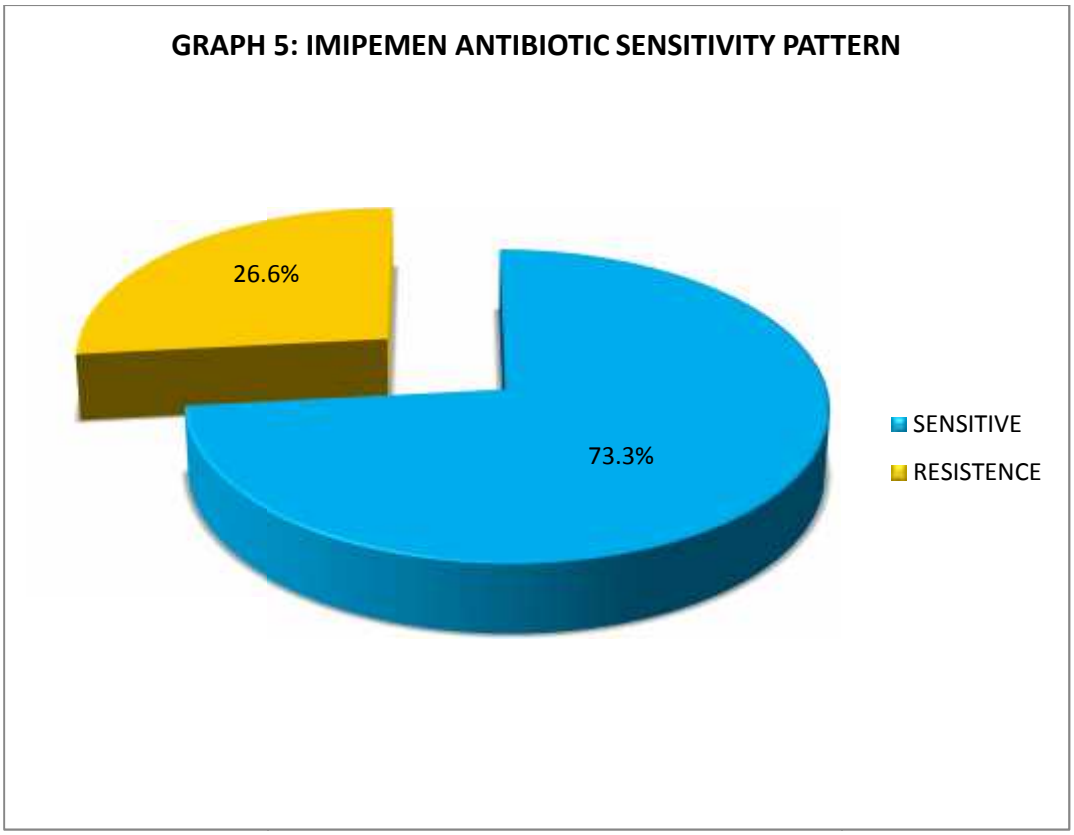
Total number of *P.aeruginosa* resistant to Ceftazidime	DDT	DDST	Negative by both methods
33	9(27%)	17 (51%)	17(51%)



Of the 33 *P.aeruginosa* resistant to ceftazidime, DDT detected 9(27%) of ESBL producers and DDST detected 17(51%). And 17(51%) did not show ESBL production by either of the methods used in the study.

TABLE 12: IMIPENEM ANTIBIOTIC SENSITIVITY PATTERN OF *P.aeruginosa*:

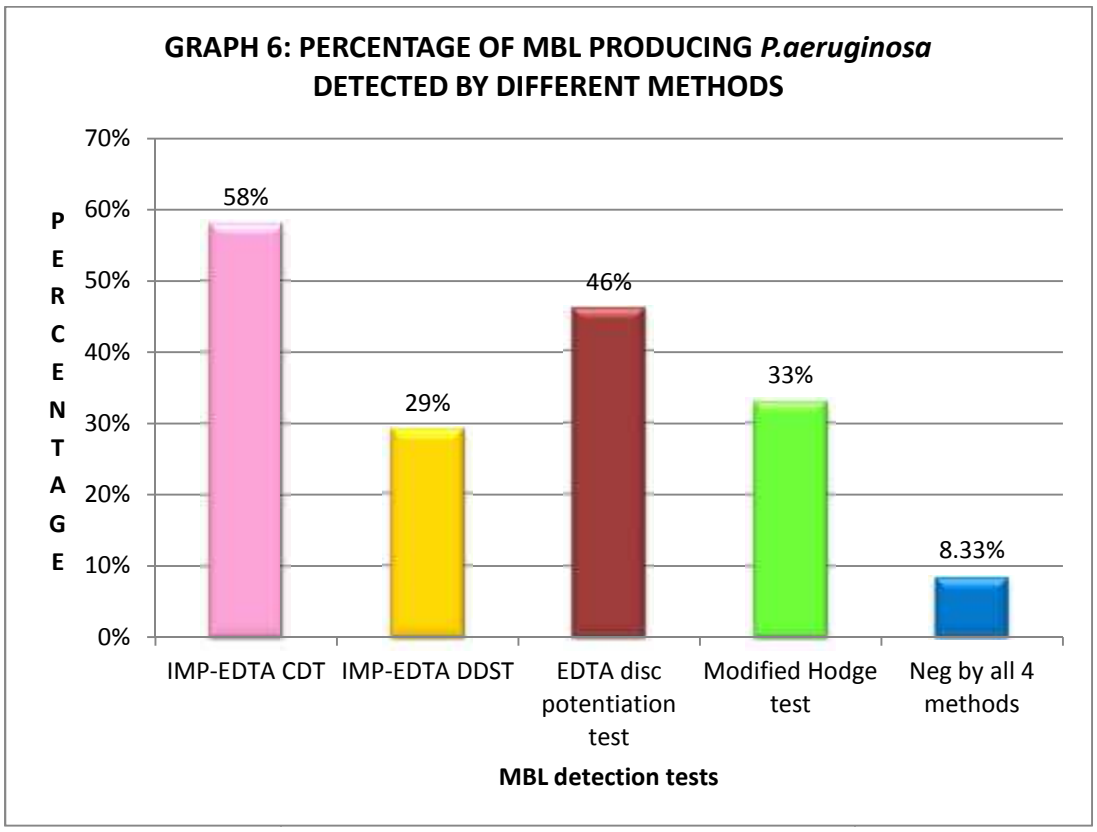
Total number of *Pseudomonas aeruginosa* isolates from pus sample	Imipenem resistant	Imipenem sensitive
90	24(26.6%)	66 (73.3%)



Out of the 90 *P.aeruginosa* isolated from pus samples, 66(73.3%) were Imipenem sensitive and 24(26.6%) were resistant.

TABLE 13: PERCENTAGE OF MBL PRODUCING *P.aeruginosa* DETECTED BY DIFFERENT METHODS:

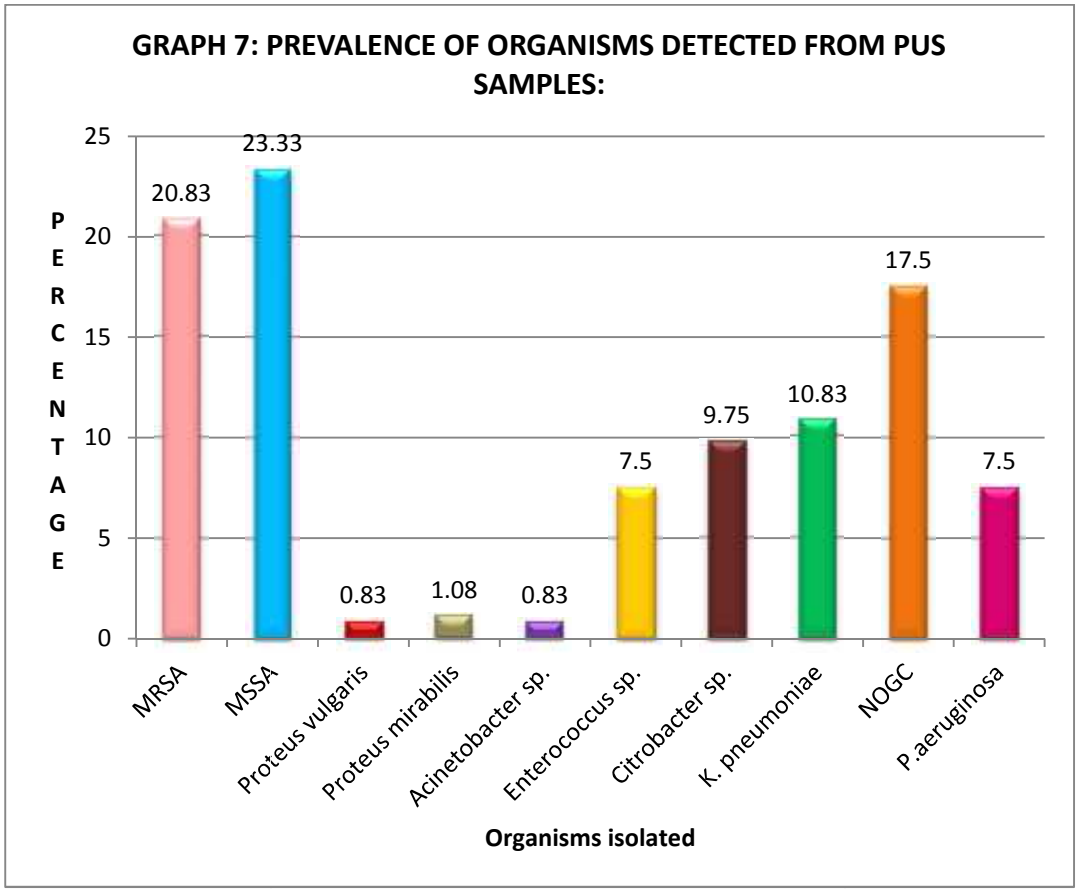
Total number of *P.aeruginosa* resistant to Imipenem	IMP-EDTA CDT	IMP-EDTA DDST	EDTA disc potentiation test	Modified Hodge test	Negative by all 4 methods
24	14(58%)	7(29%)	11(46%)	8(33%)	2(8.33%)



