Nucleic Acid Based Techniques for the Detection and Identification of Drug Resistance among Ocular Bacterial Pathogens

THESIS

Submitted in partial fulfilment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

M. SOWMIYA

2008 PHXF021P

Under the Supervision of

Dr. J. MALATHI

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

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CERTIFICATE

This is to certify that the thesis entitled ***"Nucleic Acid Based Techniques for the Detection and Identification of Drug Resistance among Ocular Bacterial Pathogens"*** *and submitted by* ***Ms. M. SOWMIYA,*** *ID No.* ***2008 PHXF021P*** *for award of* ***Ph.D Degree*** *of the Institute embodies original work done by her under my supervision.*

Signature in full of the supervisor: _____

Name in Block letters : Dr. J. MALATHI

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LIST OF ABBREVIATIONS

ABBREVIATIONS	DESCRIPTIONS
AH	Aqueous humor
ATCC	American type culture collection
BA	Blood agar
BBA	Brucella blood agar
BHIB	Brain Heart Infusion Broth
Bla	Beta-lacatamases
BLIs	Beta lactamase inhibitors
BORSA	Borderline resistant *S. aureus* strains
CA	Chocolate Agar
CA-MRSA	Community-acquired MRSA
CALT	Conjunctiva Associated Lymphoid Tissue
CaMHB	Cation adjusted Mueller Hinton Broth
CA-MRSA	Community Acquired Methicillin Resistant *Staphylococcus aureus*
CLSI	Clinical laboratory Standards Institute
CoNS	Coagulase-negative *staphylococci*
CFU	Colony Forming Unit
DCR	Donor Corneal Rim
DDST	Double Disc Synergy Test
dHPLC	Denaturing High-Performance Liquid Chromatography
DNA	Deoxyribonucleic acid
dNTPs	deoxy Nucleotide Tri-Phosphate
ddNTPs	dideeoxy Nucleotide Tri-Phosphate
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
ESBLs	Extended Spectrum of Beta-Lactamases
EVS	Endophthalmitis Vitrectomy Study

ABBREVIATIONS	DESCRIPTIONS
FC	Flucytosine
FRET	Fluorescence resonance energy transfer
FU	Fluorouracil
F Dump	Fluoro Dump
GISA	Glycopeptide Intermediate *S. aureus*
GITC	Guanidine iso Thio Cyanate
HA-MRSA	Hospital Acquired Methicillin Resistant *Staphylococcus aureus*
HBSS	Hanks Balanced Salt Solution
HIV	Human Immunodeficiency Virus
IC	Inhibition Concentration
IOL	Intra Ocular Lens
I_G	Immunoglobulin
IRB	Institutional review board
KOH	Potassium hydroxide
LB	Luria Bertani broth
MAC	MacConkey Agar
MALT	Mucosal Associated Lymphoid Tissue
MBL	Metallo-Beta-Lactams
$MgCl_2$	Magnesium chloride
MDR	Multi Drug Resistant
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
M-K medium	McCarey-Kaufman medium
MOD	Multi Organ Donor
MRS	Methicillin Resistant Staphylococci
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*

ABBREVIATIONS	DESCRIPTIONS
MRSE	Methicillin Resistant *Staphylococcus epidermidis*
MSSE	Methicillin Sensitive *Staphylococcus epidermidis*
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
MQW	Milli Q water
MIC	Minimum Inhibitory Concentration
MW	Molecular weight marker
MHA	Muller Hinton agar
nPCR	Nested Polymerase chain reaction
NaOH	Sodium hydroxide
Nacl	Sodium chloride
NC	Negative Control
NCBI	National Centre for Biotechnology Information
NDM	New Delhi Metallo-Beta-Lactamases
OD	Optical Density
PBS	Phosphate buffer Saline
PVL	Panton-Valentine Leukocidine
PBP	Penicillin-binding protein
PC	Positive Control
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction – Restriction Fragment Length Polymorphism
PDB	Protein Data Bank
PFGE	Pulse Field Gel Electrophoresis
PK	Penetrating Keratoplasty
16S rRNA	16S ribosomal ribonucleic acid
RAPD	Randomly amplified polymorphic DNA

ABBREVIATIONS	DESCRIPTIONS
Rep PCR	Repetitive sequence based PCR
RR mix	Ready reaction mix
RT	Room Temperature
SDA	Sabouraud's Dextrose Agar
snPCR	Semi nested Polymerase Chain reaction
SSCP	Single strand confirmation polymorphism
SCC *mec*	Staphylococcal cassette chromosome *mec*
Tn	Transposon
TRUST	The Resistance U.S Tracking
UV	Ultraviolet
VF	Vitreous fluids
VRSA	Vancomycin Resistant *Staphylococcus aureus*

ABBREVIATIONS	DESCRIPTIONS
bp	Base pair
fg	Femtogram
g	Grams
mM	Millimolar
mm	Millimetre
mg	Milligram
ml	Millilitre
ng	Nanogram
pg	Pictogram
µg	Microgram
µm	Micrometre
µl	Microlitre

ABSTRACT

Antibiotic resistance among ocular pathogens is increasing in consonance with the increase of resistance among bacteria and fungi associated with systemic infections. The factors contributing to the development of drug resistance among ocular bacterial isolates include overuse of antibiotics for systemic infections, increased drug resistance are improper dosing regimen, misuse of antibiotics and extended duration of therapy and finally the overuse of topical antibiotics in the eye.

The rate of prevalence of microorganism and the predominant bacterial species isolated among ocular specimens in our hospital population were studied to understand predominant and emerging ocular bacterial pathogens and to understand the current rate of occurrence of Polymicrobial infections (PMI) and a special focus was given to emerging drug resistance among them. The predominant bacterial species identified in the current study includes *Coagulase negative Staphylococci* (10.4%), *Coagulase positive Staphylococci* (6.5%) and *Enterobacteriaceae* (5.9%). *P. acnes* is the predominant anaerobe (2.6%). The rate of PMI was 3.9%

The conventional methods involved in the detection of bacterial pathogens in intraocular specimens are less sensitive due to lesser amount of available clinical specimen and low microbial threshold, laborious and time consuming techniques. Hence raise the need for rapid diagnostic molecular techniques. Polymerase Chain Reaction (PCR) based DNA sequencing along with Denaturing High Performance Liquid Chromatography (dHPLC) posed the ability to unequivocally identify the non-cultivable

pathogen and detection of more than one microbial species present in ocular clinical specimens. Our study is the first study to identify the microorganisms from culture negative, PCR positive intraocular specimens. Among 116 intraocular specimens studied, single bacterial genome was detected in 92 (79.3%) and two or more genomes in 24 (20.7%) specimens. Sixty-nine different bacteria were identified by this method of which many were uncultivable by conventional methods. Forty eight different bacteria detected in this study are being reported for first time in infectious endophthalmitis.

Methicillin resistant Staphylococci (MRS) has emerged as a dreaded organism because of its wide range of resistance to several groups of antibiotics. Its prevalence in conjunctivitis is highly variable. Situation in case of *coagulase negative staphylococci* (CoNS), a common cause of keratitis and endophthalmitis, is no less precarious. The isolation rate of ocular MRS was 63.6 % and MRS constituted a larger portion of *Staphylococcus*. Our study demonstrated that, PCR assay was superior in identifying intermediate and heterogeneous MRS in shorter duration of time & PCR-RFLP of *gap* gene being specific for speciation of *Staphylococcal spp.*

Recently, serious concern has been initiated to analyze the factors contributing for high degree of resistance shown by MDR Gram negative bacteria causing ocular infections. Hence, screening and identification of predominant drug resistance encoding ESBL genes among the ocular isolates of *Enterobacteriaceae* was carried out. *CTX-M-15* was demonstrated to be the common ESBL genotypes present among *Enterobacteriaceae* isolated in our hospital set up. The overall rate of ESBL positivity seen anong our unb was 31.5% in hospitalized patients and 68.5% in community acquired infections.

Reports on existence of *blaNDM-1* genes among bacteria causing systemic infections is being published in increasing frequency but our study proved the absence of *blaNDM-1* genes among the ocular isolates of *Enterobacteriaceae.* PCR based molecular technique for screening *blaNDM-1* genes among ocular isolates of *Enterobacteriaceae* isolates was standardized to detect the most virulent gene *blaNDM-1*. Our study proved the absence of *blaNDM-1* genes on the ocular isolates of *Enterobacteriaceae. CTX-M-15* was demonstrated to be the common ESBL genotypes. Overall rate of ESBL positivity was 31.5% in hospitalized patients and 68.5% in community acquired infections. The *blaNDM-1* gene was absent among ocular isolates of *Enterobacteriaceae.*

Most of the ocular infections arise due to external source. Hence predominant community related extra ocular specimens such as conjunctival swabs collected from multi organ donor (MOD) and Donor corneal rim specimens (DCR) were studied to understand the drug resistance exhibited by these extra ocular specimens. Prevalence of antibiotic resistance was significantly higher among Gram positive group of organisms. Gram negative isolates showed 29.1% positivity for ESBL. A *blaNDM-1* carrying *A. baumannii* was isolated from a MOD. Hence antibiotic susceptibility testing of DCR and conjunctival swabs from multi organ donor prior to transplantation will aid in understanding antibiotic pattern and initiation of appropriate antibiotic therapy following penetrating keratoplasty.

Efficiency of molecular interactions inferred through *in silico* docking studies in understanding the drug binding interactions of *blaNDM-1* gene was evaluated in this

current study. Bioinformatics assay paved the way in knowing the correlation between *blaNDM-1* positivity and biofilm production through *in silico* docking studies which proved its attribution to high level resistance. Further our study also highlighted the potential of 162-166 region in *blaNDM-1* as a vulnerable site for drug target since *blaNDM-1*, has become a significant threat to human health due to the extensive drug resistance, leaving few or no therapeutic options thus becoming a major public health problem throughout the world, particularly in India.

P. acnes is the most predominant anaerobe isolated from ocular specimens in our hospital set up. Detailed studies have not been carried out so far with *P. acnes* isolated from wide range of ocular clinical specimens. Hence phenotypic and genotypic studies to understand the pathogenicity of the bacterium, co-existent site of infection and clinical correlation with the phenotypic and genotypic results of the *P. acnes*, drug resistance and its relationship with biofilm production were initiated. Phylogenetic analysis of *P. acnes* proved that type I A, B, C were prevalent among the isolates while type II were isolated from only conjunctival isolates. PCR-Restriction Fragment Length Polymorhism (PCR-RFLP) was found to be a less expensive, sensitivity and specific technique for subtyping *P. acnes*. Biofilm producing cells possessed increased resistance. This could explain the emergence of resistance due to presence of biofilms in treatment of ocular infections. RAPD analysis was proved to be an alternative rapid, reproducible tool for genomic typing method for *anaerobes* and molecular methods proved to be faster and much earlier application of appropriate therapy and minimize empiric use of most powerful agents.

Overall our study has shown the rate of prevalence of bacterial infections along with prevalence of PMI for the first time in literature. Our study is the first of its kind to show application of PCR/dHPLC approach for detection and identification of bacterium from culture negative intraocular specimens in endophthalmitis cases. PCR assay was superior in identifying MRS species in shorter duration of time. PCR-RFLP of *gap* gene was found to be specific for typing Staphylococcal spp. This is the first extensive study to screen ocular isolates for detection of ESBL genes. *CTX-M-15* was the common ESBL genotypes and *blaNDM-1*genes was absent among *Enterobacteriaceae* isolates. Correlation between *blaNDM–1* positivity, biofilm production with molecular interactions inferred through *in silico* docking has been proven for the first time in Indian literature. Type I A, B, C genotypes were prevalent among *P.acnes* isolates and type II strains were from conjunctival swabs alone. No association was observed between genotypes of *P. acnes* and site of infection /their degree of resistance to drugs.

In conclusion, for the first time in literature, our study has proved that application of nucleic acid based molecular methods are more sensitive to detect and to identify the bacterial agent causing ocular infections compared to phenotypic methods. The study was also aided in application of these techniques in monitoring drug resistance exhibited by ocular bacterial pathogens in Indian scenario for the first time in our study. These techniques would be of great importance in any ophthalmic setting in detection and identification of ocular infections causing bacteria and their drug resistance genes. This methodology adopted in the present study can be extended to detect drug resistance genes from any other body specimens, in any part of the world.

Chapter - 1

An overview of bacterial infections of the eye, laboratory methods for detection and identification of the pathogenic bacteria associated with the disease and its drug resistance

1.1. Introduction to Antibiotic resistance in ocular infections:

Ocular surface is covered with tear film that protects, lubricates, and serves as the major refractive surface. Human tear film plays an important role in protecting the ocular surface from various pathogens (Gachon and Lacazette, 1998, O'Callaghan, et al., 2003). Mucins, produced by conjunctival goblet cells and corneal and conjunctival epithelial cells, provide a barrier to pathogen adherence and penetrance into the ocular surface epithelium. Cytokines, including epidermal growth factor, transforming growth factor-ß, and hepatocyte growth factor, in the tears also exert antimicrobial properties (Fleiszig et al., 1994, Gipson and Argueso, 2003, Berry et al., 2002).

Eye an organ of the human body is impermeable to almost all external infectious agents (Ramesh et al., 2010) though ocular surface is invariably exposed to a wide-array of microorganisms (Akpek and Gottsch, 2003). The microbial infection of the eye is via external sources or through intraocular invasion of micro-organisms that are disseminated from the blood stream (Bharathi et al., 2010) or due to breach in the ocular barriers (Ramakrishnan et al., 2009). External bacterial infections of the eye are usually localized but may frequently spread to other tissues.

The eyelid and conjunctiva have a normal microbial flora controlled by its own mechanism and by the host (Bharathi et al., 2010).

Alterations of the normal flora of the eye contribute to varied ocular diseases (Ramesh et al., 2010, Sharma, 2011). The most common ocular infection seen is bacterial conjunctivitis, which is self-limiting and largely presents as an acute infection (Hovding, 2008, Rose, 2007). Bacterial keratitis, an infection of the cornea often associated with contact lens wear, ocular trauma, or ocular surface disease, is less common but poses a risk of loss of vision (Allan and Dart 1995, Bourcier et al., 2003, Green et al., 2008). Endophthalmitis is a rare but potentially sight-threatening infective complication of intraocular surgery (primarily cataract), intravitreal injections, and ocular trauma (Callegan et al., 2007, DeCroos and Afshari 2008). Bacterial blepharitis is an inflammation of the eyelids, particularly at the lid margins, that may be associated with a low-grade staphylococcal bacterial infection (Kowalski et al., 2005). Orbital cellulitis and other periorbital infections can be caused by a variety of organisms, including bacteria, and occurs as a complication of surgery, nonsurgical trauma, or the retention of a foreign body (Tovilla-Canales et al., 2001). Dacryocystitis, or infections of the lacrimal sac, are common at all ages and occur due to acquired or congenital obstruction of nasolacrimal duct (Campolattaro et al., 1997).

Most predominant bacterial pathogens causing ophthalmic infections include *Staphylococcus aureus, Coagulase negative staphylococci, Genus Streptococcus, Corynebacterium, Bacillus, Pseudomonas aeruginosa, Enterobacteriaceae,*

Nocardia, *Non-fermentors* and in others group of bacteria (Anand et al., 2000, Bharathi et al., 2012, Kunimoto et al., 1999).

Despite the protection by the components of tear in protecting the ocular surface from various pathogens along with the blinking action of the eyelids, the resident bacteria of the conjunctival sac or the environmental bacteria establish infection resulting in the need of antibiotics intervention to treat the disease (Sharma, 2011). Although treatment guidelines for these ocular infections recommend that laboratory culture and smear tests be conducted, when possible, for determination of the causative pathogens, in practice the initial choice of antibiotic therapy is generally made without knowing the identity or susceptibility of the ocular pathogen (Bertino, 2009, DeCroos and Afshari, 2008).

The major route of infection is by the entry of conjunctival bacterial flora at the time of surgical procedure. The most widely applied method to reduce the rate of ocular infection following surgery is by the application of topical povidone-iodine or the antimicrobial agents pre-operatively to reduce microbial flora around the eye. Some surgeons use intraoperative subconjunctival or intraocular infusion of optimal antibiotics (Kim and Toma, 2012, Liesegang, 2001). The broad-spectrum antibiotic therapy for the bacterial infections is initially used in order to prevent a decline in the vision or permanent vision loss that may require surgical intervention (Callegan et al., 2007).

The optimal choice of preoperative topical antibiotics depends on many factors, including the isolated bacteria, their antibiotic susceptibility and resistance

patterns, rapidity of action, rate of penetration, and toxicity (Dawood and Ahmad, 2005). Repeated exposure of ocular flora (microbes living on or inside the body), however, may select for resistant bacterial strains and cultivate 'superbugs' with multiple-drug resistance that may considerably affect the treatment of ocular infections caused by such bacteria into the eye (Kim and Toma, 2012). Frequent or inappropriate, systemic long-term use of an antibiotic may also result in the development of bacterial antibiotic resistance (Bertino 2009).

Thus knowledge on the antimicrobial resistance is especially to commonly used antibiotics is very important. Timely institution of appropriate therapy must be initiated to control the infections and thereby minimize ocular morbidity. If they are not treated promptly, it may lead to sight threatening condition (Bharathi et al., 2010, Sharma, 2011). The factors contributing to development of drug resistance among ocular isolates include overuse of antibiotics for systemic infection as well as overuse of antibiotics in the eye (Sharma, 2011, Fong et al., 2007). As a result, periodic susceptibility testing along with molecular biological techniques for the detection of emerging antibiotic resistance among clinical isolates of bacteria is warranted to understand the prevalence of drug resistance to higher levels of drugs and also to ensure the availability of broad spectrum antimicrobials.

1.2. Anatomy of the Eye:

Orbits, Their Soft Tissue Contents and Adjacent Structures:

Orbits are pear-shaped bony cavities that contain the globes, extraocular muscles, nerves, fat and blood vessels (Figure 1a) (Klotz et al., 2000, Moseley, 1993). Walls of the orbit comprises of seven bones. Periosteal covering of orbital

bony cavity fuses anteriorly with the orbital septum and posterior with the duramater. Abscesses can localize in the subperiosteal space. The roof, medial wall, and floor of orbit separate it from adjacent paranasal sinuses, including maxillary, frontal, ethmoid, and sphenoid sinuses. The paranasal sinuses arise from and drain into the nasal cavity. Thus, an intimate anatomical relationship exists between orbit and adjacent paranasal sinuses, and latter may be the source of an orbital infection. (Klotz et al, 2000)

Eyelids:

Eyelids perform several functions for the eye, from distributing tears to protect cornea **(**Hughes, 2004). Eye lid possesses protective anatomical barriers preventing penetration of pathogens beyond anterior surface of the globe. First is orbital septum, a thin multiplayer fibrous tissue that divides orbit from eyelid and serves as a physical barrier to prevent infections. Second is conjunctiva that is reflected back on itself. This prevents material on anterior surface of globe from freely moving posteriorly (Klotz et al, 2000).

Lacrimal System:

Lacrimal system is comprised of lacrimal gland, accessory glands, and the excretory system (Van Haeringen, 1997). The lacrimal gland secretes tears that pass down over the cornea and enter the lacrimal excretory system at the puncta, which drains tears into the canalicular system that leads to the lacrimal sac, which drain in to the nose. With total nasolacrimal duct obstruction, infected material in the sac may reflux onto the ocular surface (Klotz et al., 2000, Hughes, 2004).

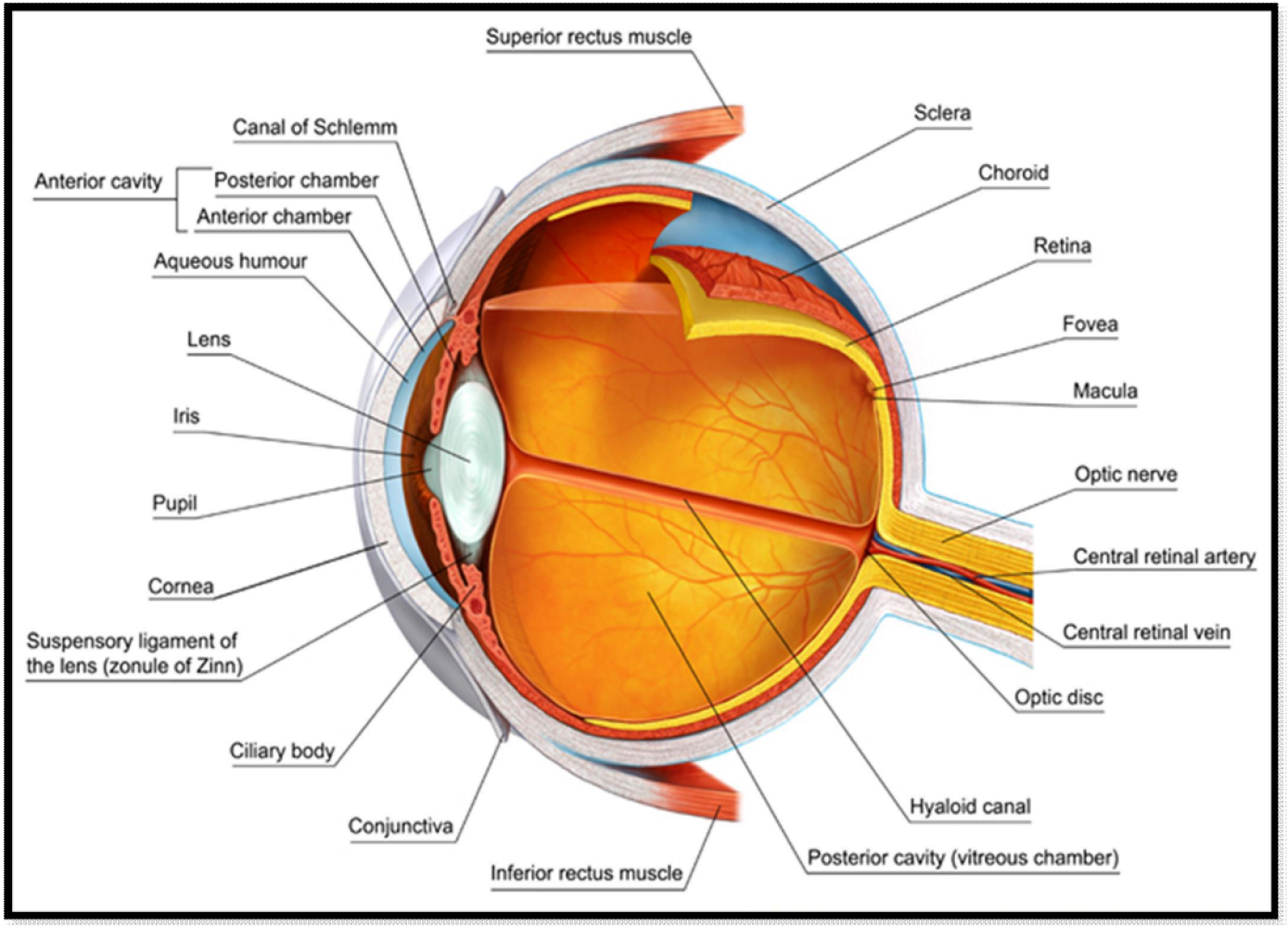


Figure 1a: Anatomy of the Eye. The human eye in situ with the tunics peeled back, exposing a portion of vasculature of retina, lens, and anterior chamber as seen from the side. (Source: From http://www.virtualmedicalcentre.com/anatomy/the-eye-and-vision/28)

The Globe:

The adult human eye is approximately 24 mm in anterior posterior length and is 6 mm^3 in volume. The basic structure of the globe consists of three concentric layers or tunics. The outermost tunic is comprised of the cornea and sclera, middle tunic is the uveal tract, which consists of choroid, ciliary body, and iris and innermost tunic is the retina (Klotz et al., 2000).