Of the 24 *P.aeruginosa* resistant to imipenem, IMP-EDTA CDT detected 14(58%) of ESBL producers, IMP-EDTA DDST detected 7(29%), EDTA disc potentiation test detected 11(46%) and Modified Hodge method detected 8(33%). And 2 (8.33%) did not show MBL production property by any of the four methods used in the study.

TABLE 14: PREVALENCE OF ORGANISMS DETECTED FROM PUS SAMPLES:

Other organism isolated from pus samples	Percentage
Methicillin Resistant *Staphylococcus aureus*	250(20.83%)
Methicillin Sensitive *Staphylococcus aureus*	280(23.33%)
Proteus vulgaris	10(0.83%)
Proteus mirabilis	13(1.08%)
Acinetobacter sp.	10(0.83%)
Enterococcus sp.	90(7.5%)
Citrobacter sp.	117(9.75%)
Klebsiella pneumoniae	130(10.83%)
No orgamisms grown in culture	210(17.5%)
Pseudomonas aeruginosa	90(7.5%)
Total samlples screened	**1200**



The MSSA and MRSA isolates were the highest to be isolated from 1200 pus samples processed with 20.83% and 23.33% respectively. 7.5% of *P.aeruginosa* isolated were included in our study to detect ESBL and MBL producers.

DISCUSSION

Pseudomonas aeruginosa is the leading cause of nosocomial infections, including wound infection, pneumonia, urinary tract infections, and bacteremia. *P.aeruginosa* exhibits intrinsic resistance to several antimicrobial agents. However, acquired resistance to anti-pseudomonal β-lactams such as Ticarcillin, Piperacillin, Ceftazidime, Cefepime, Aztreonam and Carbapenems considered as deterrent weapon that can be a major challenge in managing MDRPA infections, especially while it is associated with co-resistance with other classes of drugs namely Aminoglycosides and Fluoroquinolones.

Several mechanisms can contribute to the acquired β-lactam resistance in *P.aeruginosa*, that includes production of β-lactamases, the upregulation of efflux systems, and decreased outer membrane permeability. With respect to β-lactamase production, acquired Extended-spectrum-β-lactamases (ESBL) and Metallo-β-lactamases (MBL) are the predominant emerging resistance mechanisms in *P.aeruginosa*. The present study aimed at halucidating major resistance mechanisms.

The present study was carried out in Department of Microbiology, J. N. Medical College, Belgaum on 90 *P.aeruginosa* isolates, isolated from 1200 pus samples received at our laboratory from January 2011- December 2011.

In our study we found, highest prevalence of ESBL producing *P.aeruginosa* were from surgery ward with 36% followed by ENT ward, Paed ward, Ortho, Opthal and Maternity ward with 21%, 15%, 12%, 6% and 6% respectively.

In the present study 36.6% (33/90) *P.aeruginosa* were resistant to Ceftazidime which is similar to the study done by Aggarwal et al[14], at Haryana showing 20.27% of

Cefazidime resistant *P.aeruginosa*. Another study done by Singh et al[123], at Mysore also showed 27.2% *P.aeruginosa* resistant to Cefazidime, 20.9% by a study done by Zahra et al[125], West Bengal and 20.27% by a study by Wayne et al.[124]

Studies in some places like in Nagpur, the figures of ESBL producers were 50%. [193] and another comparatively recent study in 2005, from New Delhi, showed 68.78 % of the strains of gram negative bacteria to be ESBL producers [194] , which is high compared to our study. And studies in few other places like in Varanasi ,Upadhyay S et al showed the prevalence of ESBL producing *P.aeruginosa* was 3.3%[199] and Rodrigues C et al, in their study showed 5.9% of *P.aeruginosa* isolates harbored ESBLs in Mumbai [203],which is less in comparison to our study.

This variation in the prevalence of ESBL producing *P.aeruginosa* in different places/studies could be due to the variation in sample size studied or due to their differences in hygienic practices.

In our study of the 33 *P.aeruginosa* resistant to Ceftazidime processed for ESBL detection, Double Disc synergy test detected 17(51%) compared to Disc diffusion test which detected 9(27%) of ESBL producing *P.aeruginosa* and thus DDST proved to be better method than DDT to detect ESBL producers.

A similar study by Umadevi S et al[126], in which the the two conventional methods DDST and DDT were compared. They found, no significant differences between the ESBL detection rates by two conventional methods in *P. aeruginosa*. Their failure to detect the better performance of the double disk synergy test as compared to the disk diffusion test for the detection of ESBL production among the

Pseudomonas aeruginosa isolates could be due to the relatively small number of isolates which were tested in their study (27 isolates).

A study done by Jiang X at al, to detect ESBL producing *P.aeruginosa* from 75 isolates showed that there were no ESBL false positive detected in the ESBL-screening methods like DDST and Combined Disc test when compared to IEF (isoelectric focusing electrophoresis), PCR, and PCR product sequencing. And hence found the conventional methods to be more cost effective, easy to perform in routine clinical laboratory and are as sensitive as molecular techniques like IEF and PCR. [168]

In a study by Shukla et al, they found DDT to be more sensitive for detecting ESBL producers than the DDST. And the reason coated was the problem of optimal disc space and correct storage of the Clavulanate containing disc.[173]

The 17(51%) Ceftazidime resistant *P.aeruginosa* which gave negative results both methods used to detect ESBL producing may have other mechanism of resistance such as impermeability of outer membrane and or active efflux mechanism.

The main limitation of our studies were, due to the absence of any standard methods to detect ESBL in non-fermenters, it is difficult to comment on true or fasle ESBL producers, MIC reduction would be a better method to know the drug susceptibility, but it is a cumbersome, laborious method and PCR could have been an additional investigation to detect the genes responsible for resistant, but has the disadvantage of its high cost.

In our study we found, highest prevalence of MBL producing *P.aeruginosa* were from surgery ward with 33% followed by ENT ward, Ortho, Paedo, Opthal and Maternity ward with 20%, 20%, 16%, 4% and 4% respectively.

In the present study 26.6% (24/90) *P.aeruginosa* were resistant to Imipenem which is similar to to study done by Shobha KL et al[117], showing 30% of Imipenem resistant P.aeruginosa. Another study done by Varaiya A et al[2], showed 20.8% P.aeruginosa resistant to Imipenem. A study by John S et al[128], showed 27.7% , Zahra Tet al[125], showed 30.2% , Irfan S et al[129], showed 25% and Kumar SH et al[130], showed 32.4% Imipenem resistant *P.aeruginosa*.

Study in few other places like Bangalore, by Navneeth et al found 12% of MBL producing *P.aeruginosa.* [112] Another similar study by Mendiratta et al, Nagpur showed prevalence of 8.62% MBL producing Carbapenem resistant *P.aeruginosa* isolates.[220] and Puducherry 10.9% isolates showed resistance to carbapenems in a study [221], which is less in comparision to our study.

This variation in the prevalence of MBL producing *P.aeruginosa* in different places/studies could be due to the variation in sample size studied or due to their differences in hygienic practices.

In our study, of the 24 *P.aeruginosa* resistant to Imipenem processed for MBL detection, Imipenem(IMP)- EDTA combined disc test detected 14(58%) compared to EDTA disc potentiation using Ceftazidime, Ceftizoxime and Cefotaxime which detected 11(46%), Modified Hodge test detected 8(33%) and Imipenem(IMP)- EDTA double disc synergy test detected 7(29%) of MBL producing *P.aeruginosa* and hence IMP-EDTA CDT was a better method compared to other methods used in the study.

This is in accordance with a similar study by Behera et al[13], showed CDT is superior to DDST and DPT for detection on MBL producing *P.aeruginosa*.

Another study by Yan JJ et al[83] also showed that CDT is better method to detect MBL producing *P.aeruginosa*. Murugan et al showed DDST detected 70% and MHT detected 50% of MBL producing *P.aeruginosa* and hence showed DDST was more accurate than MHT in detecting MBL producing *P.aeruginosa*. [130]

Pragapati SB et al [131] showed DDST detected 16(27.11%) and CDT detected 30(50.84%) of MBL producers, which is similar to a study done by Boghiel et al. [132] A study done by Agrawal G et al to know the prevalence of metallo-beta-lactamase production in clinical isolates of *Pseudomonas aeruginosa* using DDST and MIC methods using 174 isolates gave same results for both the combinations, and showed that MIC reduction is a cumbersome, laborious method. Thus according to this study DDST was a performed method. And hence, recommended DDST as a screening method for detection of MBL production in *P. aeruginosa*. [171]

In a study done by Bhalerao DS et al, employing three methods for MBL detection, the incidence of MBL producing *Pseudomonas* by DDST was found to be 50% as compared to combined disc method (66.7%). And found Combined Disc test to be easier to detect only by observing for the extension of zone more than 7mm and therefore easy to interpret. The EDTA disc potentiation test with four cephalosporins was not found to be useful as it could detect only 6(11.1%) isolates as MBL producers. The combined disc method proved to be more sensitive over DDST and EDTA disc potentiation methods in detection of MBL producers.[174]

A study by Noyal MJC et al showed of the 18.9% Merapenem resistant , 16 (50.0%) were MBL producers by EDTA disk synergy test, but only 9 (28.1%) were positive for carbapenemases by modified Hodge test, and hence recommended ETDA disk synergy test over modified Hodge test. [121]

The two (8.33%) Imepenim resistant *P.aeruginosa* which gave negative results by all the four methods used to detect MBL producing may have other mechanism of resistance such as impermeability of outer membrane and or active efflux mechanism.

The main limitation of our studies were, due to the absence of any standard methods to detect MBL in non-fermenters, it is difficult to comment on true or fasle MBL producers, MIC reduction would be a better method to know the drug susceptibility, but it is a cumbersome, laborious method and PCR could have been an additional investigation to detect the genes responsible for resistant, but has the disadvantage of its high cost.

Of the total 90 patients from whom *P.aeruginosa* were isolated, those which were not ESBL and MBL producers improved with treatment by third generation Cephalosporins, Erythromycin, Ampicillin and Ciprofloxacin ear drops. 3 patients were take-up for amputation, 3 of them expired due to development of septicemia and 12 patients could not be followed up.All the patients in whom ESBL producing *P.aeruginosa* was isolated were treated with combination of Amoxicillin with Clavulanic acid and those with MBL producing *P.aeruginosa* were treated with colistin.

Thus according to this study it was found that Double Disc synergy test is a better method to detect ESBL producing *P.aeruginosa* and Imipenem(IMP)- EDTA combined disc test for MBL producing *P.aeruginosa*.

This early and accurate detection of ESBL and MBL producing *P.aeruginosa* has helped the doctors to treat the patients early with appropriate antibiotics, thereby improving the patient outcome and decreased the morbidity and mortality.

However in the absence of any CLSI guidelines for detection of MBL in Non-fermenters, we reframe from commenting on specificities of either of tests. There is a strong need for standardization/ CLSI guidelines for detection.

CONCLUSION

Total of 90 *P.aeruginosa* isolates, isolated from 1200 pus samples of hospitalized patients of K.L.E.'S DR. Prabhakar Kore Hospital and MRC, Belgaum, received at Microbiology department from January 2011- December 2011 were included in the study.

Double Disc Synergy Test is a better method to detect ESBL producing *P.aeruginosa* and IMP-EDTA Combined Disc Test for detection of MBL producing *P.aeruginosa* accurately.

Hence early and accurate detection of ESBL and MBL producers will help to start the treatment at the earliest and thus reduces mortality and morbidity due to indiscriminate antibiotic usage.

SUMMARY

A study conducted on 90 *P.aeruginosa* isolates isolated from 1200 pus samples received at Microbiology Department, KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum and Department of Microbiology, J. N. Medical College, Belgaum during the period from January 2011 to December 2011, revealed the following findings:

- Of the 33 Ceftazimide resistant *P.aeruginosa*, maximum number of isolates were from Surgery ward with 36% of cases.
- Of the 24 Imipenem resistant *P.aeruginosa*, maximum number of isolates were from Surgery ward with 33% of cases.
- Out of the 90 *P.aeruginosa* isolated from pus samples, 57(63%) were Cefazidime sensitive and 33(37%) were resistant.
- Of the 33 *P.aeruginosa* resistant to Ceftazidime, DDT detected 9(27%) of ESBL producers and DDST detected 17(51%). And 17(51%) did not exibit the ESBL producing property by either of the methods which was used in the study.
- Out of the 90 *P.aeruginosa* isolated from pus samples, 66(73.3%) were Imipenem sensitive and 24(26.6%) were resistant.
- Of the 24 *P.aeruginosa* resistant to Imipenem, IMP-EDTA CDT detected 14(58%) of ESBL producers, IMP-EDTA DDST detected 7(29%), EDTA disc potention test detected 11(46%) and Modified Hodge method detected 8(33%). And 2(8.33%) could not detect MBL producing property by any of the four methods used in the study.

- Of the total 90 patients from whom *P.aeruginosa* were isolated, 3 patients were taken up for amputation, 3 of them expired due to development of septicemia, 12 patients could not be followed up and remaining patients showed improvement.

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ANNEXURE – I

PRINCIPLES OF BIOCHEMICAL TESTS

1. Gram's stain (Hucker's modification): A thin smear from a single colony was made on a glass slide and fixed by flaming over the Bunsen burner. After fixing crystal violet was poured over the smear, care was taken to completely cover the smear, it was allowed to stand for 1min, washed with tap water. Then Gram's iodine was poured over the slide and kept for one minute, washed with water and was decolorized by 95% alcohol till the blue color of crystal violet disappeared. The slide was washed with water and counter stained by safranine for 1min. Then the slide was washed with water, dried and was observed under oil immersion objective. In all those smears, gram negative bacilli were seen, further motility of these organism were noted by hanging drop method. [21]

Quality control:

Positive control: *Staphylococcus aureus* ATCC 25923

Negative control: *Escherichia coli* ATCC 25922

2. Hanging drop: For this, a small amount of paraffin wax was placed around the lip of the well on the concavity slide. A smooth saline suspension of an individual colony was prepared with the help of a clean sterile loop and loopful of the material was placed on a cover slip. The slide was inverted and pressed over the cover slip, the guiding the drop of bacterial suspension in to the centre of the well. The slide was carefully examined by hanging drop method to know the motility of the different gram negative bacilli. In doubtful cases, motility was confirmed by inoculating onto semisolid media.

One peptone water tube was inoculated with the growth. Care was taken to select individual discrete colonies. The tube was kept at 37ºC for 2 hours and turbidity was compared with 0.5 McFarland standards. It was used for sugar fermentation tests, biochemical tests and also for antibiotic sensitivity.[21]

BIOCHEMICAL TESTS:

3. Indole test:

Principle: This test was done to demonstrate the ability of certain bacteria to decompose the amino acid tryptophan in to indole. Here tryptophan rich medium was used**.**

Procedure: Kovac's reagent method was employed.

Kovac's reagent- preparation,

Amyl or isoamyl alcohol	150ml,
β-dimethylaminobenzaldehyde	10gm,
Concentrated HCL	50ml.

Individual colonies were inoculated on to tryptophan broth were incubated at 37^{o}C for 18-24hrs. To this 0.5ml of Kovac's reagent was added and gently shaken.[119]

Interpretation: Appearance of red color was taken as indole producer.