Sclera:

The normally near-white sclera extends from the limbus to cover the rest of the globe. The function of the sclera is to provide a firm protective coat for the intraocular contents. The posterior outer layer of the globe is the sclera, which is comprised of collagen and ground substance. The sclera is essentially avascular except for superficial episcleral vessels and the intrascleral vascular plexus (Watson, 2004). The scleral width ranges from 0.3 to 1.0 mm (Klotz et al., 2000, Hughes, 2004, Watson et al., 2006).

Episclera:

Episclera, or Tenon's capsule, provides for part of the nutrition of the sclera and for the cellular response to inflammation. It acts as a synovial membrane for smooth movement of the eye and, together with muscular sheaths to which it is fused, provides a check on excessive movement (Klotz et al,. 2000, Hughes, 2004, Watson et al., 2006).

Choroid:

Choroid is a vascular tunic that comprises the posterior portion of uveal tract. The purpose of this highly vascularized tissue is to provide nutritive support to the outer

layer of the retina (Hayreh, 1975). Blood flow and oxygenation of choroid are very high compared to other tissues in the body. Because of these qualities, choroid serves as a fertile site for proliferation of hematogenously spread pathogens (Klotz et al, 2000).

Anterior Chamber and Aqueous Humor:

Anterior chamber is a space bordered anteriorly by the cornea and posteriorly by the iris diaphragm and pupil and is filled with aqueous humor (Figure 1a, left). Aqueous humor, produced by non pigmented ciliary epithelium in the posterior chamber, passes through the pupillary aperture into the anterior chamber (Klotz et al, 2000).

Cornea:

The cornea is avascular, and its stroma is composed of highly organized collagen fibrils. A tear film comprised of three layers covers the anterior surface of the cornea (Klotz et al, 2000). The cornea (and other transparent tissue) appears "clear" because water is actively pumped out of it. When it absorbs water (or is injured), it puffs up and clouds, thus affecting the appearance of a cornea. The cornea is responsible for protecting the eye against incidences such as injury and infection (Maurice, 1957).

Iris:

Iris is anterior extension of the ciliary body that forms a contractile diaphragm in front of anterior surface of the lens (Klotz et al, 2000). Iris ranges in size from 11 mm to 13 mm; visible shape of iris is determined by clarity of the clear cornea (Hughes, 2004, Morrison, 2010).

Pupil:

The central aperture in the iris is the pupil, which constantly changes size in response to light intensity. The size of the pupil is generally altered only by actions of the sphincter and dilator muscles, although it can be changed by medications and surgery. An average size can be estimated at 2 mm to 4 mm (Klotz et al, 2000, Hughes, 2004).

Limbus:

Anatomical limbus contains drainage angle, wherein resides the watery aqueous humor (produced in ciliary body and filling anterior chamber) (Hughes, 2004). Limbus contains radially oriented fibrovascular ridges known as palisades of Vogt that has stem cell population (Thomas, 2007).

Posterior Chamber, Lens and Vitreous Humor:

The posterior chamber is bordered anteriorly by the iris diaphragm and pupil and posteriorly by the lens and zonules. The lens is an avascular biconcave crystalline structure centrally located in the posterior chamber. It continues to grow throughout life, receiving nutrition from the aqueous and vitreous humors. The vitreous is a gel-like substance occupying the posterior segment of the eye. The vitreous consists of a collagen framework interspersed with hyaluronic acid. In its normal state, it is optically clear, whereas during intraocular inflammation it may become hazy (Klotz et al, 2000).

Retina and Optic Nerve:

Retina is the thin, transparent, net-like membrane that captures light energy. It comprise of 10 layers, with the layer nearest the interior of the globe containing the photoreceptors called rods and cones. The inner half of the retina receives its blood supply from the central retinal artery, and the outer half receives its blood from the choroid (Klotz et al, 2000). The inner cell layer axons in the retina exit the globe to make up the optic nerve. Optic nerves are vulnerable to infectious processes originating both within cranial vault and within the orbits (Jonas et al., 1992).

1.3. Microbial flora of the normal eye:

The human conjunctiva is first exposed to microbes when an infant passes through the birth canal. Right after birth, the eye is exposed to the environmental sources of bacteria such as air, contaminated material, and people. The most common aerobic bacteria isolated immediately after birth from the conjunctiva are the *Staphylococcus aureus* (coagulase-negative), diphtheroids and *Streptococcus spp. Propionibacterium acnes*, *Bifidobacterium*, and *Bacteroides* spp. are isolated anaerobically from the newborn conjunctiva after vaginal delivery. *Lactobacillus* spp. can also contaminate the conjunctiva from the vaginal tract (Kowalski, 2009). The conjunctiva is sterile in 20% to 87% of infants born vaginally (Isenberg et al., 1988). Conjunctiva cultured after cesarean section is sterile in 80% to 95% of infants (Lee et al., 1989). If cesarean section is delayed for more than 3 hours after the sac is ruptured, conjunctivas are sterile in 45% of newborns. During first several days of life, the infant's own cutaneous, respiratory tract, gastrointestinal flora, and the surrounding

environmental flora are important new sources of bacteria for the conjunctiva. After birth, before prophylaxis, there is a dramatic increase in frequency of culture-positive conjunctivas, from as few as 13% of newborns to 98% of 3 to 5 day old infants. Bacteria isolated include *Staphylococcus spp.*, *Streptococcus spp.* or *Moraxella (Branhamella) catarrhalis* (Isenberg et al., 1988).

Indigenous microbial flora of the ocular surface is primarily comprised of Gram-positive organisms, composed of Coagulase negative *Staphylococcus* spp. and diphtheroids. Generally, the density of isolation is limited to fewer than 10 colonies on culture, compared with the confluent growth of infection. Studies have shown that *Staphylococcus epidermidis* has developed strategies that have allowed it to overcome tear defenses and become part of the normal ocular flora. Isolates have demonstrated resistance to the action of lysozyme, lactoferrin, and other constitutive tear proteins (Leitch et al., 1997, Krohn et al., 1993). Organisms isolated from conjunctival surface, transiently include *S. pneumoniae*, *Haemophilus* spp. and *Staphylococcus aureus*, *Streptococcus* spp., are isolated more commonly from children than from adults. *Propionibacterium acnes* (*P. acnes*), diphtheroids, and gram-negative bacteria are isolated more frequent in adults. These isolates are probably transitory residents passed to the eye from other parts of the body ie.adjacent skin or by the hands. Anaerobic bacterial flora of skin and mucous membrane, including *P. acnes* and *Peptostreptococcus* spp., has been isolated from outer eye (Kowalski and Roat, 2006, Singer, 1988).

Microbial flora of normal eye:

*** AEROBIC : Gram Positive Cocci**

Staphylococcus epidermidis	30-80%
Staphylococcus aureus	3-25%
Micrococcus spp.	1-28%
Streptococcus pyogenes	0-03%
Streptococcus pneumoniae	0-03%

Gram Negative Cocci - *Moraxella catarrhalis* 2-05%

Gram Positive Bacilli - *Corynebacterium spp.* 5-83%

Gram Negative Bacilli

Haemophilus influenzae	0-01%
Klebsiella spp.	0-0.5%
Escherichia coli	0-01%
Pseudomonas aeruginosa	0-02%
Moraxella spp.	0-02%

*** ANAEROBIC :**

Propionibacterium spp.	0-33%
Peptostreptococcus	0-02%
Bacteroides spp.	0-0l%
Lactobacillus spp.	0-02%
Clostridium spp.	0-02%

*** FUNGUS :** These are transient and are those found in the environment.

*** VIRUS :** These are not normal residents.

(Source: Therese KL and Madhavan HN. Microbiological Procedures for Diagnosis of Ocular Infections (http://www.ijmm.org/documents/ocular.pdf)

1.4. Surface protection of the eye:

The normal conjunctiva and cornea are protected by a triple-layered tear film comprising an outer oily layer from the meibomian glands, an aqueous layer from lacrimal glands and an inner layer of mucus, derived chiefly from the conjunctival globlets. Blinking maintains the integrity of this protective layer (Seal, 2007)

Immunological components present in Tears:

Human tear film plays an important role in protecting the ocular surface from various pathogens. The tear film contains lactoferrin, lysozyme, and beta-lysin, which have antimicrobial effects (Londer, 1974). Immunoglobulins, complement, cytokines inflammatory mediators such as histamine and prostaglandins are also present in tears. Lysozyme attacks the mucopeptides of the cell walls of susceptible bacteria while lactoferrin has bacteriostatic properties (Hori, 2008, Seal, 1986, Lemp, 2006). Secretory IgA is the major immunoglobulin in tears. IgG is usually present in lesser amounts compared to IgM and IgE. IgA is important in promoting microbial phagocytosis, inhibiting epithelial binding by microbials, interfering with bacterial exotoxins, and facilitating antibody-dependent cell-mediated cytotoxicity (Seamone & Jackson, 2006, Johnson & Murphy, 2004).

Tears play an important role in maintaining the normal health of the ocular surface and in the repair of injury to the ocular surface.. Tears contain several water-soluble substances with antibacterial properties (Lemp, 2006, Holly, 1977). Total tear volume under relatively non-stimulated conditions is approximately 6 to 8µl (Jordan,

1976). Proteins secreted by the lacrimal glands include lysozyme, lactoferrin, free albumin, lactoperoxidase, transferrin, lipocalin, phospholipase, free albumin, and secretory IgA, epidermal growth factor, basic fibroblastic growth factor, transforming growth factor α and β, and retinols in the tears also found to exert antimicrobial properties (Hori, 2008, Seal, 1986, Lemp, 2006, Seamone and Jackson, 2006, Johnson and Murphy 2004).

Immunological components present in Conjunctiva:

Conjunctiva also provides an immunologic as well as a physical barrier to the environment. The conjunctiva contains blood vessels, lymphoid tissue, and immunoreactive cells, including lymphocytes, neutrophils, Langerhans cells, neutrophils, and mast cells. Lymphoid cell population of the conjunctiva-associated lymphoid tissue (CALT) is analogous to that of other mucosa-associated lymphoid tissues (MALT) (Franklin and Remus 1984). Substantia propria (ie. Stroma of cornea) contains T lymphocytes (helper T cell and cytotoxic T-cells). Langerhans cells are the major antigen-presenting cells of the conjunctiva and are located in the epithelium (Sacks et al., 1986).

There are approximately 50 million mast cells in the ocular and adnexal tissues of the human eye (Allansmith, 1982). In normal conditions, mast cells are concentrated in the substantia propria of the conjunctiva but not in conjunctival epithelium. Each mast cell has as many as 500,000 receptors for IgE; IgE is bound to 10% of these receptors, thus coating the mast cell surface and trigger mast cell degranulation,

resulting in type I immune reaction (Butrus, 1988). Eosinophils and basophils are not present in normal conjunctiva but are present in disease states. Conjunctival substantia propria (mostly T cells) and in lacrimal and accessory lacrimal glands (mostly B cells) releases lymphoid cells when the antigen is sensitized (Seamone and Jackson, 2006).

Immunological components present in Cornea:

The normal cornea is devoid of blood vessels, lymphatics, and inflammatory cells. Class II antigen-bearing Langerhans cells are found in corneal epithelium. Although complement components C3, C4, and C5 are found throughout corneal stroma, there is more complement in the peripheral cornea than in the central cornea. IgG is the predominant immunoglobulin in the cornea and probably the most important in microbial defense. However, IgM is found only in the peripheral cornea, probably because its large size restricts diffusion (Mondino, 1988).

1.5. Bacterial infections of the eye:

Blepharitis:

Blepharitis is a condition that is caused by bacterial infection on the surface of the eyelids and lashes. It can cause chronic infection and inflammation to the lids and surface of the eye. Treatments generally start with eyewashes to clean the skin surface of bacteria and other debris, and may include prescription drops or ointments.

Bacteria responsible for Blepharitis: The predominant bacterial agent is *Staphylococcus* spp. i.e. *S. aureus* or *S. epidermidis* (McCulley et al., 2000).

Conjunctivitis:

Conjunctivitis is the inflammation of the bulbar (covering the globe of the eye) and tarsal (lining the orbit) conjunctiva caused by bacterial infections, trauma, or autoimmune disease. Patients will complain of redness, and a foreign body sensation that is often associated with discharge. Most cases of bacterial conjunctivitis resolve spontaneously in a week to 10 days (Mannis and Plotnik**,** 2006, O'Brien, 2009).

Bacterial agents responsible for Conjunctivitis:

The various pathogens of bacterial conjunctivitis include *Staphylococci, Streptococci, N. gonorrohae,* and *C. trachomatis*. Conjunctivitis can be categorized into, Hyperacute (*N. gonorrhoeae, N. meningitides***),** Acute (pathogenic bacteria), Chronic (*S. aureus, M. lacunata, H. infleunzae, H. aegyptius)* (http://www.ijmm.org/documents/ ocular.pdf, Bertino 2009)

Scleritis:

Scleritis a severe destructive disease, sometimes leading to the loss of an eye from deteriorating vision, severe pain, or even (occasionally) perforation of globe. Such changes, when occur, are rapid and therefore an early diagnosis and effective treatments are essential. Onset of scleritis is usually gradual, building up over several days.

Bacterial causative agents of Scleritis:

Scleritis can also be the result of an infectious process caused by bacteria predominantly *Pseudomonas spp.* (Watson, 2006).

Keratitis:

Inflammation of the cornea with or without violation of its epithelium constitutes to keratitis. Patients will present with an acutely red, painful eye and often complain of foreign body sensation, tearing, and vision change. Contact lens use itself imparts a 10-fold risk of developing an infectious keratitis. Corneal abrasions may accompany a keratitis because of excessive rubbing or scratching of the affected eye. Prompt diagnosis, treatment, and identification of cause are paramount to prevent vision loss due to ulceration, necrosis, and scarring (Keay et al., 2006, Titiyal et al., 2006).

Etiological agents of Bacterial keratitis:

Bacterial keratitis is caused by *Staphylococcus* spp., *Streptococcus pneumoniae* (non–contact lens wearer) but in contact lens wearers, *Pseudomonas* spp. may predominate followed by *S. marcescens* and *Moraxella catarrhalis* in immunocompromised patients. *N. gonorrohae* and *C. trachomatis* should be considered in the sexually active patient, particularly if conjunctivitis is present (Schaefer et al., 2001, Mueller, 2008, Bourcier et al., 2003, Bertino, 2009).

Hordeolum and Chalazion:

Patients complaining of acute onset of pain, focal swelling, and lid edema should be evaluated for hordeolum or stye. A hordeolum represents purulent infection of a cilium and adjacent gland with local abscess formation (Mueller, 2008) and Chalazion are granulomatous inflammatory lesions present on the lid that occur from obstruction of a sebaceous gland (Mueller, 2008).

Bacterial agents responsible of Hordeolum and Chalazion infections:

Staphylococcal spp., are the most common causative organism. Cellulitis may accompany the hordeolum. Treatment is conservative, with warm compresses several times a day for 10 minute (Mueller, 2008).

Dacryocystitis:

Dacryocystitis is an inflammation of the lacrimal sac, which usually occurs because of obstruction of the nasolacrimal duct (Bharathi et al., 2008). Obstruction of the nasolacrimal duct from whatever source results in stasis with the accumulation of tears, desquamated cells, and mucoid secretions superior to the obstruction. This creates a fertile environment for secondary bacterial infection (Iliff et al., 1996).

Microbiology of dacryocystitis:

The most common isolates in dacryocystitis are *P. aeruginosa*, *S. aureus*, *Enterobacter aerogenes*, *Citrobacter spp.*, *S. pneumoniae*, *E. coli*, and *Enterococcus spp.*, (Briscoe et al., 2005). Acute dacryocystitis is often caused by Gram-negative group of bacteria. In chronic dacryocystitis, mixed bacterial isolates are more commonly found with the predominance of *Streptococcus pneumoniae* and *Staphylococcus spp.*, *Coagulase-negative Staphylococci* (CoNS) and *S. aureus* are the most frequently isolated organisms in lacrimal sac infections (Mueller, 2008, Bharathi et al., 2008).

Canaliculitis:

Canaliculitis occurs secondary to dacryocystitis or obstruction within the nasolacrimal passage, or after instrumentation of the nasolacrimal passages or implantation of materials such as plastic tubing or silicone (O'Brien and Hahn, 2009, Wulc and Edmonson, 2009).

Causative agents of Canaliculitis:

The most common pathogen is *Actinomyces israelii* and less commonly, *Nocardia* and *Streptomyces* spp., *Propionibacterium propionicus* and *Eikenella spp.* (O'Brien and Hahn, 2009, Wulc and Edmonson, 2009).

Preseptal Cellulitis:

In patients with preseptal cellulitis due to trauma or local infection, (*e.g.*, hordeola, acute chalazia, acute dacryocystitis, or impetigo) cultures from the wound or primary focus of infection should be obtained (Wulc and Edmonson 2009).

Microbiology of Preseptal Cellulitis:

The most common pathogen is *H. influenzae*. *S. aureus* and beta-hemolytic *Streptococci* are the predominant aerobes. Anaerobes, such as *Peptostreptococcus* and *Bacteroides* spp., are associated with infections from human or animal bites. In the absence of trauma or skin infections, preseptal cellulitis in children younger than six years is caused almost exclusively by *H. influenzae* type B or *S. pneumoniae* (O'Brien and Hahn, 2009, Wulc and Edmonson 2009).

Orbital Cellulitis:

Orbital cellulitis is an acute infection of the tissues immediately surrounding the eye, including the eyelids and eyebrow. Symptoms of orbital cellulitis may includes painful swelling of upper and lower eyelid, and possibly the eyebrow and cheek, bulging eyes, decreased vision, eye pain, especially when moving the eye along with fever (O'Brien and Hahn, 2009, Wulc and Edmonson, 2009).

Bacterial agents causing Orbital Cellulitis:

The bacterial agents most commonly responsible are *H. influenza* and *S. pneumoniae,* followed by *Bacillus spp., S. aureus, S. pyogenes* and *Streptococcus viridans.* Polymicrobial infection by a mixture of aerobic and anaerobic bacteria may occur, and orbital cellulitis from unusual anaerobes (*e.g., E. corrodens*) has been reported (O'Brien and Hahn, 2009, Wulc and Edmonson, 2009).

Endophthalmitis:

Infectious endophthalmitis is defined as an inflammation of intraocular tissues or fluids secondary to intraocular infection. Colonization of organisms inside the eye can occur through introduction of infectious agents into the eye following a breach in ocular barriers or by dissemination through the systemic blood stream. Endophthalmitis are categorized according to underlying cause as: (a) Postsurgical: acute, and delayed or chronic; (b) post traumatic; (c) bleb-related; and (d) endogenous: fungal, bacterial, and other. When infectious agents reach vitreous cavity across an opening in the globe, it is

termed as exogenous endophthalmitis and when it occurs by hematogenous spread, it is termed as endogenous endophthalmitis. Exogenous endophthalmitis usually occurs following surgical or traumatic alteration of structural integrity of the globe. Occasionally, exogenous endophthalmitis results from contagious spread of infectious microbes from ocular adnexa, especially following infections on the cornea or sclera (Meredith, 2006, Jambulingam et al., 2010, Ramakrishnan et al., 2009).

Bacterial agents causing Endophthalmitis:

Post-operative infectious endophthalmitis: S*taphylococci spp.* and *Streptococci spp.*

Post-traumatic infectious endophthalmitis: *Bacillus spp.* and *Pseudomonas spp.*

Delayed endophthalmitis: *Streptococcus* spp., *Staphylococcus* sp,. *B. cereus*, *P. acnes*

Bleb-related endophthalmitis: *Streptococcus*, *Staphylococcus* and *Enterococcus*.

1.6. Antibiotic treatment for ocular bacterial infections:

Antibiotics:

An antibiotic is a substance or compound also called chemotherapeutic agent that kills or inhibits the growth of bacteria. Most antibiotics are modified chemically from original compounds found in nature, as is the case with beta-lactams (which include penicillins, produced by fungi genus *Penicillium*, cephalosporins). Some antibiotics are still produced and isolated from living organisms, such as aminoglycosides (www.antibiotics.com). The predominant antibiotics that are used in treatment of ocular bacterial agents are shown in table 1a.

Table 1a: Predominant antibiotics used in treatment of ocular bacterial infections

Organism	Topical	Subconjunctival	Systemic
Micrococci, Staphylococci methicilin-resistant)	Vancomycin (50 mg/ml)	Vancomycin (25 mg)	Vancomycin* (2 g/day)
Streptococci	Penicillin G (100,000 U/ml)	Penicillin G (500,000 U)	Penicillin G* (2.0–6.0 MU/ 4 hr) IV
Enterococci	Vancomycin (50 mg/ml), gentamicin (14 mg/ml)	Vancomycin (25 mg) gentamicin (20 mg)	Vancomycin* (2 g/day) IV, gentamicin (3.0–7.0 mg/kg/day) IV
Anaerobic gram-positive cocci	Penicillin G (100,000 U/ml)	Penicillin G (500,000 U)	Penicillin G* (2.0–6.0 MU/ 4 hrs) IV
Corynebacteria	Penicillin G (100,000 U/ml)	Penicillin G (500,000 U)	Penicillin G* (2.0–6.0 MU/ 4 hr) IV
Mycobacterium fortuitum/ chelonae	Amikacin (40–100 mg/ml)	Amikacin (20 mg)	Amikacin*,† (5 mg/kg/day) IV
Nocardia spp.	Amikacin (40–100 mg/ml) or trimethoprim (8 mg/ml) + sulfamethoxazole (80 mg/ml)‡	Amikacin (20 mg)	Trimethoprim /sulfamethoxazole* (10–20 mg/kg/day)§ IV
Neisseria gonorrhoea Neisseria meningitidis or	Ceftriazone (50 mg/ml)	Ceftriaxone (100 mg)	Ceftriaxone (1.0–2.0 g/day)IV or IM
Pseudomonas and other gram-negative bacilli	Tobramycin‖ (14 mg/ml)	Tobramycin (20 mg)‖	Tobramycin* (3.0–7.0 mg/kg/day) IV

IM, intramuscularly; IV, intravenously.

* Use only for corneal perforation or scleral suppuration.

† Consider oral clarithromycin (250–500 mg/day).

‡ Use undiluted IV preparation.

§ Based on trimethoprim component; Consider use of oral trimethoprim/sulfamethoxazole for mild suppuration.

‖ Consider addition or substitution of ceftazidime (topical, 50 mg/ml; subconjunctival, 100 mg).

Barriers influencing the entry of drugs into the eye:

Epithelial Barriers:

The corneal epithelium restricts the entry of the water-soluble drugs into the cornea and the aqueous humour. The barrier is breached by an epithelial defect or, if the epithelium is intact, is bypassed by the subconjunctival injection.

Aqueous-vitreous Barriers:

Bulk flow of aqueous humour from the eye and presence of an intact lens and zonule retard the diffusion of drugs from anterior into vitreous humour.

Blood-aqueous Barriers:

This limits the entry into the aqueous from the blood. Epithelium of iris and ciliary body pumps anionic drugs from aqueous into blood stream.