Quality control:

Positive control: *Escherichia coli* ATCC 25922

Negative control: *Klebsiella pneumoniae*.

4. Urease test:

Principle: This was done to determine the ability of bacteria to decompose urea in ammonia. Here Christensen's urea agar medium was used.[5]

Procedure: Inoculate heavily over the entire surface of Christensen's urea agar medium with the peptone water culture and incubate at 37^{o}C. Examine after 4hrs and then overnight incubation.

Interpretation: Positive: when the indicator turned to purple-pink

Negative: no change in colour.

Quality control:

Positive control: *Proteus species*,

Negative control: *Escherichia coli.*

5. Citrate utilization test:

Principle: To determine the ability of bacteria to utilize citrate as sole source of carbon for its growth. Simmon's citrate medium was used to know the utilization of citrate.

Procedure: The citrate slant was inoculated with the suspected single colony and medium was incubated at 37^{o}C for 24 to 48hrs. A positive reaction was indicated by blue colour and streak of growth. A negative reaction is indicated if original green color and no growth is observed.

Quality control:

Positive control: *Enterobacter aerogenes*,

Negative control*: Escherichia coli.*

6. Triple sugar iron agar test:

Principle: This was done to determine the ability of bacteria to ferment carbohydrates incorporated in a growth medium and production of hydrogen sulfide**.** Triple sugar iron (TSI) agar medium contains 10 parts Lactose, 10 parts sucrose, 1 part glucose and peptone. Phenol red and ferrous sulphate serve as indicators of acidification and H_2S production respectively. With a sterile straight inoculating wire, touch the top of a well-isolated colony.

Procedure: Inoculate TSI by first stabbing through the centre of the medium to the bottom of the tube and then streaking the surface of the agar slant. Incubate the tube at 37^oC for 18 to 24 hours the results were interpreted as follows.

Interpretation:

Slant/butt	**Color**	**Utilization**
Alkaline slant / No change in butt (K/No change)	Red/No Change	Glucose, lactose, sucrose non-utilizers.
Alkaline slant / Acid butt(K/A)	Red/Yellow	Glucose only fermented; peptones utilized
Acid slant / Acid butt (A/A)	Yellow/Yellow	Glucose fermented, lactose and/or sucrose fermented
Alkaline slant / Alkaline butt (K/K)	Red/Red	No fermentation of glucose, lactose or sucrose. Peptones utilized

A black precipitate in the butt indicates production of ferrous sulphide and H_2S gas (H_2S^+). Bubbles or cracks in the media indicate the production of CO_2 or H_2.[5]

7. Methyl Red test:

Principle: To determine the ability of bacteria to produce and maintain the low pH after prolonged incubation.

Procedure: Inoculate the glucose phosphate peptone water medium with a young culture and incubate at 37^{o}C for 48 hrs. To this, add about five drops of the methyl red reagent. Mix and read immediately. Positive tests are bright red and negative tests are yellow.

Quality control:

Positive control: *Escherichia coli.*

Negative control: *Enterobacter aerogenes*

8. Voges Proskauer test:

Principle: This test was done to determine the ability of an organism to produce neutral end products like acetyl methyl carbinol or it reduction product 2,3 butylene glycol from glucose fermentation.

Medium: Glucose phosphate peptone water.

Procedure: Inoculate the glucose phosphate peptone water medium with a young culture, and incubate at 37^{o}C for 48hrs, to this, add 1ml of 40% potassium hydroxide and 3ml of solution of α-naphthol in absolute ethanol.

Interpretation: A positive reaction is indicated by the development of a pink color in 2-5min, becoming crimson in 30 min.

Quality control:

Positive control: *Enterobacter aerogenes,*

Negative control: *Escherichia coli.*

9. Sugar fermentation test:

Principle: This test was done to determine the ability of an organism to ferment a specific carbohydrate that is incorporated in a basal medium, thereby producing acid with or without visible gas.

Procedure: The test was performed on conventional culture media with test sugar. The common sugar fermentation media used for present study were glucose, sucrose, lactose, maltose, mannose, arabinose and xylose. From the peptone water tube (which was incubated for 2hrs. after inoculation) all the sugar fermentation media were inoculated with the help of a clean sterile loop. Care was taken to sterilize the loop every time, after dipping in different sugars. After different media were inoculated, these were incubated at 37^{o}C for 18-24hrs. After 24hrs, the sugar media were examined for the production of acid indicated by pink color and gas (presence of an air bubble inside the durham's tube).

Interpretation: Positive test in indicated by change in color to pink with or without gas formation in durham's tube. Negative test is indicated by growth, but no change in colour[25].

10. Oxidation/ Fermentation test (Modified Hugh and Leifson):

Principle: This test was done to know of the organism uses carbohydrate substrate to produce acid byproducts either oxidative or fermentative.

Procedure: Hugh-Liefson's basal medium prepared and carbohydrate to be added was sterilized separately and added to give final concentration of 1%. The medium was then tubed to a depth of about 4cm.

Duplicate tubes of medium were inoculated by stabbing. One tube was promptly covered with a liquid paraffin to a depth of 1cm and were incubated at 37^{o}C for 18-24hrs.[5]

Interpretation:

- Fermenting organisms produce an acid reaction throughout the medium is covered (anaerobic) as well as the open (aerobic) tube.
- Oxidizer produces an acid reaction only in the open tube.
- Organisms that cannot breakdown the carbohydrate aerobically or anaerobically produce an alkaline reaction in the open tube and change in covered tube.

11. Nitrate Reduction test:

Principle: The test is used to determine the ability of an organism to reduce nitrate. The reduction of nitrate to nitrite is determined by adding sulfanilic acid and alpha-napthylamine. The sulfanilic acid and nitrate react to form a diazonium salt. The diazonium salt then couples with α-napthylamine to produce a red, water soluble azo dye.[5]

Procedure: This liquid medium was inoculated with the suspected single colony and the medium was incubated for 18-24hrs.

Add 0.1ml of the test reagent to the test culture. The test reagent was prepared by mixing equal volumes of solution A (8.0gm of sulphanilic acid in 1liter of acetic acid 5mol/liter) and solution B (5.0gm of α-napthylamine in 1 liter of acetic acid 5mol/liter). A red color developing within a few minutes indicates the presence of a nitrate. No colour indicated that nitrate have not been reduced or reduced to product other than nitrites, such as ammonia, molecular nitrogen, nitric oxide or nitrous oxide

and hydroxylamine. Addition of zinc dust to all negative tests was necessary. Zinc ions reduced nitrates to nitrites and development of red colour after addition of zinc dust indicated the presence of residual nitrates[5].

Quality control:

Positive control: *Escherichia coli*

Negative control: *Acinetobacter baumannii*

12. Phenylalnine Deaminase test (PPA):

Principle: To determine the ability of bacteria to deaminate phenylalanine to phenyl pyruvic acid.

Procedure: This test was done to know the ability of the organism to deaminate phenylalanine with the production of phenyl pyruvic acid, which reacts with ferric salts to give green color. Inoculated the agar slope medium containing DL-phenylalanine with a fairly heavy inoculum and incubate 37^{o}C for 18-24hrs. After incubation, allow a few drops of 10% solution of ferric chloride to run down over the growth on the slope if the test is positive, a green color will develop in the fluid and in the slope.

Quality control:

Positive control: *Proteus species.*

Negative control: *Escherichia coli.*

13. Amino acid decarboxylase and Arginine dihydrolase test:

Principle: Decarboxyases are a group of substrate- specific enzymes that are capable of reacting with the carboxyl portion of amino acids, forming alkaline-reacting

amines. The conversion of arginine to citrullin is a dehydrolase, in which an NH_2 group is removed from arginine.

Procedure: Here Moellar decarboxylase broth base was used. Test organism was inoculated in the medium with straight wire then overlay tubes with sterile mineral oil to cover about 1 cm of the surface. Incubate and read daily for 4 days. The medium first becomes yellow due to acid production during glucose fermentation later if decarboxylation occurs, the medium turns to violet colour.

Quality control:

Positive control: *Pseudomonas aeruginosa.* ATCC 27853

Negative control: *Klebsiella pneumoniae*.

14. Catalase test:

Principle: The enzyme catalase mediates the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water. The presence of the enzyme in bacterial isolates is evident when a small inoculum is introduced into H_2O_2, and the rapid elaboration of oxygen bubbles occur. The lack of catalase is evident by a lack of or weak bubble.

Procedure: Presence of catalase was demonstrated by test tube method. A small amount of the culture to be tested was picked from a nutrient agar plate with a clean sterile platinum loop or a clean, thin glass rod and was inserted in to 3% hydrogen peroxide solution held in a small, clean tube.

Interpretation: The production of gas bubbles from the surface of the solid culture material indicates a positive reaction and negative reaction when there were no gas bubbles.[5]

Quality control:

Positive control: *Staphylococcus aureus*

Negative control: *Streptococcus pyogenes*

This test was used to differentiate *Staphylococcus spp.* from *Streptococcus spp*.

15. Oxidase test:

Principle: To determine the presence of an enzyme oxidase, which catalyse the transport of electrons between electron donors in the bacilli and redox dye. The dye is oxidized to indophenol blue producing deep purple colour.

Procedure: Wet filter paper method was used for this test. Strips of Whatman's No. 1 filter paper was soaked with a little freshly made 1% solution of tetramethyl-para-phenylene-diamine dihydrochloride and then with a help of sterile glass rod a single colony from the medium was rubbed over the strip.

Interpretation: A positive reaction was indicated by an intense deep purple blue, appearing within 5-10 seconds and a negative reaction by absence of colouration or by colouration later than 60seconds.

Quality control:

Positive control: *Pseudomonas aeruginosa ATCC 27583*

Negative control: *Escherichia coli ATCC 25922*

Antibiotic susceptibility testing:

Antibiotic susceptibility testing was done for all the *Pseudomonas aeruginosa* isolates under the standard CLSI guidelines

Antibiogram testing:

Antibiotic sensitivity was tested by Kirby-Bauer's disk diffusion method. Mueller-Hinton agar plate was used. One-two colonies from the culture plate were inoculated into 2 ml of peptone water and incubated at 37^{o}C for 2hours. Turbidity was compared to that of 0.5 McFarland's standard (1.5×10^{8} CFU/ml). A cotton swab was immersed rotated in this inoculum, the swab was then pressed to the sides of the tube so on to remove excess inoculum. The swab was then used to inoculate the plate of Mueller-Hinton agar, in three different directions to ensure an even and complete distribution of the inoculum over the entire plate. The antibiotic disks were applied within 15 minutes of inoculation of plate and the plate inverted for incubated for 18-24 hrs at 37ºĊ.

Commercially obtained Hi-media disks were used. The strength of disks used and their zone size interpretative standards were according to guidelines by CLSI guidelines standards.

ANNEXURE – II: PROFORMA

Topic: Comparative evaluation of phenotypic tests for identification of MBL/ESBL producing *Pseudomonas aeruginosa* from pus samples of hospitalized patients, tertiary care hospital, one year study.

Name: Dr._____

Case no.: IP/OP No.:

Name of patient: Unit/ Ward:

Age: Date of sample collection:

Sex: Time of sample collection

Address: Sample processed time:

Chief complaints: Site of pus collection:

Any other complains:

Treatment history:

Clinical diagnosis:

DAY 1:

Direct smear (Gram stain):

Blood agar:

MacConkey agar:

DAY 2:

Gram staining of smear from colony:

1. From blood agar:

2. From MacConkey agar:

Oxidase: + / - Catalase: + / -

Motility: motile / non-motile O/F Glucose: fermentative / oxidative

Nitrate: reduced / not reduced Indole: produced /not-produced

H2S: produced /not-produced MR: + / - VP: + / -

Urease: hydrolysed/ not-hydrolysed

Citrate: utilized / not-utilized

TSI:

Mannitol: fermented / not-fermented motile/non-motile

Lysine decarboxylase: decarboxylated/not-decarboxylated

Arginine dehydrolase: decarboxylated/not-decarboxylated

Ornitine decarboxylase: decarboxylated/not-decarboxylated

Identification of isolates:

Antibiogram:

FIRST LINE DRUGS: ZONE SIZE R/S

Ceftizoxime (30mcg)

Ceftriaxone (30mcg)

Cefotaxime (30mcg)

Ceftazidime (30mcg)

Gentamicin (10mcg)

Tobramycin (10mcg)

SECOND LINE DRUGS: ZONE SIZE R/S

Amikacin (30mcg)

Aztreonam (10mcg)

Cefepime (30mcg)

Ciprofloxacin (5mcg)

Levofloxacin (5mcg)

Imipenem (10mcg)

Meropenem (10mcg)

Piperacillin/Tazobactam (100mcg/10mcg)

Clavulanic acid (75mcg)

Polymyxin-B (300units)

Chloramphenicol (30mcg)

ESBL screening tests:

1. **Double disk synergy test(DDST):**

   Cefotaxime (30mcg), Ceftriaxone (30mcg), Ceftazidime (30mcg), Amoxiclav (20mcg/10mcg)

   INTERPRETATION:

2. **Disc diffusion test(DDT):**

   Ceftazidime (30mcg) alone and with combination with Clavulanic acid (30mcg).

INTERPRETATION:

MBL screening tests:

1. Imipenem (IMP)-EDTA combined disc test:

Two IMP discs (10mcg). 10mcg 0.5M EDTA (750mcg) added to one of the IMP disc.

INTERPRETATION:

2. Imipenem (IMP)-EDTA double disc synergy test (DDST):

One IMP disc (10mcg). Blank disc with 10mcg of 0.5M EDTA (750mcg)

INTERPRETATION:

3. EDTA disc potentiation using ceftazidime, ceftizoxime and cefotaxime:

Ceftazidime disc (30mcg), Ceftizoxime disc (30mcg) and Cefotaxime disc (30mcg).Blank disc with 10mcg of 0.5M EDTA (750mcg)

INTERPRETATION:

4. Modified Hodge test:

Overnight culture suspension of *E.coli* ATCC 25922. Imipenem disc (10mcg).

INTERPRETATION:

ANNEXURE – III: PRESENTATIONS

1. Poster presention done at XVI Annual Conference of IAMM Karnataka Chapter held at St.John's Medical College, Bangalore on 19th February 2012.

Abstract : COMPARATIVE EVALUATION OF PHENOTYPIC TESTS FOR IDENTIFICATION OF EXTENDED SPECTRUM BETA LACTAMASES (ESBL) AND METALLO-BETA-LACTAMASES (MBL) PRODUCING PSEUDOMONAS AERUGINOSA FROM PUS SAMPLES OF HOSPITALIZED PATIENTS OF TERTIARY CARE HOSPITAL.

Post Graduate Student-Department of Microbiology[1],

Prof depart of Microbiology[2],

Jawaharlal Nehru Medical College, Belgaum.

INTRODUCTION:

The beta-lactam antibiotics are among the most widely used antibiotics. *P.aeruginosa* is one of the most common pathogens causing nosocomial infections and an opportunistic pathogen with physiologically versatile nature. It flourishes as a saprophyte, with innate resistance to many antibiotics and disinfectants. In addition to its innate resistance acquired resistance is particularly associated with indiscriminate antibiotic usage. ESBL and MBL production are important examples of them. With world wide increase in occurance and dissemination of ESBl and MBLs, early detection is essential. This will help in timely implication of strict infection control practics as well as clinical guidance regarding potential risk of therapeutic failure.

AIMS AND OBJECTIVES:

1. To compare different phenotypic tests for identification of ESBL and MBL producing *P.aeruginosa* from pus .
2. To know the prevalence of ESBL and MBL producing *P.aeruginosa* isolated from pus samples.

MATERIAL AND METHODS:

70 consecutive *P.aeruginosa* isolates from pus samples over a period of one year were identified upto species level by standard microbiological methods and susceptibility to ceftazidime and imipenem were noted and then subjected to ESBL and MBL screening tests based on Caz and IMP respectively.

Methods for ESBL screening:

1. Double Disk Synergy Test
2. Disc Diffusion Test

Methods for MBL screening:

1. IMP-EDTA Combined Disc Test
2. IMP-EDTA Double Disc Synergy Test
3. EDTA disc potention test
4. Modified Hodge method

RESULTS:

Out of the 70 *P.aeruginosa* isolated from pus samples, 43(61.42%) were Caz sensitive and 27(38.57%) were resistant. Of the 27 *P.aeruginosa* resistant to Caz, DDST detected 6(22.22%), DDT detected 11 (40.74%) and negative by all four methods 15 (55.55%).

Out of the 70 *P.aeruginosa* isolated from pus samples, 51(72.85%) were IMP sensitive and 19(27.14%) were resistant. IMP-EDTA CDT detected 12(63.15%), IMP-EDTA DDST detected 7(39.84%), EDTA disc potention test detected 8(42.10%),Modified Hodge method detected 5(26.31%) and negative by both methods 3(15.7%).