Blood-retinal Barriers:

The blood retinal barrier limits the entry of the drugs into the eye from the systemic circulation: External, the pigment epithelial barrier; Internal, the retinal capillary endothelial barriers. There is an outward pumping of anions across the retina by the retinal pigment epithelium and endothelial cells of the retinal vessels. (Sharma S, 2011, Seal DV, 1998)

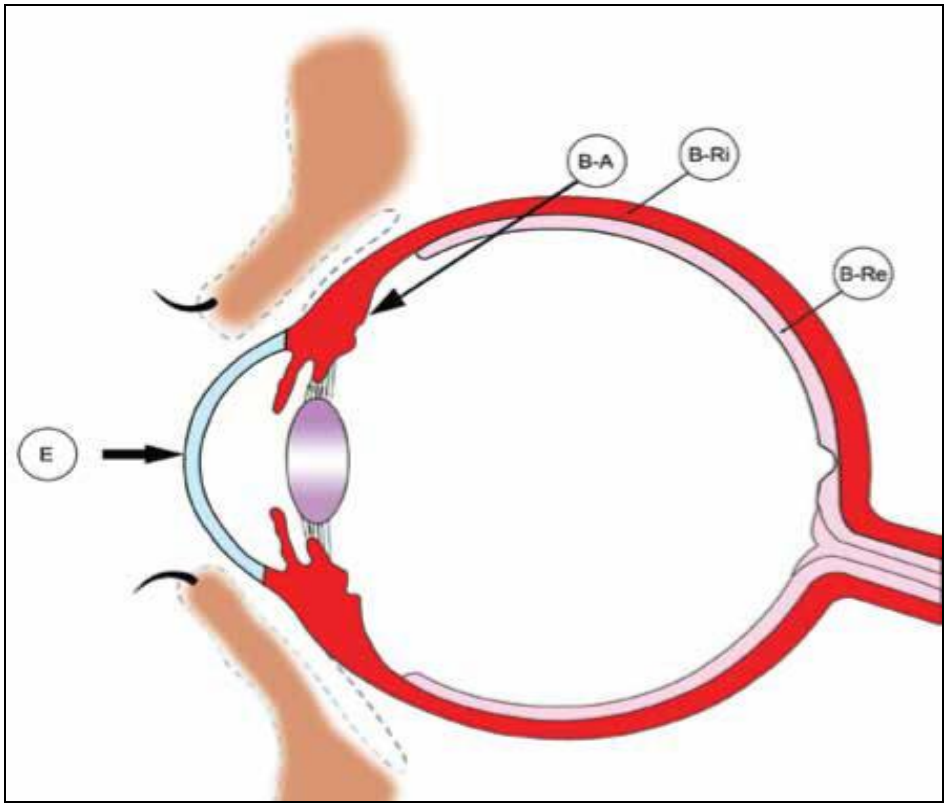


Figure 1b: Barriers influencing the entry of drugs into the eye. Entry of drugs in to eye can be blocked by various structures such as corneal epithelium (E), epithelium of the iris and ciliary body forming blood- aqueous barrier (B-A), the retinal capillary endothelium forming internal blood-retinal barrier (B-Ri) and the retinal pigment epithelium forming the external blood-retina barrier (B-Re).

(Source: Sharma S. 2011. Antibiotic resistance in ocular bacterial pathogens. *Indian J Med Micro*. 2011; 29:218-222)

1.7. Antibiotic resistance among bacteria:

The main mechanisms of drug resistance posed by the bacteria include:

a) The antibiotic may not be able to interact with the target.

b) Antibiotic may fail to reach the target. For example, narrow spectrum penicillins.

c) Bacteria may produce enzymes that inactivate the antibiotic. Examples include the penicillinases and cephalosporinases (β-lactamases)

Bacteria acquire ability to produce antibiotic-destroying enzymes by:

i. Bacteria take up extracellular DNA from their environment (*transformation*).

ii. A virus picks up bacterial DNA from one bacterium and transfers it to another (*transduction*).

iii. Plasmids (self-replicating pieces of extrachromosomal DNA) possessing genes for antibiotic resistance (R factors) are transferred to antibiotic-sensitive bacteria after sexual mating of the bacteria (*conjugation*).

iv. Bacteria posses ability to transport antibiotic out of itself actively, via "efflux pump."

Commonly studied antibiotic resistances among ocular isolates are:

- Methicillin resistant *S. aureus*
- Extended spectrum of Beta – lactamase resistance

Methicillin resistant S. *aureus* (MRSA):

Staphylococci are ubiquitous microorganisms, which are found as normal flora of skin. However, it is the most common pathogen of skin infections, ocular infection, and also act as opportunistic pathogens. Among the genus *Staphylococcus, Staphylococcus aureus* (*S. aureus*) is one of the most common causes of Nosocomial or community-based infections, leading to serious illnesses with high rates of morbidity and mortality. MRSA is by definition a strain of *Staphylococcus aureus* that is resistant to a large group of antibiotics called the beta-lactams, which include the penicillin group of drugs and the cephalosporins. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections worldwide, and hospital outbreaks caused by MRSA are common. Such endemic MRSA are difficult to eradicate, and they most likely further increase the number of infections, the costs, and the length of hospital stays. The organism is often sub-categorized as Community-Associated MRSA (CA-MRSA) or Health Care-Associated MRSA (HA-MRSA). MRSA possesses the ability to resist treatment with beta-lactam antibiotics, methicillin, dicloxacillin, nafcillin, and oxacillin. This resistance to methicillin is due to the presence of the *mec* gene in the bacteria. This gene alters the site at which methicillin binds to kill the organism. Hence, methicillin is not able to effectively bind to the bacteria (Brown et al., 2005, Rajaduraipandi et al., 2006, Salmenlinna et al., 2002, Sharma et al., 1999).

The Ocular Tracking Resistance in the U.S. Today (TRUST) program, which annually evaluates the in vitro susceptibility of *Staphylococcus aureus* along with *Streptococcus pneumoniae,* and *Haemophilus influenzae* to a number of ophthalmic

antibiotics in national samples of ocular isolates, reported a 12.1% increase in the incidence of methicillin-resistant *S. aureus* (MRSA) strains from January 2000 to December 2005, with more than 80% of MRSA resistant to fluoroquinolones (Asbell et al., 2008).

Extended Spectrum of Beta – Lactamase resistance:

Extended Spectrum of Beta-Lactamases (ESBL's) enzymes are typically plasmid–mediated, clavulanate-susceptible enzymes that hydrolyze penicillins, expanded- spectrum cephalosporins (eg. cefotaxime, ceftriaxone, ceftazidime, cefepime) and monobactams (aztreonam) but do not affect cephamycins (eg. cefoxitin and cefotetan) or carbapenems (e.g. meropenem or imipenem) This beta-lactamase enzymes are found commonly among bacteria belonging to the *Enterobacteriaceae* family *Escherichia coli, Klebsiella pneumoniae*, and *Klebsiella oxytoca* are the most common ESBL-producing pathogens (Jacoby and Monoz, 2005, Bonnet, 2004).

Extended spectrum beta-lactamases producing *Escherichia coli* are highly resistant to an array of antibiotics and infections by these strains are difficult to treat ESBL has traditionally been defined as the transmissible beta-lactamases that can be inhibited by the clavulanic acid, tazobactam or sulbactam, and which are encoded by the genes that can be exchanged between bacteria (Jacoby and Munoz-Price, 2005, Bonnet, 2004).

1.8. Current state of drug resistance by ocular bacterial isolates:

Current prevalent rate of MRSA in India:

The importance of studying ocular MRSA has been shown by the following authors. According to Rajaduraipandi, et al. (2006) 31.1% and 37.9% of *Staphylococcus aureus* were methicillin resistant among clinical and carrier samples respectively. Among these 63.6% of clinical and 23% of carrier MRSA were multidrug resistant. In another study by Blomquist, (2006) 30% of patients were considered to have acquired MRSA via nosocomial transmission and 70% to have Community acquired MRSA. Of them 1.3% had ophthalmic MRSA involvement**.**

The most common manifestation of ophthalmic MRSA infection was preseptal cellulitis and/or lid abscess followed by conjunctivitis, but sight-threatening infections, including corneal ulcers, endophthalmitis, orbital cellulitis, and blebitis, also occurred (Blomquist, 2006).

Current prevalent rate of ESBL in India:

Jones *et al.*, (2002) have recorded the incidence of ESBL positivity to be 60–68% in India, from strains isolated from major hospitals. However, the prevalence studies on ESBL producing ocular isolates from India are very few. Bharathi *et al.*, (2012) had reported the incidence of ESBL among the ocular population to be 14 %.

1.9. Antibiotic policy:

Within hospitals, overuse of antibiotics encourages the selection and proliferation of multidrug resistant bacterial strains carrying antibiotic resistant genes. Once such resistant strains are selected, they are favored by antibiotic usage and spread by cross-infection. Where resistance is encoded on transmissible plasmids, resistance can also spread between bacterial spp. There is thus a link between antibiotic use (or abuse) and the emergence of antibiotic resistant bacteria causing hospital-acquired infections. To avoid both the spread of antibiotic resistance and increase in the prevalence rate of this resistance to antibiotics by various group of bacteria we need to know about the Antibiotic policy. This requires the inclusion of an antibiotic policy in the infection control programme. Each and every hospital has its own antibiotic policy to prevent the spread of drug resistance.

The following are the benefits of the antibiotic policy:

Antibiotic policy will improve patient care by promoting the best practice in antibiotic prophylaxis and therapy and improve patient care by promoting the best practice in antibiotic prophylaxis and therapy. They reduce the spread of antimicrobial resistance in the hospital setting. They help us to initiate best efforts in hospital area as many resistant bacteria are normally generated in hospital and in particularly critical areas. Antibiotic policy paves way for the initiation of good hygienic practices so that these bacteria don't spread **(**http://www.ific.narod.ru/Manual/ABX.htm).

1.10. Gap in existing research on drug resistance among ocular isolates in India:

Antibiotic resistance among ocular pathogens is increasing in parallel with the increase of resistance in other systemic pathogens (Sharma, 2011). The development of bacterial resistance to specific antibiotics is an important consideration for clinicians treating ocular infections. The factors contributing to development of drug resistance in worldwide among ocular isolates include, overuse of antibiotics for systemic infection, inappropriate dosing of broad-spectrum antibiotics for systemic infections, exacerbated by inadequate compliance to full treatment duration (Bertino, 2009) as well as overuse of topical antibiotics in the eye. Other contributory factors may be improper dosing regimen, misuse of antibiotics for viral and other nonbacterial infections, extended duration of therapy and not in the least globalization and migration (Sharma, 2011). Timely institution of appropriate therapy must be initiated to control the infections and thereby minimize ocular morbidity. If they are not treated promptly, it may lead to sight threatening condition (Bharathi et al., 2010).

With no certain way to determine antibiotic concentration in ocular tissues during topical therapy, eye tissue-specific breakpoints are not yet available for determining the susceptibility of ocular isolates to antibiotics. Susceptibility of bacterial isolates from the eye is evaluated using Clinical and Laboratory Standards Institute (CLSI) procedures based on breakpoints derived from serum/plasma/cerebrospinal fluid levels of antibiotics. These systemic breakpoints may have limited predictive value for ocular isolates. On one hand, the concentration of antibiotic reached in external ocular

tissue on topical therapy may exceed the minimum inhibitory concentration for common ocular isolates (Varaldo, 2002). On the other hand, the high concentration of the topical antibiotic in ocular tissue may be rapidly diluted through tearing. Therefore, studies are needed to resolve the dynamics of breakpoint versus antibiotic resistance of ocular isolates and its relationship to clinical response (Sharma, 2011).

The management of bacterial resistance poses a problem when the conventional methods yield a negative result. Hence the need for a sensitive and specific technique arises to detect the presence of a etiological agent, its drug resistant genes. Molecular techniques are already proven to be rapid, sensitive and many a times more specific than conventional methods in detection and identification of bacteria from clinical specimens and its genes responsible for drug resistance.

In India, Polymerase Chain Reaction (PCR) based molecular techniques proved to be extremely sensitive to detect drug resistance among bacteria causing systemic infections. These techniques would be of great importance in an ophthalmic setting to detect and identify ocular infections causing bacteria and their drug resistance genes. Many reports on the existence and dissemination of such drug resistance genes among ocular arise in a rapid rate throughout the world and in lesser frequencies from India. Therefore, in this study we aimed to explore the predominant ophthalmic clinical isolates belonging to family of *Staphylococcus spp.*, *Enterobacteriaceae* and *P. acnes* in quest of the existence of drug resistance genes at a tertiary eye care centre in South India.

1.11. Need for molecular diagnosis:

The management of bacterial resistance poses a problem when the conventional methods yield a negative result. The need for a sensitive and specific technique arises to detect the presence of an etiological agent among ocular clinical infections.

Rapid identification from clinical specimens:

Traditional culture may take several days to allow sufficient growth of an organism so that a positive identification may be subsequently made. Often in clinical microbiology, such allowances in time result in patients being managed empirically, until culture result is known, which may allow sub-optimal management of patients. However, there is a role for molecular techniques from clinical specimens where a culture would give you a comparable result, but several days later (Millar et al., 2007).

Identification from culture-negative specimens:

There exists several situations where molecular approaches should be considered where conventional culture fails to identify the causal organism due to one or more of the following reasons including (i) prior antibiotic therapy, e.g. treatment of acute meningitis with i.v. benzylpenicillin, (ii) where organism is fastidious in nature, such as the HACEK group of organisms (*Haemophilus spp., Actinobacillus, Cardiobacterium hominis*, *Eikenella corrodens*, *Kingella kingae*) in the case of endocarditis, (iii) where organism is slow growing, e.g. *Mycobacterium spp.,* (iv) where

specialized cell culture techniques are required, e.g. *Chlamydia spp.*, *Coxiella burnetti*, (v) uncultivable bacteria (Millar, et. 2007).

For early, accurate and rapid detection of culture negative specimens, a method of detecting bacterial DNA targeting 16S r RNA gene is essential. The way to identify these pathogenic bacteria is by using rapid molecular techniques. Conventional methods involved in the detection of bacterial infections are less sensitive due to lesser amount of available clinical specimen and low microbial threshold and difficulties in isolation of fastidious and anaerobic organisms (White et al., 1990). These techniques are also laborious and time consuming. Rapid diagnosis is therefore essential where molecular methods are involved are rapid, sensitive and specific.

Molecular techniques for detection of bacteria and their drug resistant genes:

In vitro nucleic acid based amplification techniques:

In 1983, Kary Mullis envisioned a process of in vitro amplification which is eventually known as polymerase chain reaction (PCR) (Mullis, 1990). This is the hallmark of nucleic acid amplification method (Saiki et al., 1992). The ability of the PCR to amplify small amounts of specific nucleic acid has made it an important and convenient diagnostic tool with a potential to detect microorganisms rapidly and reliably. PCR techniques also allow the rapid, accurate identification of genes coding for drug resistance.

Target amplification methods:

(i) Polymerase chain reaction:

Polymerase Chain Reaction (PCR) is an enzyme-driven, primer-mediated, temperature-dependent process for replicating a specific DNA sequence in vitro. The principle of PCR is based on the repetitive cycling of three simple reactions, the conditions of which vary only in the temperature and time of incubation.

a. **Denaturation** - This is the initial step in which the double helical DNA strands are denatured to form single strand.

b. **Annealing -** In this step added primer strand that has complementary sequences to that of the single strands of DNA anneals to it at the respective target sites.

c. **Extension** - In this step the strand keep on adding the complementary bases to form a new strand that has complementary sequence to that of parent DNA.

Short comings of PCR:

Contamination with extraneous DNA fragments that could be amplified along with the sample is the most important problem which is carried over from previous amplifications (amplicons) or introduced from other sources. To eliminate this problem, adherence to careful laboratory procedures, such as rigid quality control of enzyme preparations and the use of dedicated pipettes, pre-aliquoted reagents and exposure to UV (Wilson, 1997).

Specific PCR:

Specific PCR is the simplest PCR approach of which is designed for detecting specific gene target of microbe; which many a time include spp. specific gene or a virulent gene of the microbe. This is a key point for specific PCR so that the primers should be so-designed so that they are strictly specific for the targeted microorganisms. As the result is specific for the detection of target microbes, this method can be used as a direct detection and identification method. This is the most widely used method in the diagnosis of infectious diseases (Millar, 2007).

Nested/semi-nested PCR:

In this approach, genomic template DNA is amplified with two sets of primers. The first PCR set produces a larger PCR product than that in second PCR set. The second PCR set uses the first PCR product as template DNA to amplify an internal region of DNA during the second (nested/semi-nested) amplification stage. The primers in the second PCR set can be different to the first set (nested) or one of the primers can be the same as the first set (semi-nested). This method can be used to increase the sensitivity of detection or to identify the first set PCR products when the primers in the second PCR reaction are spp.-specific (Millar, 2007).

Advantages and disadvantages of Nested PCR:

The sensitivity of most nested amplification procedures is extremely high. A single copy of target can be detected without the need for hybridization with labeled

probes Re-amplification with the second set of internal primers also serves to verify the specificity of the first round product. In addition, the transfer of reaction products from the first reaction effectively serves to dilute out inhibitors that might be present in the sample initially. The disadvantage of the nested PCR is contamination due to the aerosolized amplified DNA during the transfer of the reaction product from the first reaction into a new tube for re-amplification (Persing and Cimino 1993, Millar, 2007).

Multiplex PCR:

In multiplex PCR, two or more primer pairs are included in one reaction tube and two or more DNA templates are targeted simultaneously. This is a relatively simple molecular way to detect few different bacteria in one PCR reaction. In multiplex PCR, the primer pairs should be specific to the target gene and PCR products should be in different sizes. Critical parameters that require optimization for a successful multiplex PCR assay are: Primers – their relative concentrations, length, G+C content, amplification target size (which is easily resolvable on the agarose gel), concentration of the PCR buffer, cycling temperatures, number of cycles for amplification, balance between the concentrations of $MgCl_2$ and dNTPs (Henegariu et al., 1997)

Restriction fragment length polymorphism (RFLP):

Restriction enzymes are DNA-cutting enzymes found in bacteria (and harvested from them for use). Because they cut within the molecule, they are often called restriction endonucleases. A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA. The enzyme makes two incisions, one through

each of the phosphate backbones of the double helix without damaging the bases. Many of the procedures of molecular biology and genetic engineering rely on restriction enzymes. Many of them are palindromic; that is, the sequence on one strand reads the same in the same direction on the complementary strand. After restriction endonuclease digestions, the amplified DNA fragments are cut to different small fragments according to their DNA sequences. The resulting fragments can be separated by gel electrophoresis, and/or then transferred to a nylon membrane. RFLP usually is used as a microbe typing and epidemiological investigative method.

Nucleotide sequencing or DNA sequencing:

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. Thus far, most DNA sequencing has been performed using the chain termination method developed by Frederick Sanger. This technique uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates.

In chain terminator sequencing (Sanger sequencing), extension is initiated at a specific site on the template DNA by using a short oligonucleotide 'primer' complementary to the template at that region. The oligonucleotide primer is extended using a DNA polymerase, an enzyme that replicates DNA; included with the primer and DNA polymerase are the four deoxynucleotide bases, along with a low concentration of a chain terminating nucleotide (di-deoxynucleotide).

Limited incorporation of the chain terminating nucleotide by the DNA polymerase results in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used. Fragments are then size-separated by electrophoresis in a slab polyacrylamide gel, or more commonly now, in a narrow glass tube (capillary) filled with a viscous polymer.

Advantages and limitations of DNA sequencing:

The advantages of DNA sequencing as a genotypic method include its wide applicability, and ability to identify single nucleotide variation. The limitations however, include technical complexity, high cost of the equipment and large amount of the data which may be complex to analyze (http://en.wikipedia.org/wiki/Sequencing).

1.12. Hypothesis of the study:

Application of nucleic acid - based molecular methods are more sensitive to identify the bacterial agents causing ocular infections and to detect drug resistance among ocular bacteria compared to phenotypic method, and polymerase chain reaction (PCR) method is superior to the commonly used conventional techniques in the rapid and accurate diagnosis.

1.13. Objectives of the study:

1. **To determine the prevalence rate of bacterial pathogens causing ocular infections and the prevalence of polymicrobial ocular infections (PMI) at a tertiary care centre, South India and to determine the *in vitro* susceptibilities to commonly used antibacterial agents.**

    a. To analysis the prevalence rate of ocular infections.

    b. To determine prevalence of polymicrobial infections among ocular isolates.

    c. To determine the in vitro antibiotic susceptibilities of predominant antibacterial agents used routinely for treatment of ocular infections.

2. **To identify bacteria from intraocular specimens, from culture negative PCR positive cases by the application of dHPLC and PCR based DNA sequencing.**

    a. To apply nested PCR for detection of bacteria from intra ocular specimens by targeting eubacterial genome.

    b. To standardize dHPLC technique for the separation of mixed bacteria present in the PCR amplified product obtained from intraocular specimens collected from patients with endophthalmitis.

    c. To identify bacteria present in culture negative PCR positive patients by the application of PCR based DNA sequencing technique.

3. **To determine rate of isolation of Methicillin Resistant Staphylococci (MRS) among ocular isolates by conventional and molecular methods and to standardize, apply PCR-RFLP and DNA sequencing techniques for identification and speciation of MRS and to compare results of conventional and molecular methods in detection of Methicillin resistance (MR).**

    a. Conventional method of Detection of Methicillin resistant *Staphylococcus.*
    b. Standardization of uniplex PCR and duplex PCR for detection of *Staphylococcus aureus* and methicillin resistant *Staphylococcus spp.*
    c. Comparison of PCR results with conventional method.
    d. Detection of methicillin resistance among ocular staphylococci.
    e. Identification of Coagulase Negative Staphylococcus to spp. level by PCR-RFLP of the *gap* gene using the *Alu I* restriction enzyme.

4. **To study the prevalence of *CTX-M, OXA, TEM, SHV* and *blaNDM-1* genes that confer the Extended Spectrum of Beta-Lactamases (ESBLs) production in *Enterobacteriaceae* isolated from ocular clinical specimens from a tertiary care centre Chennai, Tamil Nadu, India along with screening of existence of *blaNDM-1* genes.**

    a. To detect the production of ESBL by conventional method using double disc diffusion test.
    b. To analyze the prevalence of Extended Spectrum of Beta-Lactamases (ESBLs) genes such as *CTX-M, OXA, TEM, SHV* and *NDM-1 g*enes among the *Enterobacteriaceae* isolated from ocular clinical specimens.
    c. To screen for the presence of virulent gene; *blaNDM-1* among ocular *Enterobacteriaceae* isolates.

5. **To screen for the prevalence of drug resistance and existence of *blaNDM-1* among microorganisms isolated from Multi organ donor and Donor corneal rim in a tertiary eye care centre, India**

    a. To study the microbial spectrum of Donor corneal rim (DCR) and Multiorgan donor (MOD).
    b. To understand antibiotic susceptibility of DCR and MOD isolates.
    c. To analyze the prevalence of Extended Spectrum of Beta-Lactamases (ESBLs) genes such as *CTX-M, AmpC, TEM* and *SHV g*enes among the *Enterobacteriaceae* isolated from DCR and MOD by PCR based DNA sequencing method.
    d. To screen for the presence of virulent MBL gene; *blaNDM-1* among ocular *Enterobacteriaceae* isolates and to confirm the same by PCR based DNA sequencing method.

6. **To analyze New Delhi Metallo-Beta-Lactamase -1 structure producing *Acinetobacter baumannii* isolated from Donor Swab in a tertiary eye care centre, India and structural analysis of its antibiotic binding interactions.**

    a. To analyze biofilm producing ability of a New Delhi Metallo-Beta-Lactamse-1 gene positive *A. baumannii* isolated from Donor Swab.
    b. To study the viability of the biofilm producing bacteria after antibacterial treatment by MTT assay.
    c. To detect the transfer of drug resistance via conjugation experiments
    d. To *in silico* characterize the NDM structure by molecular dynamic studies and by docking tests.
    e. To correlate microbiological investigations carried out to screen its level of drug resistance with that of *in silico* studies of drug interactions using bioinformatics tools.

7. **To determine the genotypic prevalence of *P. acnes* isolated from ocular specimens and to study their clinical correlation with phenotypic and genotypic characteristics, analysis of epidemiological pattern and also to determine antibiotic susceptibility profiling.**

   a. To detect *P. acnes* by conventional and molecular techniques.
   b. To validate the results of conventional by molecular techniques of *P. acnes.*
   c. To standardize PCR targeting *16S rRNA* gene and *recA* gene to understand the prevalent genotypes of *P. acnes* causing infections.
   d. Comparison of PCR results with conventional method.
   e. To understand the epidemiology of *P. acnes* by Random Amplification of Polymorphic DNA (RAPD) technique.
   f. To determine the antibiotic susceptibility pattern of ocular *P. acnes.*

Chapter - 2

Prevalence rate of bacterial pathogens causing ocular infections and a special focus on polymicrobial infections from a tertiary care centre, South India

2.1. Aim:

To determine the prevalence rate of bacterial pathogens causing ocular infections and a special focus on polymicrobial infections from a tertiary eye care centre, South India.