CONCLUSION:

To detect ESBL, DDT and for MBL, IMP-EDTA CDT is a better method. However in the absence of any CLSI guidelines for detection of MBL in Non-fermenters, we reframe from commenting on specificities of either of tests. There is a strong need for standardization/ CLSI guidelines for detection.

2. Submitted abstract for poster presentation at XXVI National Conference of Indian Association of Medical Microbiologists, MICROCON 2012, Lady Hardinge Medical College, New Delhi.

COMPARATIVE EVALUATION OF PHENOTYPIC TESTS FOR IDENTIFICATION OF METALLO-BETA-LACTAMASES (MBL) PRODUCING PSEUDOMONAS AERUGINOSA FROM PUS SAMPLES OF HOSPITALIZED PATIENTS OF TERTIARY CARE HOSPITAL.

Post Graduate Student-Department of Microbiology[1]

Prof and Head of Department of Microbiology[2],

Jawaharlal Nehru Medical College, Belgaum.

INTRODUCTION:

The Metallo-β-lactamase (MBL) is an enzyme which requires zinc for their catalytic activity. The MBL activity is inhibited by metal chelators, such as EDTA and THIOL compounds. MBL hydrolyze all beta-lactam antibiotics including, penicillins, cephalosporins and carbapenems, with the exception of aztreonam monobactam. At present Clinical and Laboratory Standards Institute (CLSI) guidelines do not describe any method for detection of this enzyme in *P. aeruginosa.*

MATERIAL AND METHODS:

90 consecutive *P.aeruginosa* isolates from pus samples in hospitalized patients of K.L.E.S DR. Prabhakar Kore Hospital and MRC, Belgaum, received at Microbiology department were identified up to species level by biochemical tests and susceptibility to Imipenem (IMP) 10µg were noted, those resistant to IMP were subjected to MBL screening.

1. Imipenem(IMP)- EDTA combined disc test
2. Imipenem(IMP)- EDTA double disc synergy test
3. EDTA disc potentiation using ceftazidime, ceftizoxime and cefotaxime
4. Modified Hodge test.

RESULTS:

Out of the 90 P.aeruginosa isolated from pus samples, 66(73.3%) were Imipenem sensitive and 24(26.6%) were resistant.

Of the 24 P.aeruginosa resistant to imipenem, IMP-EDTA CDT detected 14(58%) of ESBL producers, IMP-EDTA DDST detected 7(29%), EDTA disc potention test detected 11(46%) and Modified Hodge method detected 8(33%).

CONCLUSION:

Hence IMP-EDTA CDT is a better method for detection of MBL producers accurately. However in the absence of any CLSI guidelines for detection of MBL in Non-fermenters, we reframe from commenting on specificities of either of tests. There is a strong need for standardization/ CLSI guidelines for detection.

ANNEXURE – IV: MASTER CHART

KEY TO MASTER CHART

P.aeru	:	*Pseudomonas aeruginosa*
K.pneu	:	*Klebsiella pneumoniae*
MSSA	:	Methicillin-resistant *Staphylococcus aureus*
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
E.coli	:	*Escherichia coli*
M	:	Male
F	:	Female
CSOM	:	Chronic Suppurative Otitis Media
O/C/O	:	Operated Case Of
Surg	:	Surgery
MAT	:	Maternity
VAP	:	Ventilator-associated pneumonia
Ortho	:	Orthopaedic
MBL	:	Metallo-beta lactamase
Paedo	:	Paediatric
ICU	:	Intensive Care unit
LL	:	Lower limb
GNB	:	Gram Negative Bacilli
eGPC	:	Gram Positive Cocci
Pf	:	Pus cells-few
Pp	:	Pus cells-Plenty
Ef	:	Epithelial cells-few
Ep	:	Epithelial cells-plenty
DDST:	:	Double Disc synergy test
DDT	:	Disc diffusion test

IMP-EDTA CDT	:	Imipenem(IMP)- EDTA combined disc test
IMP-EDTA DDST	:	Imipenem(IMP)- EDTA double disc synergy test
Disc potentiation test	:	EDTA disc potentiation using ceftazidime, ceftizoxime and cefotaxime
#	:	Fracture
HTN	:	Hypertension
DM	:	Diabetes mellitus
No F/U	:	Could not be followed up
G	:	Gentamycin
Cot	:	Cotrimoxazole
C	:	Chloramphenicol
Cip	:	Ciprofloxacin
Pi	:	Pipracillin
Ak	:	Amikacin
Exp	:	Expired
Amp	:	Amputation
P	:	Positive
N	:	Negative
R	:	Resistant
S	:	Senstive

MASTER CHART

SL. NO	IP NO	LAB NO	AGE(yrs)	SEX	WARD	CLINICAL DIAGNOSIS	UNDERLYING DISEASE	SITE OF PUS COLLECTION	PRIOR ANTIBIOTIC TREATMENT	DIRECT SMEAR	MONOMICROBIAL	POLYMICROBIAL	Ceftazidime(CAZ)			Imipenem(I)					G	Cot	C	Cip	Pi	Ak	OUTCOME
													R		S	R				S							
													DDST	DDT		IMP-EDTA CDT	IMP-EDTA DDST	EDTA Disc potentiation test	Mod HODGE test								
1	473821	17965	45	F	Surg A unit	Diabetic foot	DM & HTN	Right foot	NO	Pf, Ep, GNB	P.aeru	Micrococci			S					S	S	R	S	R	S	S	Imp
2	476543	18207	32	M	ENT A unit	Right ear CSOM	NIL	Right ear	YES	Pp, Ep, GNB & GPC in pairs	P.aeru	Micrococci			S					S	S	R	R	R	R	R	Imp
3	473720	17274	11	M	ENT B unit	Left ear CSOM	NIL	Left ear	NO	Pp, Ep, GNB	P.aeru				S					S	S	R	R	R	R	R	Imp
4	477554	18340	3	M	ENT A unit	Right ear CSOM	NIL	Right ear	NO	Pf,Ep, GNB	P.aeru	E.coli			S					S	S	S	R	R	R	R	Imp
5	478084	18356	25	F	MAT C unit	LSCS wound infection	NIL	LSCS site	NO	Pp, Ep, GNB & GPC in pairs	P.aeru	K.pneumoniae	N	P						S	S	S	R	R	R	R	Imp
6	478086	18346	50	M	Ortho A unit	Chronic Osteomyelitis	DM & HTN	Left tibia	YES	Pf, Ep, GNB & GPC in clusters	P.aeru	Citrobacter sp.	P	P		N	N	P	P		R	S	R	R	S	R	Amp
7	478636	18502	12	F	ENT B unit	Right ear CSOM	NIL	Right ear	NO	Pp, Ep, GNB	P.aeru				S					S	S	S	R	R	S	R	Imp
8	478440	18410	30	M	Ortho A unit	Right tibia #, chr OM	NIL	Right tibia	NO	Pf, Ep, GNB & GPC in clusters	P.aeru	MSSA			S					S	S	S	R	R	S	R	Imp
9	480973	19144	48	F	ENT A unit	Ludwing's angina	DM	Submandibular swelling	YES	Pp, Ep, GNB	P.aeru	K.pneu			S					S	S	R	R	R	S	R	Imp
10	478086	19166	50	M	Ortho D unit	Right leg #, open wound	DM	Right leg # site	YES	Pp, Ep, GNB	P.aeru		P	P		N	N	P	P		R	R	S	R	S	R	Amp
11	482045	19592	55	M	Surg B unit	Scrotal abscess	DM	Right Scrotum	NO	Pf, Ef, GNB	P.aeru		N	P						S	S	S	S	R	S	R	Imp
12	479056	19735	33	M	Ortho A unit	Right tibia #, chr OM	NIL	Right tibia	NO	Pp, Ep, GNB	P.aeru	Citrobacter sp.	N	P		P	N	N	N		R	S	S	R	S	R	Imp
13	477340	18513	55	M	Surg C unit	Appendicitis post op wound inf	HTN	Appendicitis op site	NO	Pf, Ep, GNB & GPC in pairs	P.aeru	Micrococci			S					S	S	S	S	R	S	R	Imp
14	2114461	18595	60	F	ENT B unit	Right ear CSOM	DM & HTN	Right ear	YES	Pp, Ep, GNB	P.aeru				S					S	S	S	S	R	R	S	Imp
15	1972089	18736	12	M	ENT C unit	Left ear CSOM	NIL	Left ear	NO	Pf, Ep, GNB & GPC in clusters	P.aeru	CONS			S					S	S	S	S	S	R	S	Imp
16	1612210	19003	19	M	ENT B unit	Right ear CSOM	NIL	Right ear	YES	Pp, Ep, GNB	P.aeru				S					S	S	S	S	S	R	S	Imp
17	479589	18977	51	F	Ortho C unit	Left foot OM	DM	Left foot	NO	Pf, Ep, GNB & GPC in clusters	P.aeru	MSSA			S					S	S	R	R	S	S	S	Imp
18	428193	9245	13	F	Surg B unit	neurogenic bladder	NIL	wound site	NO	Pp, Ep, GNB	P.aeru	Citrobacter sp.	P	P						S	S	R	R	S	S	S	no F/U
19	431836	10889	55	M	Surg F unit	Right leg cellulitis	DM & HTN	Right leg	YES	Pp, Ef, GNB	P.aeru		N	N						S	S	S	R	R	S	S	Exp
20	432664	11833	65	M	Surg E unit	Non healing left LL ulcer	DM	Left leg	YES	Pf, Ep, GNB	P.aeru		N	N						S	S	R	R	S	S	S	Imp
21	431501	11871	9	M	Paed D unit	Congenital anomaly	NIL	Right hand	NO	Pp, Ep, GNB	P.aeru		N	N						S	S	S	R	S	S	S	Imp
22	430868	9916	62	M	Surg A unit	Diabetic Foot	DM	Right leg	YES	Pp, Ef, GNB	P.aeru	K.pneu	N	P						S	R	S	R	S	S	R	no F/U
23	430183	9758	13	M	ENT A unit	viral encephalitis	NIL	tracheostomy wound	YES	Pf, Ep, GNB	P.aeru		P	P						S	S	S	R	R	R	R	no F/U
24	399148	63	2	M	Opthal B unit	Left eye lid abscess	NIL	Left lower eye lid	NO	Pf, Ep, GNB & GPC in clusters	P.aeru	MSSA	P	P						S	S	S	R	R	S	R	Imp
25	423966	6036	65	F	Surg F unit	Diabetic foot	DM	Right foot	YES	Pp, Ep, GNB	P.aeru		N	N						S	S	S	S	R	R	R	no F/U
26	428294	3367	16	M	Surg D unit	Appendicitis post op wound inf	NIL	Pus frm surgical site	NO	Pf, Ep, GNB & GPC in pairs	P.aeru	Enterococci	N	P						S	R	S	S	S	R	R	no F/U
27	431878	4317	58	M	ENT A unit	Left ear CSOM	DM	Left ear	NO	Pp, Ep, GNB	P.aeru		P	P						S	R	S	S	S	R	R	Imp
28	431664	11583	30	M	ENT A unit	Right ear CSOM	NIL	Right ear	NO	Pf, Ep, GNB	P.aeru		N	N		P	P	P	N		R	S	S	S	R	S	no F/U
29	431501	12147	11	M	Paed D unit	Right empyema thorax	NIL	ICD tube	YES	Pp, Ef, GNB	P.aeru		N	P		N	P	P	N		R	S	S	S	R	S	Imp
30	430647	11837	40	M	Surg C unit	Diabetic foot	DM	Right leg	NO	Pp, Ep, GNB	P.aeru		N	N		P	P	P	N		R	R	S	S	S	S	no F/U
31	442935	19395	12	M	Surg A unit	60% thermal burns	NIL	Abdominal burns	YES	Pf, Ep, GNB	P.aeru		P	N		P	P	P	P		R	R	S	S	S	S	Imp
32	446957	19397	32	F	Surg B unit	50% deep burns-infected	NIL	Right arm	NO	Pf, Ep, GNB & GPC in pairs	P.aeru	Micrococci	N	N		P	P	P	P		S	R	S	S	S	S	no F/U