2.2. Review of literature:

2.2.1. Ophthalmic infections:

Ophthalmic infections can cause damage to structures of the eye, which can lead to vision loss and even blindness if left untreated (Snyder, 1994). Ocular infections are commonly categorized into 3 groups. Infections affecting the cornea and conjunctiva often present with eye pain and a red eye; noninfectious aetiologies can have a similar presentation. Infections inside the eye (endophthalmitis) often have devastating consequences and usually occur following penetrating ocular trauma or after intraocular surgery. Prompt referral to an ophthalmologist is crucial. Infections in the soft tissue namely ocular adnexa and orbit can involve the eye indirectly and can spread from the orbit to the brain (Donahue, 1996). Because of changes in ocular normal flora, wide range of extraocular and intraocular diseases such as blepharitis, conjunctivitis,

canaliculitis, dacryocystitis, orbital cellulitis, keratitis, infectious endophthalmitis, including panopthalmitis etc., occurs (Hori et al., 2008, Ramesh et al., 2010, Sharma, 2011, Tuft, 2000). Most common microorganisms causing ocular infections include *Staphylococcus aureus, Coagulase negative staphylococci, Genus Streptococcus, Coynebacterium, Bacillus, Nocardia, Pseudomonas aeruginosa, Enterobacteriaceae, Nonfermentors* and others (Anand et al., 2000, Bharathi et al., 2012, Kunimoto et al., 1999). Infection of the eye leads to increased incidence of morbidity and blindness worldwide (Sherwal, 2008). The spectrum of micro-organisms responsible for ocular bacterial infections varies according to geographical locations (Carmichael, 1985).

2.2.2. Polymicrobial infections (PMI) of the eye:

Polymicrobial diseases are caused by combinations of viruses, bacteria, fungi, and parasites and are being recognized with increasing frequency (Brogden, 2005). In polymicrobial infections, the presence of one micro-organism generates a niche for other pathogenic micro-organisms to colonize; one micro-organism predisposes the host to get colonized by other micro-organisms, or two or more non-pathogenic micro-organisms together can cause the disease (Brogden, 2005). Polymicrobial or mixed infections provide a complex environment in which a variety of interactions may occur between causal pathogens. Organisms may share virulence determinants, allowing an organism that is usually non-pathogenic or weakly pathogenic to cause disease. The presence of two or more pathogens may trigger the synergic release of cytokines, which may subsequently increase the severity of the infection (Solomkin, 2004).

Polymicrobial diseases in animals and humans are more common than generally realized, and many perceived "single etiologic agent diseases," when examined closely, contain polymicrobial etiologies. These include respiratory diseases, gastroenteritis, conjunctivitis, keratitis, hepatitis, Lyme disease, multiple sclerosis, genital infections, intra-abdominal infection (Brogden, 2002).

2.2.3. Need for this study:

Though moderate number of reports on prevalence of bacteria causing ocular infections exists in India, as a very few studies only have been published on polymicrobial infections (PMI) of eye. Hence this study was undertaken to study the bacterial pathogens causing ophthalmic infections and to find the prevalence of PMI in our population.

2.3. Objectives:

To determine the prevalence rate of bacterial pathogens causing ocular infections and the prevalence of polymicrobial ocular infections at a tertiary care centre, South India and to determine the *in vitro* susceptibilities to commonly used antibacterial agents.

a. To analysis the prevalence rate of bacterial pathogens causing ocular infections.

b. To determine the prevalence of polymicrobial infections among ocular isolates.

c. To determine in vitro antibiotic susceptibilities of predominant antibacterial agents used routinely for treatment of ocular infections.

2.4. Materials and methods:

A retrospective analysis included all patients with clinical suspicion of bacterial ocular diseases namely as blepharitis, conjunctivitis, canaliculitis, dacryocystitis, orbital cellulitis, keratitis, infectious endophthalmitis and panopthalmitis, screening of Donor corneal rim (DCR) and swab from Multi organ donor (MOD) during the period of three months from January 2010 – March 2010 from a total of 384 specimens including both extraocular and intraocular received for microbial culture at L & T Microbiology Research Centre, Kamalnayan Bajaj Research Centre, Sankara Nethralaya, a tertiary eye care centre at Chennai, Tamil Nadu, India.

After the ocular examinations, using standard microbiological techniques, specimens for culture and smear were obtained. Conjunctival specimens were collected by the laboratory staffs by wiping a Hanks Balanced Salt Solution (HBSS) / broth-moistened swab across the lower conjunctival sac in conjunctivitis cases. Apart from conjunctival swabs other specimens were collected by the ophthalmologist. Thick, tenacious purulent punctal discharge was collected from the canaliculus by pressure applied over the area of the eyelid that overlies the canaliculus in cases of canaliculitis. For infectious keratitis, specimens were collected by scraping the edge of the ulcer with a Bard Parker blade No. 15' after removal of debris or discharge in the vicinity by anoph. In case of blepharitis, specimen was collected from the eyelid margin, using a sterile broth-moistened cotton swab or by scraping the lid ulcers. Specimens from cases of suppurative scleritis were collected by scraping and swabbing the area of the suppurative abscess. In cases of external and internal hordeolum, the abscesses were

incised and the drained pus was obtained. For cases of dacryocystitis, purulent material was collected from everted punta by pressure applied over the lacrimal sac area, and surgically excised lacrimal sac was also collected. Specimens from cases of pre-septal cellulitis were obtained after stab incision or through an open wound or drainage site, if present. From patients suspected of infectious endophthalmitis, anterior chamber and vitreous fluids were obtained (Brinser and Burd, 1996, Wilhelmus, 1994, Therese and Madhavan, 1996, Ramesh, 2010). The detailed methodology of collection and processing of ocular specimens is given in Appendix I.

The bacteria was isolated from the ocular specimens by inoculating the specimen onto sheep Blood agar (BA), Mac Conkey agar (MAC), Chocolate agar (CA), Brucella blood agar (with 5% sheep blood, Haemin and Vitamin K) (BBA), Sabouraud's dextrose agar (SDA), Thioglycollate medium and Brain heart infusion broth (BHIB), and direct microscopic examinations namely KOH-Calcofluor, Gram stain, Giemsa stain was carried out to detect bacteria and fungus. For suspected cases of *Mycobacteria* spp., *Nocardia* spp., and *Actinomycetes* spp., Acid-fast stain was carried out. The BA, MAC and BHIB culture media's were subjected for the isolation of aerobic bacterial pathogens by incubating at 37^{o}C; CA were incubated at 37^{o}C with 5 – 10 % CO_2 for isolation of fastidious organisms; anaerobic bacteria were isolated using BBA and Thioglycollate medium; SDA was used for fungal isolation. The isolated organisms were identified to spp. level using standard biochemical tests (Brinser and Burd, 1996, Wilhelmus, 1994, Therese and Madhavan, 1996). A positive culture was defined as growth of the microorganism in two or more than two media. In case of

corneal scrapping and intraocular specimens, confluent growth at the site of inoculation is considered significant, if similar organism is also seen previously in direct smear examination. Study protocol was approved by institutional ethics sub-committee (IRB).

2.5. Results:

During the study period of three months 384 specimens were processed for detection of infectious etiological agent causing diseases namely conjunctivitis (n=26, 6.7%), keratitis (n=129, 33.6%), endophthalmitis (n=127, 33.0%), trauma (n=18, 4.6%), buckle and suture infection (n=12, 3.1%), graft infection (n=10, 2.6%), abscess (n=11, 2.9%), graft rejection (n=6, 1.5%), iris prolapse (n=4, 1.04%), scleritis (n=4, 1.04%), canalicularitis (n=2, 0.52%), granuloma (n=2, 0.52%), implant (n=3, 0.78%), swab from multiorgan donor (n=6, 1.5%) donor corneal rim (n=8, 2.08%). Graphical representation of specimen wise distribution of the ocular specimens is shown in figure 2a.

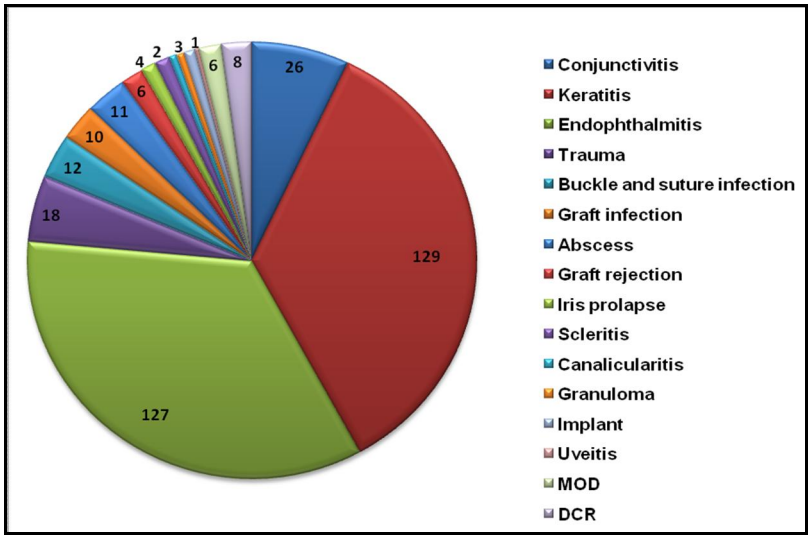


Figure 2a: Graphical representation of distribution of ocular specimen according to disease conditions.

2.5.1. Prevalence of bacterial infections:

Bacterial growth was seen in 36.4% cases. Out of 384 specimen processed, a total of 139 specimens were positive for the presence of bacterial pathogens. The bacterial pathogens were isolated from, bacterial conjunctivitis (n=22, 15.8%), bacterial keratitis (n=34, 24.5%), endophthalmitis (n=33, 23.7%), trauma (n=10, 13.9%), buckle and suture infection (n=8, 5.7%), graft infection (n=7, 5.03%), abscess (n=7, 5.03%), graft rejection (n=3, 1.5%), iris prolapse (n=2, 1.4%), canalicularitis (n=1, 0.7%), granuloma (n=2, 1.4%), implant (n=3, 2.1%), swab from multiorgan donor (n=4, 2.8%) and donor corneal rim (n=3, 2.1%).

The most frequently recovered bacterial isolates were Gram-positive cocci (74 of 139; 19.27%) followed by Gram-negative bacilli (48 of 139; 12.5%) and Gram-positive bacilli (13 of 139; 3.4%).

Predominant bacterial spp. isolated was *Coagulase negative Staphylococci* (40 of 384; 10.4%) followed by *Coagulase positive Staphylococci* (25 of 384; 6.5%), *Pseudomonas spp.* (23 of 384; 5.9%), *Enterobacteriaceae* genus (19 of 384; 4.9%) and *Streptococcus spp.* (9 of 384; 2.3%). The rate of isolation of anaerobic organisms was 1.0%. The predominant anaerobic bacteria isolated were *Propionibacterium acnes* (90%). Bacterial isolates recovered from ocular specimens are presented in Table 2a.

Table 2a: Prevalence of various bacteria isolated from 384 ocular specimens collected from different ocular infections

Types of bacteria	**No. of bacteria (%) grown in 3 months**
Bacterial Growth negative	245/384 (63.8%)
Bacterial Growth positive	**139/384 (36.2%)**
Gram positive cocci	**N = 74 (19.27%)**
Coagulase Negative Staphylococci	40 (10.4%)
Coagulase Positive Staphylococci	25 (6.5%)
Streptococcus spp.	9 (2.3%)
Gram positive bacilli	**N= 13 (3.4%)**
Bacillus spp.	6 (1.5%)
Diptheroids	3 (0.8%)
Nocardia spp.	3 (0.8%)
M. tuberculosis	1 (0.3%)
Gram negative cocci - *Neisseria spp.*	1 (0.3%)
Gram negative bacilli	**N= 48 (12.5%)**
Enterobacteriaceae	19 (4.9%)
Pseudomonas spp.	23 (5.9%)
Other Non fermentors	6 (1.5%)
Anaerobic organisms	**N= 4 (1.0%)**
P. acnes	3 (0.8%)
F. nucleatum	1 (0.3%)
TOTAL	**N=139 (36.2%)**

2.5.2. Prevalence of fungal infections:

Fungal growth was seen in 47 specimens out of 384 specimens (12.2%). Culture positive fungus were isolated predominantly from keratitis patients (n=29, 61.7%), followed by endophthalmitis (n=12, 25.5%), abscess (n=4, 8.5%) and buckle infection (n=2, 4.2%). Predominant fungal spp. isolated was *Fusarium spp.* (40 of 384; 10.4%) followed by *Aspergillus spp.* (25 of 384; 6.5%). Fungal isolates recovered from eyes are presented in Table 2b.

Table 2b: Prevalence of fungus isolated during 3 months (n=47/384)

Types of fungus	**No. of fungus (%) grown in 3 months**
Fungal Growth negative	337/384 (88.5%)
Fungal Growth positive	**47/384 (11.5%)**
Filamentous Fungus	**N = 44 (11.4%)**
Fusarium spp.	19 (4.9%)
Aspergillus spp.	11 (2.8%)
Corynespora spp.	2 (0.5%)
Bipolaris spp.	1 (0.25%)
Curvularia spp.	1 (0.25%)
Paecilomyces spp.	1 (0.25%)
Penicillium spp.	2 (0.5%)
Scedosporium spp.	1 (0.25%)
Unidentified fungus	6 (1.5%)
Yeast	**N = 3 (0.75%)**
Total	**N = 47 (11.5%)**

2.5.3. Prevalence of Polymicrobial infections (PMI):

Fifteen out of 384 specimens were positive for polymicrobial infections (PMI). PMI were predominantly observed from conjunctivitis cases (25%) followed by keratitis (12%), buckle infection (12%), implant infection (12%) and one case of PMI from each acute post operative endophthalmitis, graft infection, pus from abscesses, dacryocystitis and injury (6.6%).

Coagulase negative Staphylococci (50%) followed by *Coagulase positive Staphylococci* (32%), *Pseudomonas aeruginosa* (25%), *Enterobacteriaceae* (19%) and *Corynebacterium spp.* (13.3%) were the predominant bacteria were found to be associated with more than one spp. / genus of bacteria in case of polymicrobial infections. *P. acnes* was associated in two cases of conjunctivitis were polymicrobial infections was encountered (n=2, 13.3%). Association of fungus in case of PMI was not encountered in any of the cases.

Three pathogens namely MSSA, *Escherichia coli* and *Citrobacter koseri* were isolated from a single specimen collected from an orbital injury specimen. The most prevalent combination of PMI was the Gram positive cocci along with Gram negative bacilli (n=6, 40%), followed by Gram positive cocci with Gram positive bacilli (n=3, 25%). *P. acnes* along with the Gram positive cocci were isolated in two specimens (13.3%). The prevalence of PMI among ocular specimens was shown in the table 2c.

Table 2c: Prevalence of PMI isolated during 3 months (n=15/384)

Types of bacteria	**No. of bacteria (%) grown**
PMI Growth negative	369/384 (96.1%)
PMI Growth positive	**15/384 (3.9%)**
MSSA + *Escherichia coli* + *Citrobacter koseri*	**N = 1**
Streptococcus pneumoniae + *Serratia marcenscens*	**N = 1**
Staphylococcus* + *Streptococcus / Enterococcus	**N = 2**
MSSA + Viridans Streptococci	**1**
MRSE + *E. Faecalis*	**1**
GPC + GPB	**N = 3**
MRSE + Corynebacterium spp.	2
MSSE + *Bacillus cereus*	1
GPC + *Propionibacterium acnes*	**N = 2**
MRSE + *Propionibacterium acnes*	1
MSSA + *Propionibacterium acnes*	1
GPC + GNB	**N = 6**
MRSA + *Pseudomonas aeruginosa*	2
MRSE + *Pseudomonas aeruginosa*	1
MRSE + *H. parainfluenzae*	1
MRSA + *Citrobacter koseri*	1
MSSA + *Enterobacter aerogenes*	1
TOTAL	**15**

MSSA - Methicillin Sensitive *S. aureus*, **MSSE** - Methicillin Sensitive *S.epidermidis*, **MRSA** - Methicillin Resistant *S. aureus*, **MRSE** - Methicillin Resistant *S. epidermidis*, **GPC** – Gram Positive Cocci, **GPB** – Gram Positive Bacilli, **GNB** – Gram Negative bacilli.

2.5.4. Antibiotic susceptibility pattern:

Most of the bacterial isolates were susceptible to gatifloxacin (86.8%), followed by gentamicin (73.4%) and ciprofloxacin (69.9%). Higher degree antibiotic resistance was exhibited by the ocular pathogens to tobramycin (46.7%), followed by cefazolin (43.2%) and cefotaxime (36.5%).

2.6. Discussion:

The present study clearly showed that the most prevalent pathogen isolated among our ocular clinical specimens was *Coagulase negative Staphylococci* (40 of 384; 10.4%) followed by *Coagulase positive Staphylococci* (25 of 384; 6.5%), *Pseudomonas spp.* (23 of 384; 5.9%) and *Enterobacteriaceae*. In a study conducted by Ramesh et al., from the south Indian population, the predominated bacterial pathogen isolated in their study is *S. aureus* (25.13%) followed by *S. pneumoniae* (21.78%) and *Coagulase negative Staphylococci* (18.29%). In case of conjunctivitis, the common pathogen isolated is *Coagulase negative Staphylococci.* In contrast to the study by Ramesh et al., (2010) where *S. pneumoniae* is found to be the predominant cause of keratitis, in this study the predominant pathogen is *P. aeruginosa.* The pathogens isolated in case of keratitis are in concordance with the study conducted by Hagan et al., (1995) and Houang et al., (2001) in Ghana and Hong Kong, where *P. aeruginosa* was the primary pathogen. In case of bacterial pathogens causing endophthalmitis, the predominant pathogen is *Pseudomonas* followed by *Enterobacteriaceae* group. *P. acnes* was the predominant anaerobic bacteria isolated in 2.6% specimens.

The occurrence of PMI in our study period is 3.9%. PMI were predominantly observed from conjunctivitis cases (25%) followed by keratitis (12%), buckle infection (12%), implant infection (12%). *Coagulase negative Staphylococci* (50%) followed by *Coagulase positive Staphylococci* (32%) were isolated predominantly from PMI. Bourcier et al., (2003) reported PMI in 2% keratitis cases. Tuft et al., (2000) also have reported on PMI by bacteria in case of keratitis, whereas in our study higher rate of PMI is seen among conjunctivitis cases. From an orbital injury specimen, three pathogens namely MSSA, *Escherichia coli* and *Citrobacter koseri* were isolated. *P. aeruginosa* was the considered as pathogen in conjunctivitis cases; *E. faecalis* and *P. aeruginosa* were the predominant pathogens encountered in PMI of keratitis cases. *B. cereus* was the pathogen in endophthalmitis case. Combination of Gram positive cocci and Gram negative bacilli were seen in 40% cases. In case of other PMI the appropriate pathogen is unclear. The study period should be further extended to have a correct knowledge about PMI.

2.7. Conclusion:

Based on the current study, we conclude that predominant bacterial pathogens isolated in this study was *Genus Staphylococcus* (19.2%), followed by *Enterobacteriaceae* (12.5%). *P. acnes* was the important anaerobe isolated in 2.6% of our specimens. The rate of bacterial infections among ocular specimens was 36.2%. The prevalence of fungal infections among ophthalmic specimens was 11.5%. The rate of occurrence of PMI in our study period is 3.9%.

2.8. Future prospects:

Rapid and early identification techniques for the detection of the most predominant ocular bacterial pathogens namely *Genus Staphylococcus, Enterobacteriaceae* and *P. acnes* is therefore essential in our study population. Both early detection of the pathogens and its drug resistant mechanism is crucial for timely institution of appropriate therapy and thereby to minimize ocular morbidity. The study period should also be further extended to have a more accurate knowledge about prevalence of pathogens among ocular clinical specimens and the rate of occurrence of PMI.

Chapter - 3

Identification of bacteria from culture negative and polymerase chain reaction (PCR) positive intraocular specimen collected patients with infectious endophthalmitis by Denaturing High-Performance Liquid Chromatography (dHPLC)

3.1. Aim:

To identify the bacteria from culture negative and polymerase chain reaction (PCR) positive intraocular specimen collected patients with infectious endophthalmitis by Denaturing High-Performance Liquid Chromatography (dHPLC).

3.2. Review of literature:

3.2.1. Infectious endophthalmitis:

Infectious endophthalmitis is a devastating, sight threatening ocular inflammation resulting from the introduction of a bacterial or fungal agent into the eye, including involvement of the vitreous and /or aqueous humors. Most cases of Infectious endophthalmitis are exogenous, resulting from inoculation of organisms from the outside - for example, from trauma, intraocular surgery, or as an extension of keratitis (corneal infection). In such cases, the aqueous humor may be seeded first before extension into the vitreous. The remaining cases are endogenous, resulting from bacteremic or fungemic seeding of the eye. In endogenous endophthalmitis, organisms usually seed the highly vascular choroids first, and then extend anteriorly into the vitreous (Callegan et al. 2002).

The Endophthalmitis Vitrectomy Study (EVS) demonstrated that most isolates causing clinical endophthalmitis are introduced into the eye from the patient's conjunctival flora. Once clinical infection occurs, damage to ocular tissues is believed to occur due to direct effects of bacterial replication as well as initiation of a fulminant cascade of inflammatory mediators. Endotoxins and other bacterial products appear to cause direct cellular injury while eliciting cytokines that attract neutrophils, which enhance the inflammatory effect. Thus, recent efforts in controlling the damaging effects of endophthalmitis in experimental models have focused on identifying not only appropriate antibiotics for control of the infectious agent but also on anti-inflammatory agent that might disrupt the immunologic events that occur after infection and to elucidate the molecular and cellular events that contribute to the damage that occurs in intraocular infection (Cruciani et al., 1998).

3.2.2. Causative agents of Infectious endophthalmitis:

Severe complication of intra-ocular surgery results in acute post-operative endophthalmitis; most frequently due to *S. epidermidis, S. aureus, Streptococcus spp.*, and gram-negative organisms (Hykin et al., 1994). The occurrence of delayed post-operative endophthalmitis has been recognized increasingly in recent years and typically follows extracapsular cataract extraction with posterior chamber lens implantation. It occurs 1-11 months after surgery, is characterised by repeated episodes of low grade inflammation-which in the early stages may respond to topical steroid treatment-and clinically by large keratoprecipitates, hypopyon, white plaques on the posterior capsule, a capsular abscess and anterior vitritis. Although *P. acnes,*

P.granulosum, P. propionicus, S. epidermidis, A. israelii, A. viscosus and *Corynebacterium* have been reported in such cases, the majority are culture negative. Reasons for this include small numbers of causative organisms present within the eye, the possible localized nature of the infection leading to sampling error and the fastidious growth requirements of *Propionibacterium spp.* This results in a clinical dilemma in which post-operative inflammation due to phaco-anaphylactic uveitis, idiopathic post-operative inflammation or delayed post-operative endophthalmitis cannot be distinguished easily. Despite aggressive therapeutic and surgical intervention, endophthalmitis generally results in partial or complete loss of vision, often within a few days of inoculation (Scott et al., 1996).

3.2.3. Need for the molecular methods:

For early, accurate and rapid detection of these organisms, a method of detecting bacterial DNA in aqueous / vitreous samples is essential. The ideal way to identify these bacteria is by using rapid and reliable molecular techniques. The conventional methods involved in the detection of bacterial infections are less sensitive due to lesser amount of available clinical specimen and low microbial threshold (White et al., 1991). These techniques are also laborious and time consuming. Rapid diagnosis is therefore essential where molecular methods are involved which possess high sensitivity and specificity. Most common molecular techniques usually involved includes Polymerase Chain Reaction (PCR) is a technique which involves amplification of target gene which is unique among specific organism followed by PCR based DNA sequencing which involves the identification of the causative organism and finally Denaturing high-

performance liquid chromatography (dHPLC) is a method used for the separation of bacterial PCR amplified fragments where more than one target could be present in clinical specimens. It has potential advantages in that only a very small sample is required for analysis, minute numbers of bacteria can be detected and conventional microbial culture is not required. The disadvantage of the PCR targeting the eubacterial genome (*16SrRNA* gene using universal primers) is that, the specimen may contain more than one organism and therefore PCR based DNA sequencing may not give a good identity due to the presence of mixed organisms.