SL. NO	IP NO	LAB NO	AGE(yrs)	SEX	WARD	CLINICAL DIAGNOSIS	UNDERLYING DISEASE	SITE OF PUS COLLECTION	PRIOR ANTIBIOTIC TREATMENT	DIRECT SMEAR	MONOMICROBIAL	POLYMICROBIAL	Ceftazidime(CAZ)			Imipenem(I)					G	Cot	C	Cip	Pi	Ak	OUTCOME
													R		S	R				S							
													DDST	DDT		IMP-EDTA CDT	IMP-EDTA DDST	EDTA Disc potentiation test	Mod HODGE test								
33	442935	19021	12	M	Burns ward	58% deep thermal burns	NIL	Abdominal burns	YES	Pf, Ep, GNB	P.aeru	K.pneumoniae	N	N		N	P	N	N		S	R	S	S	S	S	no F/U
34	428551	8256	30	M	Ortho C unit	Infected post op non-union right tibia	NIL	Right tibia	YES	Pp, Ep, GNB	P.aeru		N	N		P	N	N	N		S	R	R	R	S	S	no F/U
35	428982	8381	10	M	Paed D unit	Right hydropneumothorax	NIL	Right ICD	YES	Pf, Ep, GNB	P.aeru		N	N		P	N	N	N		S	R	R	R	S	S	no F/U
36	430595	10109	29	F	Mat A unit	LSCS wound infection	NIL	LSCS site	YES	Pp, Ef, GNB	P.aeru	E.coli	P	P		N	N	P	P		R	R	R	R	S	S	Imp
37	424424	6330	4 m	F	Paed B unit	CHD	NIL	Right leg	YES	Pp, Ep, GNB	P.aeru	K.pneu	N	N		P	N	N	P		R	R	R	S	S	S	Imp
38	425672	4827	40	M	ENT D unit	Right ear CSOM	NIL	Right ear	NO	Pf, Ep, GNB	P.aeru		N	N		P	P	P	N		R	R	R	S	S	S	no F/U
39	425671	6232	42	F	Surg B unit	?VAP	NIL	tracheostomy wound	YES	Pp, Ef, GNB	P.aeru	K.pneu	N	N		P	N	N	N		R	R	R	S	S	R	Imp
40	426065	6214	9	F	Paed A unit	Right hydrothorax	NIL	ICD tube	YES	Pf, Ep, GNB	P.aeru		N	P		N	N	N	N		R	S	R	S	S	R	Imp
41	1521790	3393	12	M	ENT C unit	Right ear CSOM	NIL	Right ear	YES	Pf, Ep, GNB	P.aeru		N	N		P	N	N	N		R	S	R	S	R	R	Imp
42	415722	5176	60	M	Opthal B unit	Rt eye evisceration	DM & HTN	right eye	NO	Pp, Ep, GNB	P.aeru		N	N		P	N	N	N		S	S	R	S	R	R	Exp
43	950501	3841	11	M	ENT C unit	Right ear CSOM	NIL	Right ear	NO	Pf, Ep, GNB	P.aeru		N	P		N	N	N	N		R	S	R	S	R	R	Imp
44	1607891	3748	29	F	ENT B unit	Left ear CSOM	NIL	Left ear	NO	Pp, Ep, GNB	P.aeru		N	N		P	N	N	N		R	S	R	S	R	R	Imp
45	1838615	19176	32	M	ENT B unit	Right ear CSOM	NIL	Right ear	YES	Pf, Ep, GNB	P.aeru	E.coli			S					S	S	S	S	S	R	R	Imp
46	1758623	12322	4	F	ENT A unit	Left ear CSOM	NIL	Left ear	NO	Pf, Ep, GNB	P.aeru	E.coli			S					S		S	S	S	R	R	Imp
47	433224	12011	32	M	Ortho B unit	infected # rt tibia	NIL	Right tibia	YES	Pp, Ef, GNB	P.aeru				S					S	S	S	S	S	S	R	Imp
48	431945	11180	62	M	Surg C unit	Right foot nonhealing ulcer	DM & HTN	Right foot	YES	Pp, Ep, GNB	P.aeru				S					S	S	S	S	S	R	R	Imp
49	427553	9430	38	M	Surg A unit	Left leg cellulitis	NIL	Left leg	YES	Pf, Ep, GNB	P.aeru				S					S	S	S	S	S	S	R	Imp
50	435962	13898	70	M	Surg C unit	Diabetic foot	DM & HTN	Right foot	NO	Pp, Ef, GNB	P.aeru				S					S	S	S	S	S	S	R	Imp
51	431945	1305	65	M	Surg C unit	Diabetic foot	DM	Right foot	NO	Pf, Ep, GNB	P.aeru				S					S	S	R	S	S	S	S	Imp
52	435649	13884	14	M	ENT A unit	B/L CSOM	NIL	Right ear	NO	Pp, Ep, GNB	P.aeru				S					S	S	R	S	S	S	S	Imp
53	1788365	14945	24	M	ENT C unit	Left ear CSOM	NIL	Left ear	YES	Pf, Ep, GNB	P.aeru	K.pneu			S					S	S	R	S	S	S	S	Imp
54	1495065	15161	8	M	ENT A unit	B/L CSOM	NIL	Right ear	NO	Pf, Ep, GNB	P.aeru	Acinetobacter sp			S					S	S	R	S	S	S	S	Imp
55	399106	69	24	M	Ortho A unit	Chronic Osteomyelitis	NIL	Left leg	YES	Pp, Ef, GNB	P.aeru				S					S	S	R	S	S	S	S	Imp
56	Forensic	349	26	F	Forensic	Death due to burns	NIL	Burn site	YES	Pp, Ep, GNB	P.aeru				S					S	S	R	S	S	S	S	Imp
57	400765	407	18	M	Surg D unit	Non healing rt leg ulcer	NIL	Right foot	YES	Pf, Ep, GNB	P.aeru				S					S	S	R	S	S	S	S	Imp
58	434788	16239	43	F	Surg F unit	O/C/O AK amputation	DM & HTN	amputed stump	YES	Pp, Ef, GNB	P.aeru				S					S	S	R	S	S	R	S	Imp
59	1873359	19702	58	M	ENT A unit	Right ear otitis externa	DM	Right ear	NO	Pf, Ep, GNB	P.aeru				S					S	R	R	S	R	R	S	Imp
60	447789	19696	26	M	ENT B unit	B/L CSOM	NIL	Left ear	NO	Pf, Ep, GNB	P.aeru				S					S	R	R	S	R	R	S	Imp
61	4183145	4720	3	M	Paed C unit	TB meningitis with ET tip infection	NIL	ET tip	YES	Pp, Ep, GNB	P.aeru				S					S	R	R	S	R	R	S	Imp
62	424555	6228	50	M	Surg C unit	Post op colostomy with drain infection	DM & HTN	Drain	NO	Pf, Ep, GNB	P.aeru				S					S	R	R	S	R	R	S	Imp
63	431027	10631	58	M	Surg B unit	Diabetic foot	DM & HTN	Left foot	YES	Pp, Ep, GNB	P.aeru				S					S	R	R	S	R	R	S	Imp
64	430674	9754	35	F	Surg C unit	Gangrenous appendicitis	NIL	op site	YES	Pf, Ep, GNB	P.aeru				S					S	S	R	S	R	R	S	Imp
65	431018	10325	45	M	Surg C unit	Left LL necrotizing fascitis	DM	Left leg	YES	Pf, Ep, GNB	P.aeru				S					S	S	R	S	R	R	R	Imp
66	410485	2907	30	M	ENT C unit	B/L CSOM	NIL	Left ear	NO	Pf, Ep, GNB	P.aeru				S					S	S	R	S	R	R	R	Imp
67	409995	2804	18	F	ENT D unit	left CSOm	NIL	left ear	YES	Pp, Ep, GNB	P.aeru				S					S	S	R	S	R	S	R	Imp