3.2.4. Denaturing high-performance liquid chromatography (dHPLC):

Denaturing high-performance liquid chromatography (dHPLC) has been commonly used since 1990s in medical research to detect DNA mutations and polymorphisms (Le Maréchal et al., 2001). First step towards the development of dHPLC was taken ten years ago with synthesis of alkylated non-porous poly (styrene-divinylbenzene) particles 2 microns in diameter (Huber et al., 1993, Huber et al., 1996). In combination with a hydro organic eluent containing alkylammonium ions, this stationary phase material enabled, fornfirst time, size dependent liquid chromatographic separation of double-stranded DNA fragments with a degree of resolution only previously attained by capillary electrophoresis (Goldenberg et al., 2007)

In 1995, it was discovered that double-stranded DNA fragments up to 1500 base pairs in length containing a single-base mismatch, (i.e., a base pair other than one of the two typical Watson-Crick base pairs A-T and G-C, respectively), will be retained less

on the aforementioned chromatographic support than a perfectly matched DNA homoduplex at column temperatures sufficiently high to result in partial denaturation of the analysts (Premstaller et al., 2003) This approach requires that the two sequences to be compared are denatured and reannealed prior to HPLC analysis to allow the formation of the original homoduplices as well as that of heteroduplices between the sense and antisense strands of either homoduplex. Formation of heteroduplices is not required for a more recent embodiment of dHPLC, referred to as completely denaturing HPLC (Oefner et al., 2000). It is based on the ability of the stationary phase to resolve isomeric single stranded nucleic acids up to 100 nucleotides in length from each other even when they differ only in a single base. More recently, DHPLC protocols were adapted for the separation of bacterial PCR-amplified fragments (Goldenberg et al., 2007).

3.2.5. Advantages of DHPLC:

DHPLC is quick, economical, and capable of screening large numbers of samples in a relatively short period of time. Over the years, DHPLC has been employed in a wide range of applications, including nucleotide polymorphism analysis, gene mapping, analysis of genes, screening for mutations, analysis of primer extension products, and quantification of gene expression. Recent new application of this technique is the identification of bacteria.

By the application of PCR based DNA Sequencing and DHPLC, it was possible to differentially identify bacteria from various specimens, from the culture negative

cases thus aiding in initiation of appropriate therapy. The actual identification of bacteria by these techniques is also useful to understand the epidemiology and pathogenesis of bacteria.

3.3. Objectives:

To identify bacteria from intraocular specimens, from culture negative PCR positive cases by the application of DHPLC and PCR based DNA sequencing.

- **a.** To apply nested PCR for detection of bacteria from intra ocular specimens by targeting eubacterial genome.
- **b.** To standardize DHPLC technique for the separation of mixed bacteria detection through DNA sequenced product obtained from intraocular specimens collected from patients with endophthalmitis.
- **c.** To identify bacteria present in culture negative PCR positive patients by the application of PCR based DNA sequencing technique.

3.4. Materials and Methods:

3.4.1. Patients and clinical specimens:

One hundred sixteen intraocular aspirates consisting of 54 aqueous humor aspirates (AH) and 62 vitreous fluids (VF) from patients with post-operative endophthalmitis were included in this study. All the specimens were negative when tested by culture for the presence of bacteria, but were positive by nested polymerase chain reaction (nPCR) method (Therese et al., 1998) for eubacterial genome indicating the presence of bacterial genome in them. Informed consent was taken from the patients

and the study was approved by the institutional review board (IRB). The clinical details of these patients were collected from their clinical records. Period of study was from September 2009 – August 2010.

3.4.2. Nucleic acid-based molecular methods for identification of the bacterium:

3.4.2.1. Nested PCR for *eubacterial genome*:

DNA was extracted from aqueous humor aspirates (AH) and vitreous fluids (VF) using Qiagen DNA Mini Kit (Cat no. 51304) as per the manufactures' instructions (Appendix 2). The eluted DNA was stored at -20º C until further processed. Nested PCR to detect eubacterial genome was done as described by us earlier (Therese et al., 1998) with first round product of the size of 766 bp and second round with 470 bp. The primers had broad specificity for Gram-positive and -negative bacteria known to cause infectious endophthalmitis.

3.4.2.2. Denaturing high-performance liquid chromatography:

The DHPLC was carried out in a WAVE ® 4500 Nucleic Acid Fragment Analysis System (Transgenomic, United States (Figure 3a). It is equipped with an optional chiller, three plate auto samplers, oven UV detector and a separation cartridge (DNA Sep Cartridge). The cartridges are filled with a non-porous matrix consisting of polystyrene – Divinylbenzene (PS – DVB) copolymer beads.

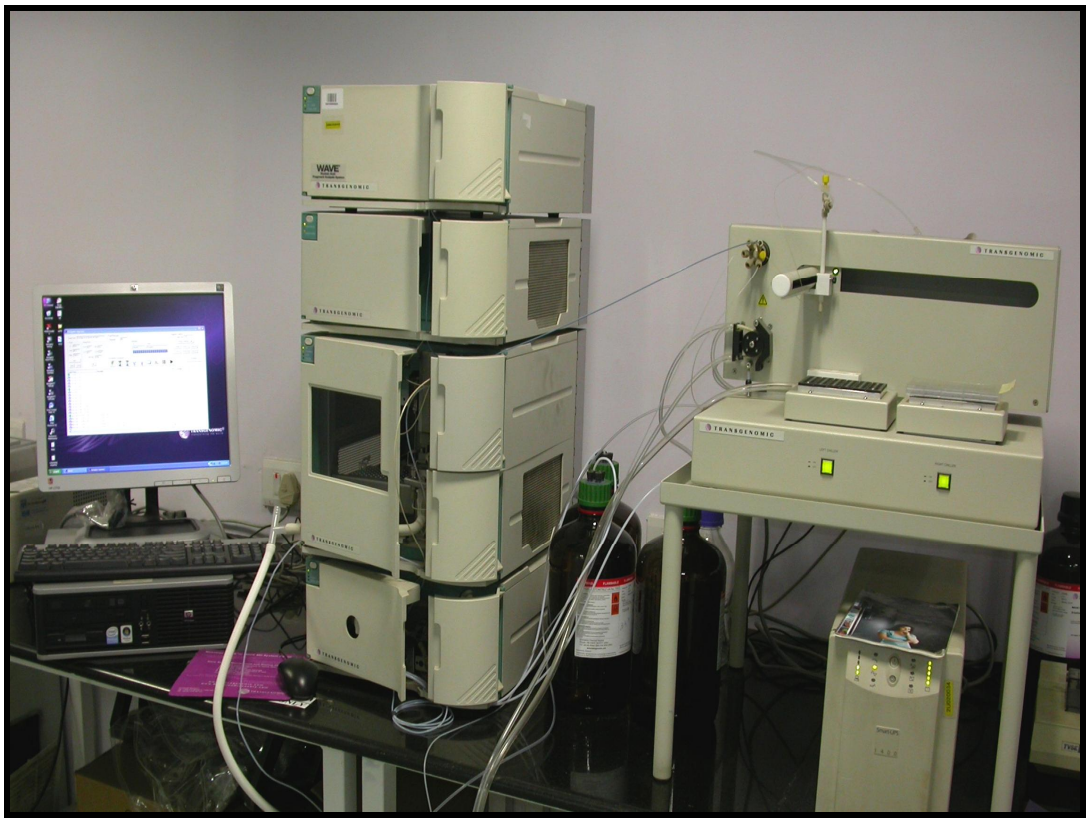


Figure 3a: WAVE ® 4500 Nucleic Acid Fragment Analysis System for DHPLC analysis.

By nature the beads within the cartridge are electrostatically neutral and hydrophobic and do not readily react with nucleic acids. Triethylammonium acetate (TEAA) is the bridging molecule to aid in the adsorption of nucleic acids to the beads. The cartridge matrix is the stationary phase. The mobile phase consists of buffers that elute the DNA off the cartridge. A 0.1M solution of TEAA mixed with 25% acetonitrile (WAVE optimized ® buffer B) is used to elute the DNA. As increasing concentrations of acetonitrile flow across the cartridge matrix, the hydrophobic interaction between the cartridge and the DNA / TEAA is broken so that the DNA elutes. The process of

separation is referred to as reverse phase ion – pairing liquid chromatography. Separation conditions were adapted by temperature, flow rate, and gradient. The optimal separation conditions are listed in Table 3a. The detection of the fragments was carried out without any need of fluorescent dyes at a wavelength of 260 nm is shown in figure 3b. All buffer solutions were obtained from WAVE ® 4500 Transgenomics and results were analyzed using Navigator TM software version.

Table 3a: Optimized conditions for the identification of bacterium using WAVE ® 4500 Nucleic Acid Fragment Analysis System.

Time (min)	% A	% B
0	50	50
0.5	50.2	49.8
3.6	54	56
6.8	42.7	57.3
9.9	30.4	69.6
13.0	30	70
13.1	0	100
13.6	0	100
13.7	55	45
14.6	55	45

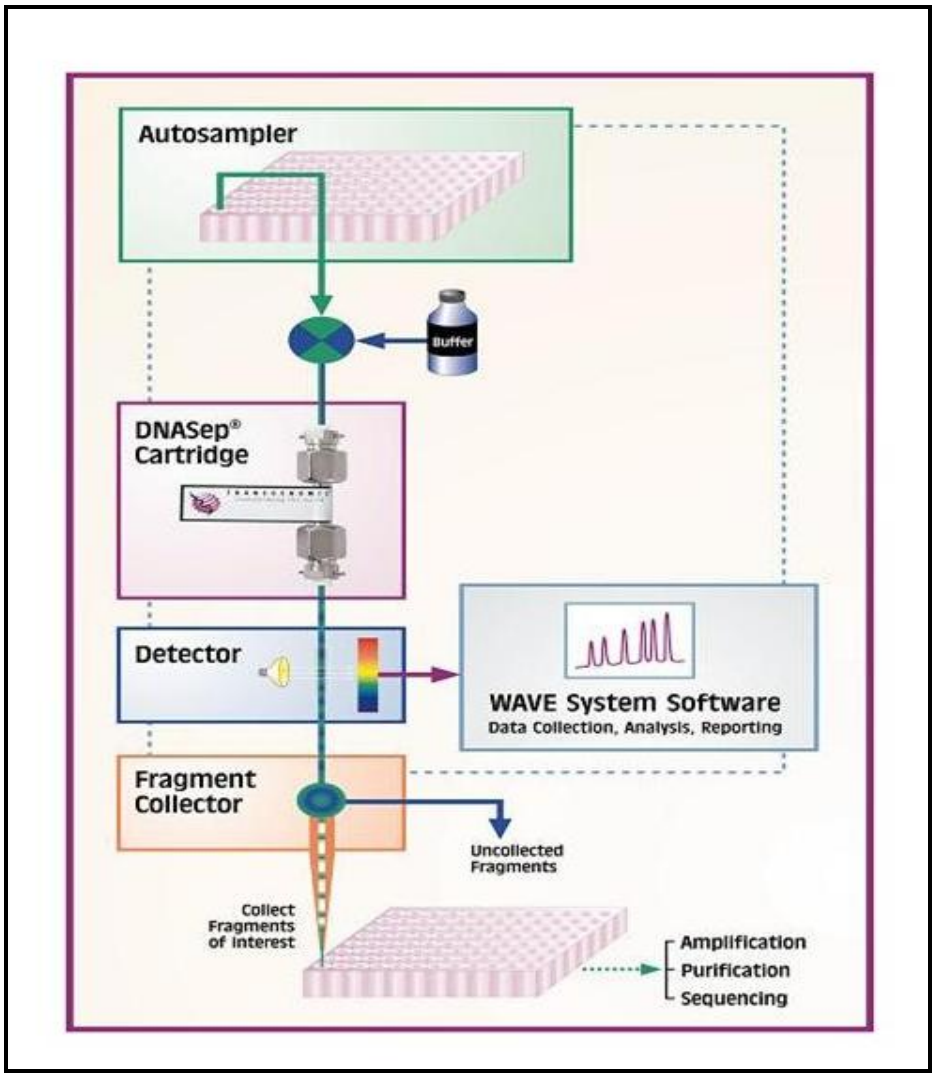


Figure 3b: **Principle involved in separation of samples by DHPLC.** DHPLC technology uses liquid chromatography technique (HPLC) both in high-pressure closing liquid flow path, the DNA sample is automatically injected, and in buffer carrying downflow patent DNA separation column, by different gradient of buffer, in different temperature conditions of separation column to achieve a different analysis of DNA; DNA sample is separated by UV detector and automatic DNA fragment collector automatically collects after DNA sample is separated, (Source: http://www.transgenomic.com.cn/DHPLCjishuyuanli.htm)

3.4.2.3. Direct sequencing of PCR products:

PCR positive amplified products of ocular specimen namely vitreous aspirate, aqueous tap were subjected for DNA sequencing. One hundred and sixteen clinical intraocular specimens (62 vitreous aspirates, 54 aqueous humor) that were positive by PCR for eubacterial genome but were culture negative were subjected to cycle sequencing reaction, consisting of 2µl of RR mix, 2µl of sequencing buffer, 2µl of amplified product, 2 picomoles/µl of forward or reverse primer and 2µl of deionized water. PCR profile consisted of denaturation at 96°C for 1min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min and final hold of 4°C (Appendix 3). Products were purified according to standard protocol, loaded onto ABI 3100 Genetic Analyzer (Applied Biosystems, USA.) with polymer POP7 and sequenced. Sequences were analyzed using BIO EDIT, (downloaded from *http://www.mbio.ncsu.edu/BioEdit/bioedit.html*), and finally blasted with National Centre for Biotechnology Information (NCBI) Blast website *http://blast.ncbi.nlm.nih.gov/Blast.cgi* to identify spp. and DNA homology.

3.4.3. Standardization of dHPLC parameters:

Initially dHPLC separation was standardized with known ATCC standard strains *E. coli* (4157) and *Salmonella typhi* (Lab isolate confirmed with DNA Sequencing) with their genus specific PCR amplified products. Equal volumes (5µl of each product) of the PCR products were mixed and run at a series of four temperatures on the range of 2 degrees over and 2 degrees under the Tp. The chromatograms obtained at varying temperatures are shown in figure 3c.

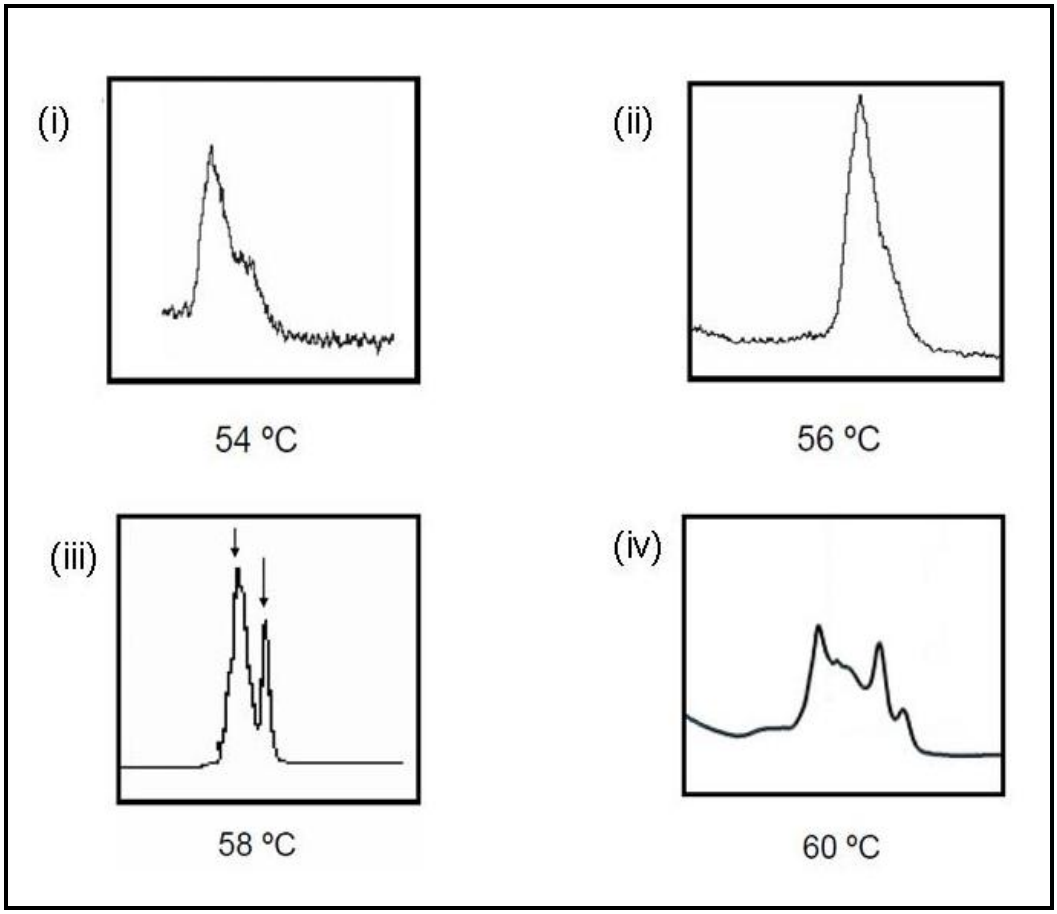


Figure 3c: Chromatograms obtained at varying temperatures. Results of PCR amplified products of *E. coli* and *S. typhi* mixed together and subjected to dHPLC analysis at a series of four temperatures on the range of 2 degrees over and 2 degrees under the prediction temperature (Tp).

(i), (ii) - There was not complete separation of the peaks.

(iii) - proper separations of two peaks were observed.

(iv) - Improper separations of the peaks were seen.

Hence temperature of 58 ºC where proper separation of peaks seen was used for further analysis.

After standardization the oven temperature was kept at 58ºC and the samples were injected at this optimized temperature. The resulting peaks were collected in the fragment collector and concentrated in the Savant SpeedVac DNA 120 concentrator, USA. It was then suspended in 30µl of sterile Milli Q water and subjected to eubacterial PCR followed by sequencing to show the fractions collected were *S. typhi* and *E. coli.* Every time before injecting the samples a high mutation standard (provided with the instrument) and the standard strain *E. coli* (PCR product) was run as controls to check the sensitivity and specificity is shown in Figure 3d.

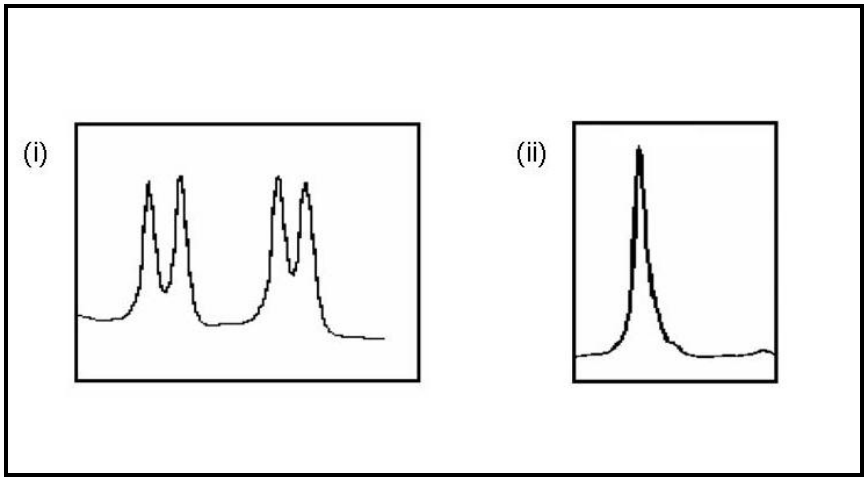


Figure 3d: Results of PCR amplified products of *E. coli* (standard strain) and High mutation standard subjected to dHPLC analysis.

A – High mutation standard provided with the instrument

B – Standard strain *E. coli* (PCR product) were subjected to dHPLC analysis before subjected the run of samples where a single peak was obtained and further confirmed by DNA Sequencing after the elution of the product.

After confirmation of sequencing results the analysis was carried out in the PCR positive cases of infectious endophthalmitis. Before subjecting those products to dHPLC, they were first sequenced to determine the occurrence of more than one bacterium. The electrophoretogram of the sequence indicative of mixture of bacteria and wherever the sequencing results that are shown as uncultured bacterium were injected into dHPLC to separate out the bacterium. The resulting peaks were collected in the fragment collector as mentioned earlier concentrated in the DNA concentrator, amplified and sequenced. Also a few specimens obtained as a single peak is shown in figure 3e were also collected, amplified and sequenced.

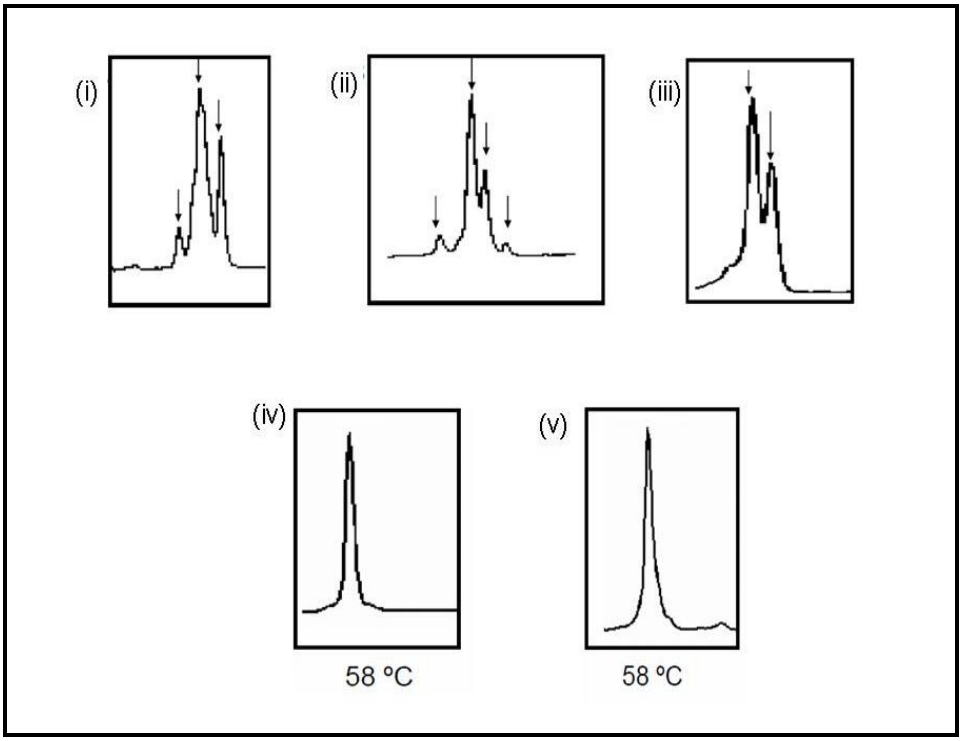


Figure 3e: Results of dHPLC peaks with mixed and single amplicons. Eight µl of Eubacterial PCR amplified product of specimen was injected at an oven temperature of

58ºC with a flow rate 0.9ml/min. If 2,3 & 4 peaks were obtained at end of the run which were further DNA sequenced. PCR product of one vitreous and aqueous aspirate was also injected at the above mentioned conditions where a single bacterium was identified.

The program was run at 58ºC and at a flow rate of 0.9ml/min.

A - 0.1 M triethylammonium acetate;

B - 0.1 M triethylammonium acetate in 25% acetonitrile.

3.5. RESULTS:

3.5.1. Direct DNA sequencing of eubacterial genome PCR products of intraocular specimens:

A total of 116 intraocular samples (vitreous and aqueous aspirates) were subjected to PCR amplification using universal eubacterial oligonucleotide primers targeting the highly conserved region of *16S rRNA* to generate PCR products of 470 bp. All the amplified PCR products were subjected to DNA sequencing. Among the 116 samples, in 92 samples a clear DNA sequence electrophoretogram was obtained. On BLAST analysis these 92 samples showed 100% identity with bacterial DNA sequences submitted in the data bank. Ninety-two different bacteria have been identified from vitreous and aqueous aspirates of which the predominant ones were Gram-positive bacteria.

Of the results obtained in these samples by direct DNA sequencing, 77 bacteria (84%) were known to cause infections in the human including endophthalmitis (table 3b & 3c), 15 bacteria (16%) were of soil and environmental origin (not reported as pathogens in literature) but reported in infectious endophthalmitis patients 30 (33%) of them (shown in table 3d) were both culture and PCR positive.

Predominant pathogens identified by the direct DNA sequencing were *P. acnes*, followed by *S. haemolyticus*, *S. epidermidis*, other *Staphylococcus* spp, *S. maltophila* and *Bacillus spp.*

Thirty three different bacteria known to cause infectious endophthalmitis were identified from these intraocular aspirates. Along with these, 25 bacteria known to cause other human infections and 21 bacteria of environmental or soil origin (shown in table 3d) were also identified in the vitreous and aqueous aspirates of infectious endophthalmitis cases.

Table 3b: List of bacteria identified by dHPLC based DNA sequencing in the intraocular specimens that were known to cause infectious endophthalmitis.

GRAM POSITIVE BACTERIA	GRAM NEGATIVE BACTERIA
Staphylococcus sp.	*Stenotrophomonas maltophila*
Staphyloccus hominis	*Haemophilus influennzae*
Staphyloccus haemolyticus	*Pseudomonas fulva*
Staphyloccus epidermidis	*Enterobacter sp*
Staphyloccus cohnii	*Enterobacter hormaechei*
Micrococcus luteus	*Schelegella aquatica*
Streptococcus sp	*Alcaligenes sp*
Streptococcus mitis	*Achromobacter xyloxidans*
Enterococcus casseliflavus	*A.calcoaceticus*
Enterococcus faecium	
Enterococcus cecorum	
Bacillus endophyticus	
Bacillus thermoamylovorans	
Bacillus horikoshii	
P.acne	
Actinomyces	
Abitrophia sp	
Corynebacterium sp	
Corynebacterium diphtheriae	
Corynebacterium jeikeium	
Williamsia muralis	
Proteobacterium	
Actinobacterium	
Microbacterium	

Table 3c: List of bacteria identified by dHPLC based DNA sequencing in the intraocular specimens that were known to cause infections other than endophthalmitis.

GRAM POSITIVE BACTERIA	GRAM NEGATIVE BACTERIA
Gemella morbillorum	
Cellulomonas hominis	
Micromonospora sp	
Clostridium sp	
Gemella hemolysans	
Granulicatella adiacens	*Neisseria pharyngis*
Brevibacilllus agri	*Comamonas sp*
Mycobacterium monacense	*Neisseria sp*
Geobacillus sp	*Brevundimonas sp*
Clostridium difficle	*Kaistobacter sp*
Rhodococcus erythropolis	*Sphingomonas sp*
Janibacter limosus	
Planococcus sp	
Clostridium lituseburense	
Clostridium longisporum	
Clostridium aurantibutyricum	
Gordonia defluvii	

Table 3d: List of bacteria identified by dHPLC based DNA sequencing in the intraocular specimens that are of environmental origin not known to cause any human infections.

GRAM POSITIVE BACTERIA	GRAM NEGATIVE BACTERIA
Nonomuraea sp	
Planotetrospora sp	*Bradyrhizobium sp*
Epulopiscium sp	*Methylocystis sp*
Serinicoccus	*Pannonibacter phagmitetus*
Candidatus Rhodoluna lacicola	*Tepidiphilus sp*
Curtobacterium flaccumfaciens	*Empedobacter bravis*
Crocinobacterium jejui	*Ronia tepidophila*
Pavlova lutheri	*Azoarcus sp*
Krasilnikovia cinnamonea	*Labrenzia sp*
Phycibacter jejuensis	*Phyllobacterium myrsinacearum*
Leucobacter komagatae	*Delftia sp*
Leifsonia pindariensis	*Hydrogenophaga sp*
Pimelobacter sp	
Methanobrevibacter ruminantium	

3.5.2. dHPLC based DNA sequencing:

Amplified products of the remaining 24 PCR positive samples (11 VH and 13AF) where DNA electrophoretogram showed presence of more than one sequence were subjected to dHPLC for analysis suspecting the presence of mixture of bacteria. Appearance of more than one peak (Fig 1e) in the dHPLC confirmed the same. Of these 24 specimens, 15 showed presence of 2 peaks, 6 showed presence of three peaks and 3 showed 4 peaks which were eluted further and sequenced to identify each of these bacteria. List of bacteria identified in each patient is shown in table 3e.

Table 3e: **List of bacteria identified in each patient by dHPLC.**

VITREOUS ASPIRATE (11)	*AQUEOUS ASPIRATE (13)*
Brevundimonas sp *Staphylococcus sp.*	*Staphylococcus haemolyticus* *Staphylococcus sp.*
Streptococcus sp. *Azoarcus sp.*	*Staphylococcus sp.* *Staphylococcus haemolyticus*
Pannonibacter phragmitetus *Candidatus Rhodoluna lacicola* *Propionibacterium acne*	*Pimelobacter sp* *Staphylococcus sp.*
Micromonospora sp *Staphylococcus haemolyticus* *Staphylococcus epidermidis*	*Janibacter limosus* *Staphylococcus cohnii*
Gemella morbillorum *Streptococcus sp* *Granulicatella adiacens* *Abiotrophia sp*	*Sphingomonas sp.* *Kaistobacter sp*
Methylocystis sp *Corynebacterium diphtheriae* *Empedobacter brevis*	*Labrenzia sp* *Stenotrophomonas maltophilia*
Bradyrhizobium sp *Epulopiscium sp* *Bacillus thermoamylovorans* *Enterococcus cecorum*	*Bacillus horikoshii* *Phycibacter jejuensis* *Geobacillus sp* *Staphylococcus epidermidis*
Micrococcus luteus *Staphylococcus hominis*	*Planococcus sp* *Crocinobacterium jejui* *Propionibacterium acne*
Clostridium aurantibutyricum *Clostridium longisporum* *Staphylococcus epidermidis*	*Clostridium lituseburense* *Clostridium difficile* *Staphylococcus haemolyticus*
Staphylococcus hominis *Staphylococcus sp*	*Ronia tepidophila* *Staphylococcus conii* *Achromobacter xylosoxidans*
Leifsonia pindariensis *Leucobacter komagatae* *Staphylococcus epidermidis* *Microbacterium sp*	*Williamsia muralis* *Gordonia defluvii*
	Phyllobacterium myrsinacearum *Bacillus thermoamylovorans* *Staphylococcus hemolyticus*
	Pavlova lutheri *Mycobacterium monacense*

These bacteria are of environmental origin and many of those were known to cause human infections. These are probably not cultivable by conventional bacteriological methods and require special media or optimal temperature for its growth.

3.6. DISCUSSION:

The present study was undertaken to emphasize on the technique for the identification and separation of mixed bacteria's present in the PCR amplified product obtained from intraocular specimens from patients with infectious endopthalmitis. In addition, bacteria identified by PCR-based DNA sequencing particularly using dHPLC separation of PCR products in Polymicrobial infections were found to be those known to cause human infections and also those not reported to have caused any human infections. Those of soil / environmental origin that are not reported to cause any human infections, dHPLC were used to separate the PCR products for further processing to identify these polymicrobial agents.

A large number of patients were referred to us from other hospitals with infectious endopthalmitis and the reason for the presence of these environmental bacteria were not known to us since no history of presurgical prophylactic management of the eyes have not been available prior to the collection of aqueous aspirate or the vitreous. *Gemella morbillorum* and *Gemella hemolysans* are Gram-positive coccal commensal organisms of the mucous membranes of humans and predominantly causes

endovascular infections and they can be incorrectly identified as viridans streptococci or *Neisseria* spp. because they are easily decolorized during Gram staining or left unidentified (Scola et al., 1998). *Rhodococcus erythropolis* which was not only found in the soil but also considered as opportunistic pathogen in the case of responsible for bacteremia in immunocompromised hosts (Vernazza et al., 1991). *Janibacter limosus* proposed to have been isolated from sludge (Martin et al., 1997) has been identified in an aqueous aspirate which had infection with Staphylococcus *cohnii* has been reported to cause bacteremia (Loubinoux et al., 2005) and *Methanobrevibacter ruminantium* were reported to have been isolated from patients with endodontic infections although they are not the potential pathogens (Vianna et al., 2006). *Brevibacillus agri* and other spp. of Bacillus identified in our study were known to cause nosocomial infections and also food poisoning (Vianna et al., 2006). *Mycobacterium monacense* was found in another aqueous aspirate sample which was reported to have been isolated from a post traumatic wound infection (Reischl et al., 2006).

3.7. Conclusion:

Based on the current study, we conclude that the molecular biological methods described in this study may replace the time-Consuming and occasionally challenging culture-based identification of bacterial spp.. Assuming that DNA extraction requires 30 minutes, PCR 3 hours, and DHPLC (WAVE system) based DNA sequencing needs 3 hours, results could be obtained in two working days. The applications of the WAVE microbial analysis system presented here capitalize on the ability of the system to

unequivocally identify more than one bacterial spp. in a given sample. Our study is the first study to identify the microorganisms from culture negative PCR positive intraocular specimens.

3.8. Future prospects:

The dHPLC technique will aid in rapid detection of unidentified culture negative bacteria to spp. level from intraocular specimens where the availability of specimens is very low. This approach will help in rapid and prompt installation of intravitreal antibiotics for eradication of the pathogenic agent and pave way for the restoration of sight.

Chapter - 4

Determination of the rate of isolation of Methicillin Resistant Staphylococci (MRS) among ocular isolates by conventional and molecular methods and Application of PCR based – RFLP for spp. identification of ocular isolates of MRS

4.1. Aim:

To determine the rate of isolation of Methicillin Resistant Staphylococci (MRS) among ocular isolates by conventional and molecular methods and application of PCR based - RFLP for spp. identification of ocular isolates of MRS.

4.2. Review of literature:

4.2.1. *Staphylococci:*

Staphylococci are ubiquitous microorganism, which is found as normal flora of the skin. However, it is the most common pathogen of skin infections, ocular infection, and also act as opportunistic pathogens. Among the genus *Staphylococcus, Staphylococcus aureus* (*S. aureus*) is one of the most common causes of Nosocomial or community based infections, leading to serious illnesses with high rates of morbidity and mortality (Brown et al., 2005, Brown et al., 2001, Velasco et al., 2005). *S. aureus* has been known to be a major pathogen causing a wide spectrum of clinical manifestations, such as wound infections, pneumonia, septicemia, and endocarditis, with beta-lactam antibiotics being drugs of choice for therapy (Grisold et al., 2005).

4.2.2. TAXONOMY: (Parker et al., 1990)

Scientific classification:

Kingdom: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Spp.: *S. aureus, S. epidermidis, S. saprophyticus, S. haemolyticus.*

4.2.3 Phenotypic Characteristics of *S. aureus:*

Staphylococcus aureus are Gram-positive organisms arranged in grape like clusters. They are non-motile, non-spore forming facultative anaerobe occasionally capsulated when isolated directly from specimen. They possess the property of fermentation of glucose, mannitol (which distinguishes from *S. epidermidis)*. They are catalase positive, coagulase positive, DNase positive. *S. aureus* produces golden yellow coloured colonies on nutrient agar.

4.2.4 Identification of *S. aureus:*

Speciation of isolates is essential to distinguish *S. aureus* from coagulase-negative *Staphylococci* (CONS). Molecular methods have been developed more recently in identification, characterization and speciation of *S. aureus*. Quality control of tests should use positive and negative control strains while testing.

The following various tests are used for the differential identification of *S. aureus* includes:

a) Tube coagulase test:

Tube coagulase is the "gold standard" test for the identification of *S. aureus*. Free (extracellular) coagulase clots plasma in the absence of calcium. The tube coagulase test with plasma and examination of tubes after incubation for 4 h and 24 h is the standard test for routine identification of *S. aureus.* Tests negative at 4 h should be re-examined at 24 h because a small proportion of strains.

b) Slide coagulase test:

Clumping factor (bound coagulase) differs from free coagulase in that it is cell-bound and requires only fibrinogen. The slide agglutination test for clumping factor is very rapid but up to 15% of *S. aureus* strains are negative, so isolates negative in slide tests should be confirmed with a tube coagulase test.

c) Latex agglutination tests:

Commercial latex agglutination tests used for the detection of *S. aureus,* targets the protein A and/or clumping factor which is specific for *S. aureus.*

f) Molecular tests:

Rapid molecular tests for determining susceptibility to methicillin/ oxacillin can be combined with simultaneous detection of an *S. aureus*-specific target to allow rapid identification of MRSA isolates. Therefore most of molecular methods for identification

of *S. aureus* and its methicillin resistance are PCR based (Unal et al. 1994, Gerberding et al. 1991, Bignardi et al. 1991, Wallet et al. 1996, Coudron et al. 1986).

4.2.5 Classification of *S. aureus* based on susceptibility to methicillin:

Methicillin-resistant *Staphylococcus* is a major problem in hospital settings. The bacterium is resistance to penicillin group of drugs, including methicillin and other narrow-spectrum β-lactamase-resistant penicillin antibiotics. Resistant strain, MRSA was first discovered in UK in 1961 and it is widespread, particularly in hospital setting where it is commonly termed a superbug. Structure of methicillin is shown in figure 4a.

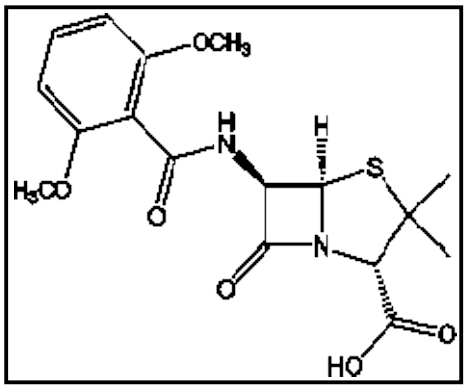


Figure 4a: Structure of methicillin (Source: http://en.wikipedia.org/wiki/Meticillin)

a) Methicillin-Resistant *S. aureus (MRSA):*

MRSA are able to grow in the presence of beta-lactams and its derivatives, including cephalosporins. Methicillin resistance is mediated by penicillin-binding protein (PBP) 2a. PBP 2a and the gene encoding for the same have been found in all methicillin-resistant strains of *Staphylococci* reported to date and only in these strains. PBP 2' (or PBP 2a), which, unlike the intrinsic set of PBPs (PBP 1 to 4) of *S. aureus*,

has remarkably reduced binding affinities to beta-lactam antibiotics. Despite the presence of otherwise inhibitory concentrations of beta-lactam antibiotics, MRSA can continue cell wall synthesis solely depending upon the uninhibitory activity of PBP 2a. A *mecA* gene located on the chromosome of MRSA encodes PBP 2a (Brown et al., 2005, Velasco et al., 2005, Brown et al., 2001, Salmenlinna et al., 2002). As *S. aureus* has gained resistance to methicillin, the drug is not used now for the treatment of the same. Later introduction of oxacillin as an alternative to methicillin resulted in development of oxacillin resistant *S. aureus.*

b) Borderline Resistant *S. aureus :*

Borderline resistant or low-level MRSA can result from the production of lesser amounts of beta-lactamases, or increased production and or modified penicillin-binding capacity of normal PBPs .Such borderline resistant *S. aureus* strains (BORSA) have minimal inhibitory concentration (MIC) of methicillin exceeding 4µg/ml (Gerberding et al., 1991 and Bignardi et al., 1996).

c) Pre MRSA:

Some methicillin-resistant *S. aureus* carry *mecA* but are sensitive to methicillin. The explanation is that *mecI* which is one of the regulatory gene involved in the *mecA* gene expression and is a powerful repressor of the transcription of *mecA*. Its repression is not removed by the addition of an inducer such as methicillin. A pre-MRSA strain develops into an MRSA strain when mutations, including deletions, of *mecI* or the site to which *mecI* binding occur (Lewis et al., 2000 and Weller et al., 1999).

d) Methicillin-Resistant *S. epidermidis*:

Similar to methicillin-resistant *S. aureus*, methicillin-resistant *S. epidermidis* was also found to contain *mecA* gene that codes for methicillin resistance.

e) Community-acquired MRSA (CA-MRSA) :

Although MRSA has traditionally been seen as a hospital-associated infection, community-acquired MRSA (CA-MRSA) strains have emerged in recent years. CA-MRSA infections have occurred in absence of health care-associated risk factors traditionally associated with MRSA infections and have predominantly involved in skin and soft tissue infections. "New" strain pulsed-field gel electrophoresis (PFGE)-type "US-300," which carries the *mec IV* gene conferring resistance to methicillin CA-MRSA isolates frequently contains genes encoding for Panton-Valentine Leukocidine (PVL), a leukocyte-killing exotoxin linked to development of furncules, cutanoeus abscesses, and severe necrotic infections. CA-MRSA infection presents most commonly as relatively minor skin soft tissue infections, but severe invasive disease, including necrotizing pneumoniae, severe osteomyelitis, and a sepsis syndrome with increased mortality have also been described in children and adults (Siegel et al., 2006).

f) Vancomycin Resistant *S .aureus* (VRSA)/ glycopeptide intermediate *S. aureus* (GISA):

Vancomycin resistance bacteria emerged initially among gut flora and they have latter become resistant to glycopeptide antibiotic vancomycin. Vancomycin (or teicoplanin) is often a treatment of choice in infections with MRSA. Vancomycin Resistant *S. aureus* (VRSA) isolates show resistance to meropenem and imipenem. It is

also termed GISA (glycopeptide intermediate *S. aureus*) indicating resistance to all glycopeptide antibiotics. These resistant bugs bypass vancomycin interference by thickening peptidoglycan mesh in bacterial cell wall without resorting to cross-linking is shown in Figure 4b (Foster et al., 2004).

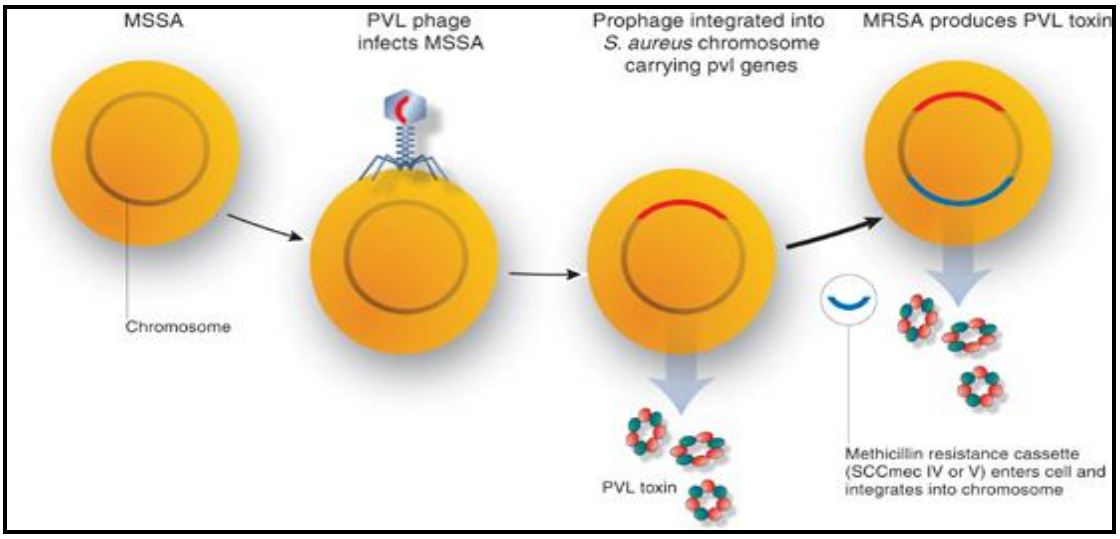


Figure 4b: Model for emergence of PVL producing CA-MRSA: A MSSA strain is infected and lysogenized by a phage (phiSLT) that harbors lukS-PV and lukF-PV genes (*pvl*) encoding the PVL. Subsequently, a methicillin resistance cassette (*SCCmec* IV, V or V_T) carrying the *mecA* gene is horizontally transferred into the *pvl*-positive MSSA strain and integrates into the genome in a location that is distinct from that of phiSLT integration site. (Source: Boyle-Vavra S and Daum RS. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton–Valentine leukocidin. *Laboratory Investigation*. 2007;87**,** 3–9)

4.2.6. GENETIC ORGANISATION OF METHICILLIN RESISTANCE:

4.2.6.1. *Staphylococcal* Casette Chromosome *mec (SCCmec):*

The *mecA* gene, is carried on mobile genetic element, staphylococcal cassette chromosome *mec* (*SCCmec*), found only in methicillin-resistant strains, *SCCmec* is located in same region in *S. aureus* chromosome, near protein A. Four structurally different *SCCmec* types (*SCCmec* I-IV) and a few variants have been identified. All *SCCmec* types contain common features, including *mecA* gene and its regulatory region, and *ccrA* and *ccrB* genes (cassette chromosome recombinase, required for site and orientation specific integration and excision of the *SCCmec*) shown in figure 4c (Enright et al., 2002, Chongtrakool et al., 2006, and Salmenlinna et al., 2002).

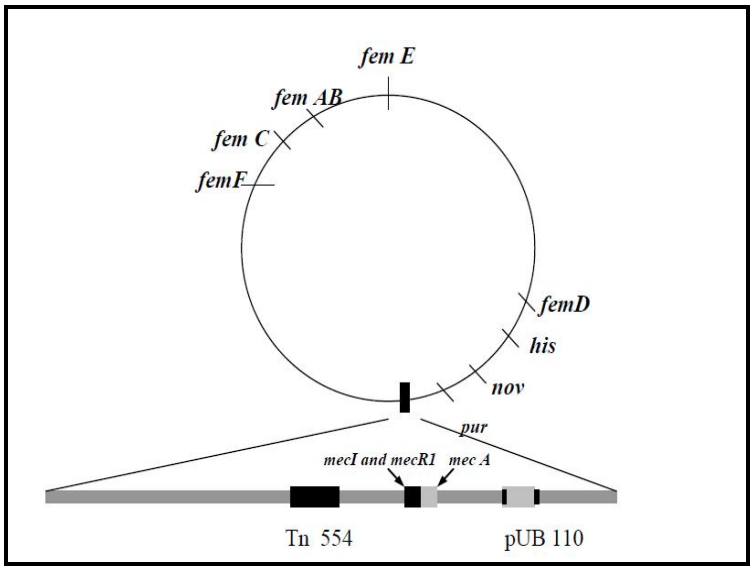


Figure 4c: Molecular organization of the approximately 50-kb *mec* region and its chromosomal location relative to *fem* factors and *pur-nov-his*. Tn554 is a transposon containing *ermA*, encoding inducible erythromycin resistance.

4.2.6.2 The *mec* DNA complex:

Approximately 30 to 50 kb of additional chromosomal DNA is added to the staphylococci. This *mec,* not found in susceptible strains of *Staphylococci* is present in methicillin-resistant strains only. It is always found near the *pur-nov-his* gene cluster on the *S. aureus* chromosome.

Components present in this *mec* DNA complex *are*

a) *mecA*, the structural gene for penicillin binding protein 2a (PBP 2a)*;*
b) *mecI* and *mecR1*, regulatory elements controlling *mecA* transcription;
c) 20 to 45 kb of *mec* associated DNA.