SL. NO	IP NO	LAB NO	AGE(yrs)	SEX	WARD	CLINICAL DIAGNOSIS	UNDERLYING DISEASE	SITE OF PUS COLLECTION	PRIOR ANTIBIOTIC TREATMENT	DIRECT SMEAR	MONOMICROBIAL	POLYMICROBIAL	Ceftazidime(CAZ)			Imipenem(I)					G	Cot	C	Cip	Pi	Ak	OUTCOME
													R		S	R				S							
													DDST	DDT		IMP-EDTA CDT	IMP-EDTA DDST	EDTA Disc potentiation test	Mod HODGE test								
68	398553	7	56	M	Ortho C unit	Rt femur infected #	DM	Rt leg	NO	Pf, Ep, GNB & GPC in clusters	P.aeru	MSSA			S					S	S	R	S	R	S	R	Imp
69	426449	6287	28	M	Med A unit	Wound infection	NIL	Wound site	YES	Pp, Ef, GNB	P.aeru				S					S	S	S	S	R	S	R	Imp
70	398269	235	60	M	Surg D unit	Diabetic foot	DM & HTN	Knee amputation site	NO	Pp, Ep, GNB	P.aeru				S					S	S	S	R	S	S	R	Imp
71	399198	71	60	M	Surg C unit	Diabetic foot	DM & HTN	Left foot	YES	Pf, Ep, GNB	P.aeru				S					S	S	S	R	S	S	R	Imp
72	397983	44	24	F	Gynac D unit	Episiotomy wound infection	NIL	episiotomy wound	YES	Pp, Ef, GNB	P.aeru				S					S	S	S	R	S	S	R	Imp
73	patho staff	5948	62	F	Self	Chronic tonsilitis	DM	Ulcer on tonsil	YES	Pf, Ep, GNB	P.aeru				S					S	S	S	R	S	S	R	Imp
74	1691619	5996	20	F	ENT C unit	Right ear CSOM	NIL	Right ear	NO	Pf, Ep, GNB & GPC in clusters	P.aeru	MSSA			S					S	S	S	R	S	S	R	Imp
75	423351	6875	51	M	Surg B unit	Diabetic foot	DM & HTN	Right foot	NO	Pp, Ep, GNB	P.aeru				S					S	S	S	R	S	S	S	Imp
76	433661	6929	3	F	ENT A unit	Right ear CSOM	NIL	Rt ear	NO	Pf, Ep, GNB	P.aeru	Proteus mirabilis			S					S	S	S	S	S	S	S	Imp
77	407750	2810	37	M	Ortho A unit	Infected stump site	NIL	Stump site	YES	Pp, Ep, GNB	P.aeru				S					S	R	S	S	S	S	S	Imp
78	420700	5249	32	F	FLR	LSCS wound infection	NIL	LSCS site	NO	Pp, Ef, GNB	P.aeru	K.pneu			S					S	R	S	S	S	S	S	Imp
79	412726	3458	16	F	ENT C unit	Post MRM Rt ear	NIL	Rt ear	YES	Pp, Ep, GNB	P.aeru				S					S	R	S	S	S	S	S	Imp
80	498270	656	45	F	ENT A unit	B/L CSOM	NIL	Left ear	YES	Pf, Ep, GNB	P.aeru				S					S	R	S	S	S	S	S	Imp
81	401812	584	50	F	Surg C unit	Cellulitis	DM, HTN &CRF	Rt leg	YES	Pp, Ef, GNB	P.aeru				S					S	R	S	S	S	R	S	Imp
82	414338	5332	80	F	Surg F unit	Post op wound inf	DM & HTN	Op. site	NO	Pf, Ep, GNB	P.aeru				S					S	R	S	S	S	R	S	Imp
83	950501	3841	11	M	ENT C unit	Rt ear CSOM	NIL	Rt ear	NO	Pp, Ep, GNB	P.aeru				S					S	R	R	R	S	S	S	Imp
84	1603643	3716	17	M	ENT A unit	Rt ear CSOM	NIL	Rt ear	NO	Pf, Ep, GNB	P.aeru				S					S	R	R	S	R	S	S	Imp
85	1607891	3748	29	M	ENT D unit	Left ear CSOM	NIL	Left ear	YES	Pp, Ep, GNB	P.aeru				S					S	R	R	S	R	S	S	Imp
86	422178	5857	27	M	Ortho A unit	Right leg OM	NIL	Rt thigh abscess	YES	Pp, Ep, GNB & GPC in clusters	P.aeru	MRSA			S	N	N	P	P		R	R	S	R	S	S	Imp
87	425609	6166	60	M	Surg C unit	Necrotizing fascitis	DM & HTN	Rt leg	NO	Pp, Ep, GNB	P.aeru				S	P	N	N	N		R	R	S	R	S	S	Exp
88	424555	6228	50	M	Surg C unit	Post op colostomy with drain infection	DM & HTN	Drain	NO	Pp, Ep, GNB	P.aeru				S	N	N	P	P		R	R	S	R	S	S	Imp
89	431027	10631	58	M	Surg B unit	Diabetic foot	DM & HTN	Left foot	NO	Pp, Ep, GNB	P.aeru				S	P	N	N	N		R	R	S	R	S	R	Amp
90	431945	11180	62	M	Surg C unit	Right foot nonhealing ulcer	DM & HTN	Right foot	YES	Pp, Ep, GNB & GPC in clusters	P.aeru	MSSA			S					S	R	R	R	R	S	R	Imp