4.2.6.3 *mec A*, gene that codes for Methicillin resistance & its penicillin binding proteins:

The *mecA* is highly conserved among staphylococcal spp., *mecA* gene encodes for PBP 2a, an inducible 76-kDa PBP which confer resistance of staphylococci to methicillin. There is no *mecA* homolog in susceptible strains. Both susceptible and resistant strains of *S. aureus* produce four major PBPs, PBPs 1, 2, 3, and 4, with approximate molecular masses of 85, 81, 75, and 45 kDa. PBPs are membrane bound DD-peptidases that have evolved from serine proteases, and their biochemical activity is mechanistically similar to that of the serine protease. These enzymes catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall.

4.2.6.4. Role of PBP against action of Beta lactam antibiotics:

Beta lactam antibiotics are substrate analogs that covalently bind to the PBP active-site serine, inactivating enzyme at concentrations that are approximately the same as the MICs. PBPs 1, 2, and 3, which have high affinity for most beta-lactam antibiotics, are essential for cell growth and for the survival of susceptible strains, and binding of Beta-lactams by these PBPs is lethal.

In methicillin-resistant cells, PBP2a, with its low affinity for binding beta-lactam antibiotics can substitute for the essential functions of high-affinity PBPs at concentrations of antibiotic that are otherwise lethal.

The expression of *mecA* gene and the resulting production of PBP2a is regulated by proteins encoded by penicillinase-associated *blaR1-blaI* inducer–repressor system and the corresponding genomic *mec R1-mec I* element is described in figure 4d (Salmenlinna et al., 2002, Lim et al., 2002, Bignardi et al., 1996, Weller et al., 1999, Chongtrakool et al., 2006 and Kobayashi et al., 1998).

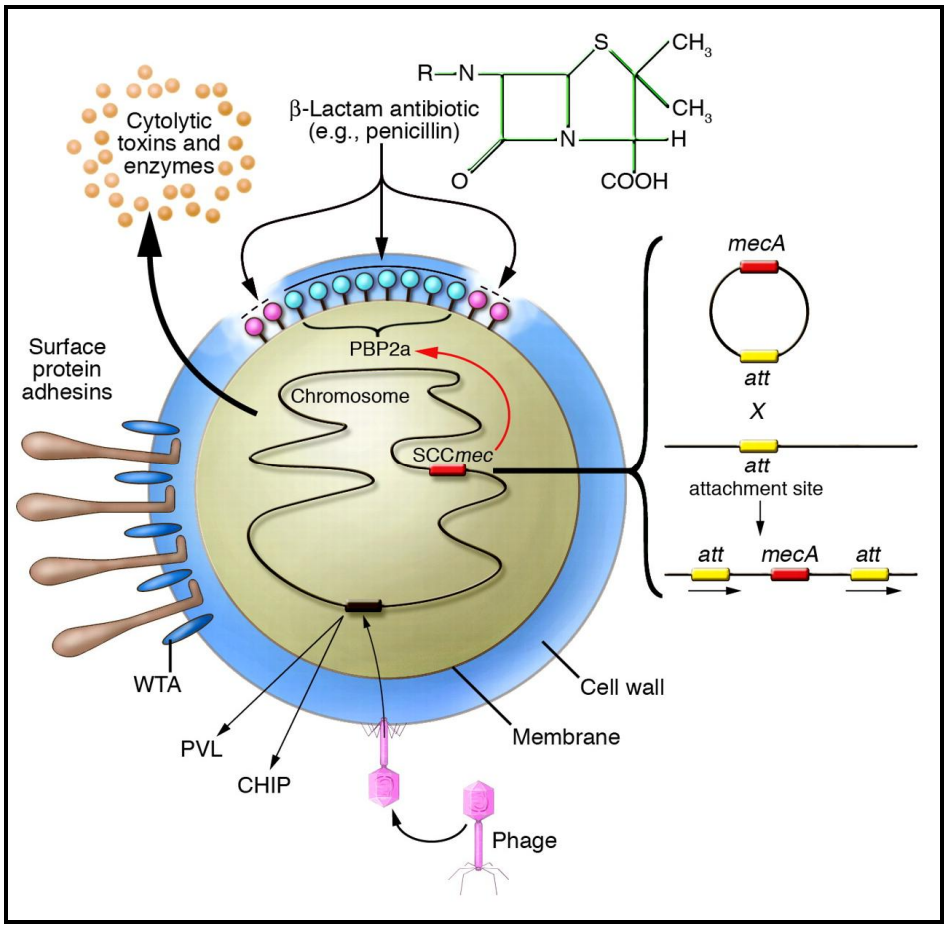


Figure 4d: Schematic diagram illustrating how *S. aureus* acquires resistance to methicillin and its ability to express different virulence factors. Bacterium expresses surface protein adhesions, wall teichoic acid (WTA) many toxins, enzymes by activation of chromosomal genes. Resistance to methicillin is acquired by insertion of a horizontally transferred DNA element called SCC*mec*. *mecA* gene encodes novel ß-lactam–insensitive penicillin binding protein, PBP2a, which continues to synthesize new cell wall peptidoglycan even when normal penicillin binding proteins are inhibited. (Source: Foster TJ. The *Staphylococcus aureus* "superbug". *J Clin Invest.* 2004; 114:1693-1696).

4.2.6.5 The *mec R1-mec I:*

The *mecA*-positive staphylococci also carry the *mecR1* and *mecI* genes, which are involved in the regulation of mecA. When present, they are situated within the *mec* region immediately upstream of *mecA.* They are, however, transcribed in the opposite direction. The *mecR1* protein consists of two regions, a membrane spanning domain and a penicillin-binding domain. It is a slow inducer of methicillin resistance; in contrast, the *mecI* protein is a strong repressor (Enright et al., 2005, Salmenlinna et al., 2002, Chongtrakool et al., 2006 and Kobayashi et al., 1998).

4.2.6.6 The *bla I* and *bla R1:*

Resistance to, Beta-lactam antibiotics in *S. aureus* is primarily due to beta lactamase, an enzyme that is induced by, Beta-lactam antibiotics. The beta lactamase gene (*blaZ*) is maintained on a plasmid, and two loci closely linked to *blaZ* are involved in the regulation of beta lactamase production. Two open reading frames located upstream of and in the opposite orientation to *blaZ* have recently been sequenced and identified as *blaI* and *blaRl* (Chambers et al., 1997).

4.2.7 Heteroresistant resistance:

In clinical methicillin-resistant *S. aureus* isolates, intrinsic methicillin resistance is due to the production of a unique, additional, low-affinity PBP, PBP 2a, which is encoded by *mecA.* In recent years, the increase in the number of bacterial strains that show resistance to methicillin (MRSA) has become a serious clinical and

epidemiological problem because this antibiotic is considered as the first option in the treatment of Staphylococcal infection. Among these organisms a few clinical isolates of MRSA express homogeneous resistance (i.e., 1 in 10^2 cells express high-level resistance); the majority of isolates have heterogeneous drug resistance (heteroresistance) to penicillin group of drugs. These "heteroresistant" bacteria was also recognized as problematic organisms for antimicrobial susceptibility tests .The problems are basically due to the occurrence of two populations, one susceptible and one resistant to methicillin, in every culture. The resistant population generally grows much slower than the susceptible population, and thus, a culture may appear susceptible because the resistant population has not grown enough to be seen macroscopically. It was further reported that varying the test conditions had major effects on the detection of resistance (Merlino et al. 2002, Sakoulas et al. 2001).

4.2.8 Laboratory diagnosis of Methicillin resistant *S. aureus*:

a) Dilution methods:

Agar dilution, tests on MH or Columbia agars with 2% NaCl and an inoculum of 10^5 CFU/ml, will distinguish most resistant form susceptible strains With the British Society of Antimicrobial Chemotherapy method either medium or Muller Hinton broth can be used or supplemented with varius compounds eg. Horse blood (5%) thiamine, tween 80, Potassium chloride or NaCl with Clinical Laboratory Standards Institute (CLSI) method only MH with 2% NaCl is permitted both methods require incubation for at 30^{o}C, while CLSI requires 33-35°C. In both methods an oxacillin MIC of $\leq$ 2

mg/L indicates that the strain is susceptible and $\geq$ 2 mg/L resistant and a methicillin MIC of $\leq$ 4 mg/L indicates that the strain is susceptible and > 4 mg/L resistant.

Broth microdiultion: CLSI method requires use of MH broth with 2 % NaCl, an inoculum of 5 X 10^5 CFU/ml and incubation at 33-35^{o}C for 24 hrs, is the only defined method in general use.

b) Etest method :

This method gives an MIC result and is affected by test condition in a similar way to other MIC and diffusion methods. Test conditions recommended by manufacturer are based on providing results comparable with CLSI methods and include MH agar with 2% NaCl, an inoculum density equivalent to 0.5-1.0 McFarland standards, application of inoculum with a swab and incubation at the 35^0C.

c) Breakpoint methods:

Breakpoint methods include both agar and broth methods and use essentially similar to dilution MIC methods but test only the breakpoint concentration (2 mg/L oxacillin, 4 mg/L methicillin)

d) Agar screening method:

Here the suspending the test organism to the density of a 0.5 McFarland standard and inoculating MH agar containing 4% NaCl and 6 mg/L oxacillin with a spot or a streak of organism. Plates are incubated at 35^0C or less for 24 h and any growth other than the single colony are indicative of resistance. To minimize the chance of missing methicillin resistant coagulase negative *Staphylococcus*, micro dilution test should be

incubated for 48 h before a strain is called susceptible and it should be confirmed with either disk diffusion or agar screen methods. Thus MIC is determined.

d) Latex agglutination:

Successful development of a slide latex agglutination assay for direct detection of PBP 2a from *S. aureus* isolates after a rapid extraction procedure which takes about 10min for a single test (Brown et al., 2005, Brown et al., 2001, Merlino et al., 2002, Sakoulas et al., 2001, Locatelli et al., 2003, Geha et al., 1994, Unal et al., 1994, Gerberding et al., 1991, Wallet et al., 1996, Coudron et al., 1996, Peterson et al., 1992, Reynolds et al., 2003.)

e) Molecular methods:

Polymerase Chain Reaction:

Kary Mullis invented PCR technique in 1985 while working as a chemist at Cetus Corporation, a biotechnology firm in Emeryville, California. This requires placing a small amount of the DNA containing the desired gene into a test tube. A large batch of loose nucleotides, which link into exact copies of the original gene, is also added to the tube. A pair of synthesized short DNA segments, that match segments on each side of the desired gene, is added. These "primers" find the right portion of the DNA, and serve as starting points for DNA copying. When enzyme *Thermus aquaticus* (*Taq*) is added, the loose nucleotides lock into a DNA dictated by the sequence of that of target gene located between the two primers.

4.2.9 Advantages of PCR:

The ability of PCR to amplify small amounts of specific nucleic acid has made it an important and convenient diagnostic tool with a potential to detect microorganisms rapidly and reliably Specificity and sensitivity of PCR to amplify small amount of specific nucleic acid has made it one of the most sought after tools to be used for diagnostic purposes**.**

4.2.10. Need for this study:

Rajaduraipandi *et al.,* 2006, found 31.1 and 37.9 percent of *S. aureus* exhibiting methicillin resistant among clinical specimens and carrier samples. In another study 30 per cent of patients was considered to have acquired MRSA via nosocomial transmission and 70 per cent to had community acquired MRSA. Of them, 1.3 per cent had ophthalmic MRSA involvement**.** The most common manifestation of ophthalmic MRSA infection was preseptal cellulitis and/or lid abscess followed by conjunctivitis, but sight-threatening infections, including corneal ulcers, endophthalmitis, orbital cellulitis, and blepharitis are occurred (Blomquist et al., 2006).

Coagulase-negative *Staphylococci* (CoNS) are the most common pathogens causing endophthalmitis (Locatelli et al., 2003) and most cases of infectious endophthalmitis occurring after cataract surgery are due to bacteria entering the eye at the time of surgery (Kamalarajah et al., 2002). Laboratory diagnosis and susceptibility testing are crucial in treating, controlling, preventing MRS infections (Brown et al. 2005).

MRS has ability to grow in presence of derivatives of beta-lactams (Brown et al., 2005, Perez-Roth et al., 2001, Tang et al., 2007, Geha et al., 1994). Methicillin resistance (MR) is transferred to susceptible strains through horizontal transfer of *mecA* gene (Chambers et al., 1997). The *mecA* gene encodes penicillin binding protein, PBP2a (Perez-Roth et al., 2001, Weller et al., 1999) and it is a useful molecular marker of putative MR (Perez-Roth et al., 2001).

Detection of MR by conventional method is time consuming, influenced by antibiotics, culture medium, NaCl concentration, temperature and time of incubation. PCR-based methods for detection of MR by *mecA* gene, is considered 'gold standard' and the results can be obtained quickly (Brown et al., 2005, Ubukata et al., 1992).

The *femB* gene codes for an enzyme important in cross-linking peptidoglycan in various *Staphylococci.* Specificity of the *femB* gene PCR primers used for DNA amplification in *S. aureus* has been demonstrated previously (Jonas et al., 2002).

Since phenotyping methods are not efficient and are highly dependent on growth conditions, it is essential to use molecular techniques to stop spreading of multiple-antibiotic resistant *S. aureus*. PCR techniques therefore allow the rapid, accurate identification of *Staphylococci* and their resistance type. Thus fast, sensitive, and specific molecular methods will be the essential diagnostic tool for microbial laboratories.

4.3. Objective:

To determine the rate of isolation of MRS among ocular isolates by conventional and molecular methods and to standardize, apply PCR-RFLP and DNA sequencing techniques for identification and speciation of MRS and to compare the results of conventional and molecular methods in the detection of MR.

- **a.** Conventional method of Detection of Methicillin resistant Staphylococcus.
- **b.** Standardization of uniplex PCR and duplex PCR for detection of *Staphylococcus aureus* and methicillin resistant *Staphylococcus spp.*
- **c.** Comparison of PCR results with conventional method.
- **d.** Identification of Coagulase Negative Staphylococcus to spp. level by PCR-RFLP of the *gap gene* using the *Alu* I restriction enzyme.

4.4. Materials and Methods:

4.4.1. Bacterial strains:

One hundred and ten consecutive staphylococcal isolates recovered from ocular clinical specimens (conjunctival swab - 46, donor corneal rim - 36, corneal scraping - 9, anterior chamber tap - 1, corneal button - 6, vitreous aspirate - 1, others - 11) received at L & T Microbiology Laboratory, Sankara Nethralaya, a tertiary eye care centre at Chennai, India, during November 2005 - August 2006, was included in this study. The study protocol was approved by the institutional ethics sub-committee. Clinical specimens were processed as described elsewhere (Folkens et al. 1996). Gram-positive

cocci isolated from the ocular clinical specimen in more than one medium were included in the study.

4.4.1. Identification of methicillin resistance by conventional method:

All the staphylococcus isolates were subjected for the detection of methicillin resistance by dilution methods and disc diffusion methods.

4.4.1.1. Microdilution method:

Minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

Quality control – Operational techniques that are used to ensure accuracy and reproducibility.

Oxacillin, (Sigma-Aldrich Company, USA) stock solution was prepared at concentrations of 1280µg/ml in deionized water. Antibiotic was serially diluted in Muller-Hinton broth with 2.0 per cent NaCl to give working concentrations of 64 - 0.125 µg/ml (Merlino et al. 2002) and bacterial suspension of 0.5 McFarland turbidity standards (containing approximately 1 to 2 x 10^8 CFU/ ml) was added to all the tubes and was incubated for 24 h at 35 ± 2ºC18. The MIC breakpoint of oxacillin (1 µg/ml) for *S. aureus* is > 4.0 - < 2.0 (µg/ml) and for CoNS it is > 0.5 - < 0.25 (µg/ml). The procedure is briefly as follows.

4.4.1.1.1. Preparation of Antimicrobial Agents:

Antimicrobial agents were prepared from the commercially available antimicrobial powders based on their potency.

4.4.1.1.2. Potency calculation:

Potency = (Assay purity) x (Active fraction) x (1- Water Content)

Potency = (998) x (1.0) x (1- 0.121)

Potency = 877 µg/mg or 87.7%

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} * \text{Concentration (µg/mL)}}{\text{Potency (µg/mg)}}$$

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} * \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}}$$

4.4.1.1.3. Preparing Stock Solutions:

Antimicrobial agent stock solutions were prepared at concentrations of at least 1000 µg/mL (example: 1280 µg/mL) or ten times the highest concentration to be tested. It is dissolved in minimum amount of solvent to solubilize the antimicrobial powder. Final stock concentration was made up with water because microbial contamination is extremely rare. However, solutions were sterilized by membrane filtration, dispensed in small volumes into sterile glass, polyethylene vials; carefully sealed and stored at -20°C. Stock solutions of most antimicrobial agents can be stored at -60°C or below for six months or more without significant loss of activity.

4.4.1.1.4. Inoculum Preparation for Dilution Tests:

1) Direct Colony Suspension Method:

Direct colony suspension is the recommended method for testing the Staphylococci for potential methicillin or oxacillin resistance. The colony was prepared by making a direct broth of isolated colonies selected from an 18- to 24-hour agar plate (nonselective medium, such as blood agar, should be used). After 15 minutes of incubation, suspension was matched with 0.5 McFarland turbidity standards. This results in a suspension containing approximately 1 to 2 x 10^{8}CFU/mL. Agar dilution MIC tests were performed in duplicate as recommended by Clinical Laboratory Standards Institute (CLSI). Oxacillin / methicillin may be used, although oxacillin is most commonly used because of its stability, availability, & reproducibility of tests.

PROCEDURE:

a. Twelve micro dilution wells are needed for determination of MIC for each isolate.
b. Hundred µl of Muller Hinton broth with 2% Nacl was added to all 12 wells and 12^{th} well was labeled as medium control.
c. Hundred µl oxacillin solutions of 16µg/ml were added to the 1^{st} well.
d. Mixed well and 100µl was transferred from 2^{nd} well to 3^{rd} well till the 10^{th} well and from last well 100µl of it was discard.
e. Hundred µl of 1 to 2 x 10^{8}CFU/mL inoculum was added to all well excluding 12^{th} well and the 11^{th} well act as growth control.
f. Micro wells was incubated at 35 ^{0}C for 24- 48 hrs & MIC is found out. (Table 1, 2)

4.4.1.2. Disk diffusion methods:

4.4.1.2.1. Recommended interpretive criteria:

1. Susceptible (S): The "susceptible" category implies that isolates are inhibited by usually achievable concentrations of antimicrobial agent when the recommended dosage is used for site of infection.

2. Intermediate (I): The "intermediate" category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates.

3. Resistant (R): The "resistant" category implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

4.4.1.2.2. Reagents for disc diffusion method:

a) Mueller-Hinton Agar (MHA):

MHA was prepared from a commercially available dehydrated base. To it 5% Nacl was added and given for autoclaving. Immediately after autoclaving, agar is allowed to cool to 45 to 50°C by placing in water bath and poured into sterile perti dishes. Plates were kept for sterility checking. Before use if excess surface moisture is present on the plates, they was kept in an incubator or a laminar flow hood at Room Temperature (RT) with lids ajar until excess surface moisture is lost by evaporation.

b) Storage of Oxacillin/ Methicillin Antimicrobial Disks:

The disk was stored at 8 ℃ or below, or freezes at -14 ℃ or below until needed. Two hours before using disk was taken from refrigerator and allowed to come to room temperature. The disc was not advised to use when they reach the expiration date.

c) Inoculum Preparation by Direct Colony Suspension Method:

Broth suspension of turbidity equivalent to a 0.5 McFarland turbidity standard was prepared. This results in a suspension containing approximately 1 to 2 x 10^{8}CFU/mL.

4.4.1.2.3. Procedure for Performing the Disk Diffusion Test:

1) Inoculation of Test Plates:

After 15 minutes of incubation, a sterile cotton swab was dipped into the adjusted suspension, rotated the swab several times and pressed firmly on inside wall of tube above the fluid level. This will remove excess inoculum from swab. Lawn culture was made with the swab. After the broth is completely absorbed Methicillin disc (5μg) discs are placed. Plates were incubated at 35 ± 2ºC for 18-24 hours before reading the result. MSSA (ATCC 6538) and MRSA (ATCC 33591) was included as controls.

2) Reading Plates and Interpreting Results:

Transmitted light was used to examine the methicillin zones for light growth of resistant colonies within apparent zones of inhibition. Any discernable growth within zone of inhibition is indicative of methicillin resistance (Prasad et al., 2002, CLSI, M2-A9. 2000 and CLSI, M7-A7. 2000).

Interpretative criteria for disc diffusion method for *S. aureus*: resistant < 9mm, intermediate resistance 10-13mm and sensitive >14mm (CLSI, M2-A9. 2000). In order to distinguish strains exhibiting "Intermediate resistant" from that of "heterogenous strains" the sensitivity plates with strains exhibiting resistance was incubated for an additional 24 h. At the end of 48 h of incubation, heterogenous strain turned sensitive whereas intermediate resistant strains remained resistant.

All coagulase negative staphylococci was tested for antibiotic susceptibility against cefazolin (30 μg), ciprofloxacin (5 μg), moxifloxacin (5 μg), Norfloxacin (10 μg), gentamicin (10 μg), tobramycin (10 μg), ofloxacin (5 μg), and penicillin G (10 units) by Kirby bauer disc diffusion method. All the antibiotics were obtained from Hi-Media Laboratories Private Limited, Mumbai (CLSI, M2-A9. 2000).

4.4.2. Molecular methods for detection of *Staphylococcus aureus* using *femB* and identification of MRS using *mecA* primers:

4.4.2.1. DNA extraction:

DNA was extracted using a single colony from an overnight culture of Staphylococci by modified guanidine iso thiocyanate (GITC) method (Malathi et al., 2002) with modification where proteinase K and lysostaphin 1μg/μl (Sigma-Aldrich-L0761 - *Staphylococcus simulans* (USA) was added for enhancing digestion at the initial step). The protocol followed is described in Appendix 2.

4.4.2.2. Primer sequence:

The primer sequences, used for the amplification of *femB* and *mecA* genes and the amplified product size are described in the table below (Perez-Roth et al., 2001) with slight modifications. Table 4a shows primer sequences of *femB* and *mecA* genes.

Table 4a: Primer sequences of *femB* and *mecA* genes

Gene	Primer sequence	Size of product
fem B	*femB1* 5'-TTA CAG AGT TAA CTG TTA CC-3' *femB2* 5'-ATA CAA ATC CAG CAC GCT CT-3'	651 bp
mec A	*mecA1* 5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3' *mecA2* 5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3'	310 bp

4.4.2.3. PCR Thermal Profile: (for Uniplex PCR for each *femB* and *mecA* gene)

Initial denaturation	94°C - 5 minutes	
Denaturation	-94°C - 30 seconds	**10 cycles**
Annealing	-64°C - 30 seconds	
Extension	- 72°C - 45 seconds	
Denaturation	- 94°C - 45 seconds	**35 cycles**
Annealing	- 50°C - 45 seconds	
Extension	- 72°C - 60 seconds	
Final extension	- 72°C - 10 minutes	
Hold	- 22° C - ∝ (Room Temperature)	

4.4.2.4. PCR Thermal Profile: (for duplex PCR combining both *femB* and *mecA*)

Initial denaturation	- 94°C - 5 minutes	
Denaturation	- 94°C - 30 seconds	**10 cycles**
Annealing	- 64°C - 30 seconds	
Extension	- 72°C - 45 seconds	
Denaturation	-94°C - 45 seconds	**35 cycles**
Annealing	- 50°C - 45 seconds	
Extension	- 72°C - 60 seconds	
Final extension	-72°C - 10 minutes	
Hold	- 22° C - $\propto$ (Room Temperature)	

Both the uniplex and duplex PCRs was carried out with ATCC controls in every PCR reaction. i.e. Methicillin Sensitive *S. aureus* (ATCC 6538), Methicillin Resistant *S.aureus* (ATCC 33591)

All PCR reagents used for amplification including primers was procured from Bangalore Genei, Ltd. Bangalore, India. All PCR amplifications were carried out using PCR thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, Massachusetts, USA).

4.4.2.5. Specificity of PCR: -

Specificity of PCR for *S. aureus* ***(femB)*** and methicillin resistance ***(mecA)*** was tested with DNA extracted from *Mycobacterium tuberclosis* (H37RV) , *M. xenopi* (lab isolate), *M. fortuitum* (lab isolate), *M. chelonale* (lab isolate), *Candida albicans* (ATCC 24433) *Aspergillus spp.* (lab isolate), *Cytomegalovirus* (CMV), *Acanthamoeba spp.* (lab isolate), *Toxoplasma gondii* (RH Strains), *Corynebacterium xerosis* (lab isolate), *Nocardia asteroids* (lab isolate), *Actinomyces spp.* (lab isolate), *Haemophilus influenzae* (ATCC 10211), *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 10211), *Streptococcus pneumoniae* (ATCC 6301), *Bacillus cereus* (lab isolate), *Acinetobacter calcoaceticus* (ATCC 9956), *Enterococcus faecalis* (ATCC 49149) *E.coli* (ATCC 4157) , *Pseudomonas aeruginosa* (ATCC 9742), *Propionibacterium acnes* (ATCC 11828).

4.4.2.6. Sensitivity of PCR:

From the extracted DNA 5µl was mixed with 45 µl of Milli Q water. Serial 10 fold dilutions were carried out. From that 5 µl was taken for amplification and the amount of DNA present was quantified using nanodrop.

4.4.2.7. Detection of amplified product:

Amplified product was subjected to electrophoresis on 2% agarose gel incorporated with 0.5µg/ml ethidium bromide (Appendix 1) for visualization by Ultra Violet (UV) transilluminator.

4.4.3. Molecular methods for identification of various spp. of MRS:

In order to speciate MRS, the DNA was first amplified using primers targeting *gap* gene followed by Restriction Fragment Lengh Polymorphism (RFLP) analysis of amplified products using restriction enzymes.

4.4.3.1. PCR of *gap* gene:

PCR for *gap* gene was performed only for 70 methicillin resistant isolates using primers described (Geha et al. 1994 and Geha et al. 1994). PCR was carried using 5 µl of DNA extracted added to 45 µl of PCR mixture consisting of 5 µl 10X PCR buffer containing 15 mM $Mgcl_2$, 200 µM dNTPs, 5 picomoles of forward and reverse primers, 30 µl deionized water of and 1.25U of *Taq* polymerase. Thermal profile followed was initial denaturation at 94°C for 2 min, followed by 40 cycles consisting of denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec, and extension at 72°C for 40 sec and a final extension at 72°C for 5 min.

4.4.3.2. RFLP of *gap* gene:

RFLP of the *gap* gene amplified product was done using the restriction enzyme *Alu*I (5' AG-CT 3', 3' TC-GA 5'). Reaction mixture (30µl) consisted 17µl deionized water, 10µl PCR product, 3µl buffer, 0.4µl restriction enzyme (10 units/µl, Fermentas Life Science, USA) and incubated at 37°C water bath for 2 h followed by snap freezing. RFLP products were analyzed using 4 per cent gel electrophoretogram. Staphylococcal isolates with identical RFLP pattern was grouped together and one isolates from each

group and three reference strains (*S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228) and *S. saprophyticus* - lab isolate) was sequenced using forward, reverse primers of *gap* gene following the protocol described by Weller et al. 1999, except DNA template taken is 20-30 ng.

4.4.3.2. Cycle sequencing and purification:

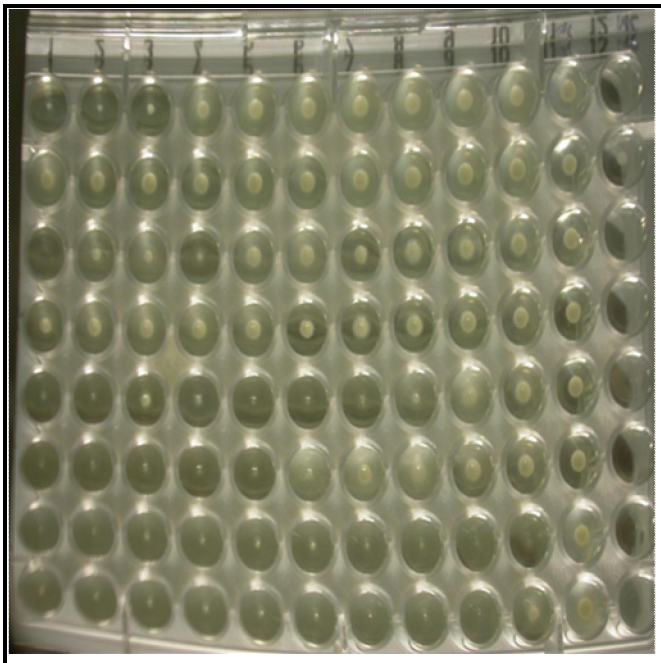
All the amplified products was then cycle sequenced, purified and loaded into the ABI 3100 Genetic Analyzer (Applied Biosystem, USA) and the resulting sequences was analyzed. The protocol followed is described in Appendix 3.

4.5. RESULTS:

Of 110 staphylococci isolated from varied ocular specimens, maximum number of MRS isolates was from conjunctival swab and donor corneal rim. All the isolates were Gram positive and catalase positive, utilized glucose. Of the 110 isolates, 23 was identified as *S. aureus* based on the results of tube coagulase, mannitol fermentation and growth in 7.5 per cent NaCl and the remaining 87 was CoNS.

4.5.1. Results of microbroth dilution:

By microbroth dilution method, of the 110 staphylococcal isolates, 8 (7.2%) had MIC of 32µg/ml by oxacillin, 22 (20%) had 16µg/ml, 10 (9.1%) each had 8, 4 and <2µg/ml. Ten isolates (9.1%) had MIC <0.5µg/ml and remaining 40 (36.3%) had MIC <0.125µg/ml (MSS). Figure 4e shows the result of microbroth dilution test.



1. MIC of 16μg/ml - 50 μL innoculum (M-7355/06)
2. MIC of 64 μg/ml - 100 μL innoculum (M-7355/06)
3. MIC of 8μg/ml with 50 μL inoculum (M-2348/06)
4. MIC of 64μg/ml with 100 μL inoculum (M-2348/06)
5. MIC of 1μg/ml with 50 μL inoculum (M-1324/06)
6. MIC of 4μg/ml with 100 μL innoculum (M-1324/06)
7. MIC of with 50 μL of *S. aureus* (ATCC 6538)
8. MIC of with 100 μL of *S. aureus* (ATCC 6538)

4e: Detection of MIC of test isolates by Microdilution method: MIC results of oxacillin of the ocular isolates Staphylococcal carried out by microbroth dilution method.

4.5.2. Results of disk diffusion test:

Among the 23 *S. aureus*, 18 (78.2%) was MRS with inhibition zone of <6 mm disc diffusion method for methicillin and 5 was (21.73%) methicillin sensitive with an inhibition zone of > 14mm. Three *S. aureus* isolates exhibited intermediate resistance at 24 h incubation, became resistant without the zone of inhibition, following an additional 24 h of incubation. Figure 4f shows the result of disk diffusion test**.**

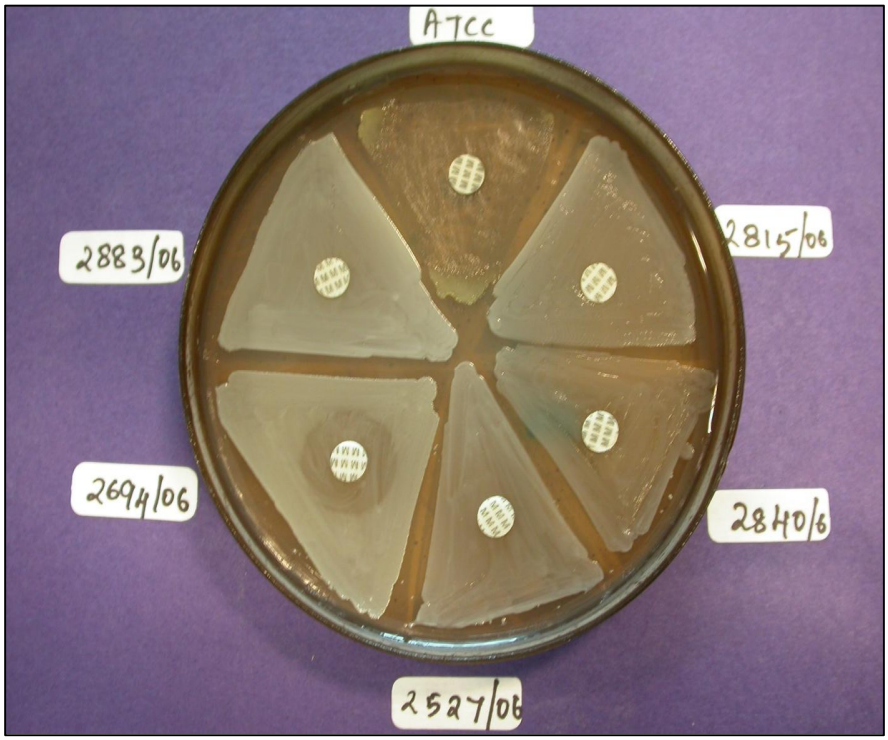


Figure 4f: Disk Diffusion results of 5 test isolates along with MSSA ATCC 6538 after 24 h of incubation. All 5 isolates showed no zone of inhibition. The isolate M-2694/06 showed tiny colonies in the region of zone of inhibition. *S.aureus* (ATCC 6538) showed 30 mm diameter of zone of inhibition

Tables 4b and 4c give comparative results of conventional and molecular methods in detection of *S. aureus* and methicillin resistance. Maximum number of resistance was observed for tobramycin, followed by norfloxacin, gentamicin. In our study 90 per cent of isolates showed resistance to more than two antibiotics (Table 4d).

Table 4b: Comparative results of conventional method with PCR performed on 110 ocular isolates of *Staphylococcus spp.* for the identification of *S. aureus.*

Total no of Isolates	No of strains positive for *S. aureus*		No of strains negative for *S. aureus*	
	Conventional Method (coagulase test)	PCR for *femB* gene	Conventional Method (coagulase test)	PCR for *femB* gene
N=110	23 (20.9%)	23 (20.9%)	87 (78.1%)	87 (78.1%)

Table 4c: Comparative results of conventional method with the microbroth dilution performed on 110 ocular isolates of Staphylococcus spp. for detection of MR.

Total no of isolates	No of strains positive for methicillin resistance			No of strains negative or methicillin resistance		
	Conventional method		PCR for *mecA* gene	Conventional method		PCR for *mecA* gene
	Disc diffusion method	Micro broth dilution method		Disc diffusion method	Micro broth dilution method	
N=110	70 (63.6%)	70 (63.6%)	70 (63.6%)	40 (36.4%)	40 (36.4%)	40 (36.4%)

¶ = MIC break point of Oxacillin (1 µg/mL): For *S. aureus* = ≥ 4.0 - ≤ 2.0 (µg/mL)
For CoNS = ≥ 0.5 - ≤ 0.25 (µg/mL)

Table 4d: Results of antibiotic susceptibility testing showing number of Methicillin Resistant *Staphylococci spp.* isolated.

(No of resistant strains/ total no of strains)

Species	Cefazolin	Ciprofloxcin	Moxifloxacin	Norfloxacin	Gentamicin	Tobramycin	Ofloxacin	Penicillin
S.aureus	10/18	11/18	2/18	15/18	16/18	17/18	8/18	18/18
S.haemolyticus	11/27	11/27	3/27	24/27	22/27	24/27	4/27	27/27
S. epidermidis	1/11	5/11	2/11	8/11	7/11	7/11	1/11	11/11
S. cohnii subspp. urealyticum	2/6	2/6	0/6	3/6	3/6	3/6	0/6	6/6
S. equorum	1/6	3/6	0/6	4/6	5/6	5/6	3/6	6/6
S. hominis	0/1	0/1	0/1	0/1	1/1	1/1	0/1	1/1
S. xylosus	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1
TOTAL	**25/70**	**32/70**	**7/70**	**54/70**	**54/70**	**57/70**	**16/70**	**70/70**

4.5.3. Results of Sensitivity of uniplex PCR for *femB* gene*:*

Sensitivity of uniplex PCR for *femB* gene was 1.3ng DNA of MSSA-ATCC 6538 is shown in figure 4g. Sensitivity was increased from 10.0 ng to 1.3ng by increasing $Mgcl_2$ from1.5 mM to 3.0 mM, annealing temperature was increased to 64°C, number of cycles increased from 25 to 35

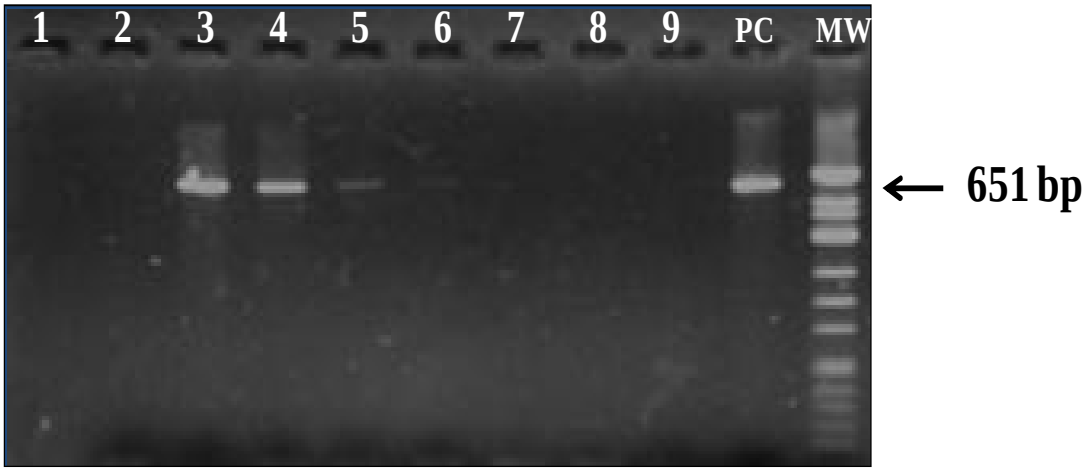


Figure 4g: Agarose gel electrophoretogram showing results of sensitivity of *femB* gene PCR. **Lane 1:** Negative control **Lane 2:** Extraction control, **Lane 3:** *S. aureus* DNA standard strain ATCC 6538 10 µg (undiluted) **Lane 4 - 9:** Serial 10 fold dilutions of *S. aureus*, **PC:** Positive control MSSA ATCC 6538, **MW:** Molecular weight marker: Phi X 174 bacteriophage DNA *Hinf* – I digest.

4.5.4. Results of Sensitivity of uniplex PCR for *mecA* gene:

Sensitivity of uniplex PCR for *mecA* gene it was 0.77 ng DNA of MRSA ATCC 33591 is shown in figure 4h. The sensitivity was originally 8.0 ng of *S. aureus* DNA. It was increased to 0.77ng primer was able to detect upto 0.77 ng of DNA, increasing $Mgcl_2$ from1.5mM to 3.0mM, annealing temperature was increased to 64°C, number of cycles was increased from 25 to 35 cycles.

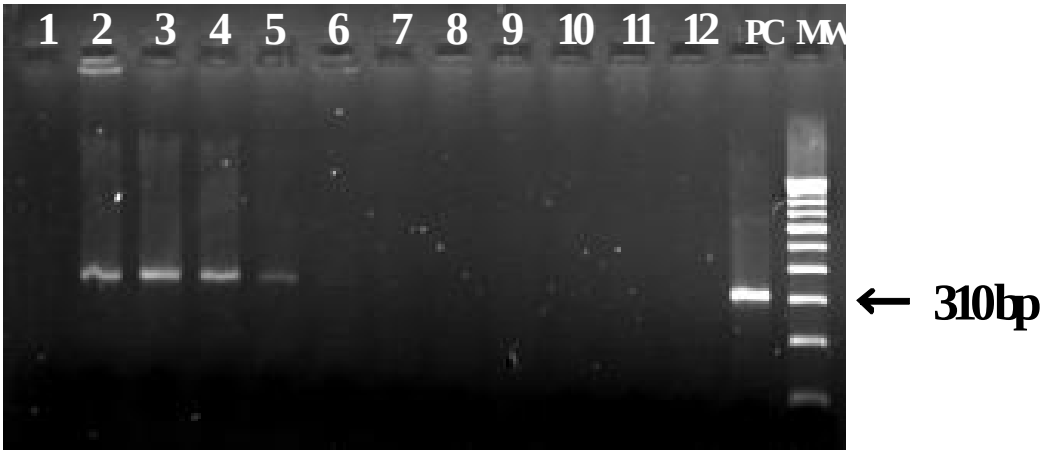


Figure 4h: Agarose gel electropheretogram showing results of sensitivity of *mecA* gene PCR. Lane 1: Negative control **Lane 2:** *S. aureus* DNA standard strain MRSA ATCC 33591 10 µg (undiluted) **Lane 3 - 12:** Serial 10 fold dilutions of MRSA, **PC:** Positive control MRSA ATCC 33591, **MW:** Molecular weight 100 bp ladder.

4.5.5. Results of Specificity of uniplex PCR for *femB* and *mecA* gene PCR:

The *femB* gene was specific for detection of *S. aureus* is shown in figure 4i and and *mecA* gene was specific for the detection of MR is shown in figure 4j.

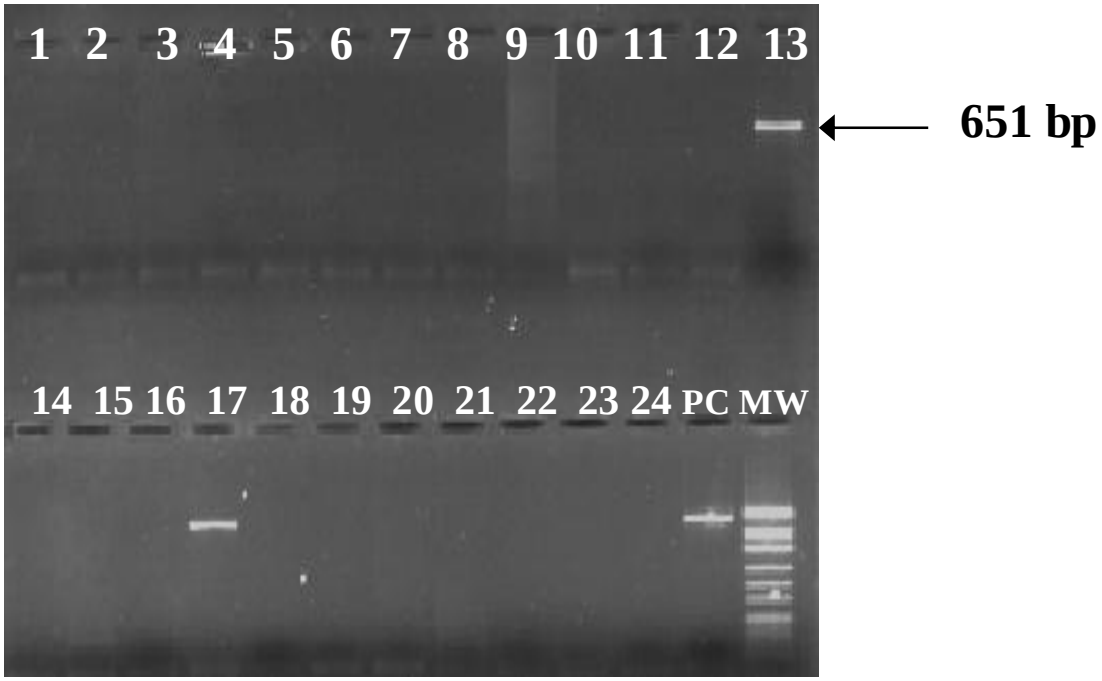


Figure 4i: Agarose gel electrophoretogram showing results of specificity of *fem B* PCR. **Lane 1:** Negative control**, Lane 2:** Extraction control**, Lane 3:** *M. tuberclosis***, Lane 4:** *M. xenopi***, Lane 5:** *M. fortuitum***, Lane 6:** *M. chelonae***, *Lane* 7:** *C. albicans***, Lane 8:** *Aspergillus spp*.**, Lane 9:** Cytomegalovirus**, Lane 10:** *Acanthamoeba spp.* **Lane 11:** *T. gondii* **, Lane 12:** *C. xerosis***, Lane 13:** MSSA ATCC 6538, **Lane 14:** *N. asteroides***, Lane15:** *Actinomyces spp***, Lane 16:** *H. influenzae***, Lane 17:** *S. aureus***, Lane 18:***S. epidermidis***, Lane 19:** *S. pneumoniae***, Lane 20:** *B. cereus***, Lane 21:** *A. calcoaceticus***, Lane 22:** *E. faecalis***, Lane 23:** *P. aeruginosa***, Lane 24:** *P. acne*s**, Lane 25:** Positive control MSSA ATCC 6538, **Lane 26:***Hinf I digest*

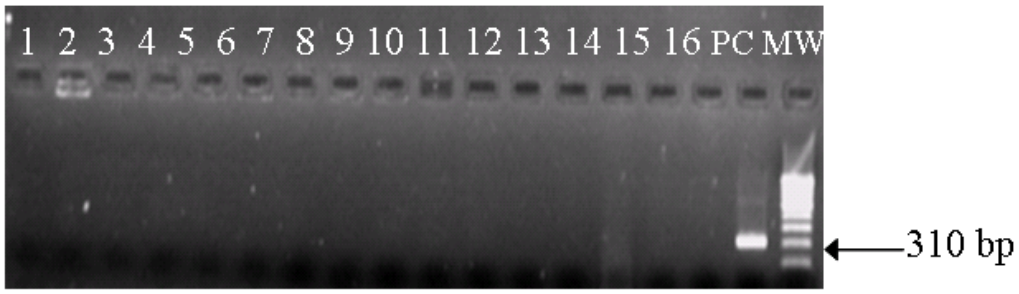


Figure 4j: Agarose gel electrophoretogram showing results of specificity of *mecA* gene PCR. Lane 1: Negative control**, Lane 2:** Extraction control**, Lane 3:** *M. tuberclosis***,** **Lane 4:** *B. cereus***, Lane 5:** *A.calcoaceticus***, Lane 6:** *E. faecalis***, *Lane* 7:** *Candida albicans***, Lane 8:** *Aspergillus spp.***, Lane 9:** Cytomegalovirus**, Lane 10:** *Acanthamoeba spp.* **Lane 11:** *T. gondii* **, Lane 12:** *C. xerosis***, Lane 13:** *S. epidermidis,* **Lane 14:** *N.asteroides***, Lane15:** *Actinomyces spp***, Lane 16:** *H. influenzae***, PC:** MRSA ATCC 33591**, MW:** 100 bp ladder.

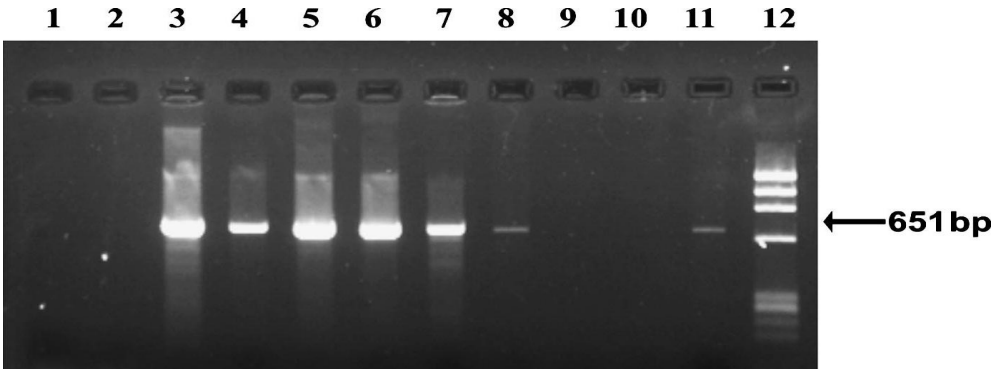


Figure 4k: Agarose gel electrophoretogram showing results of uniplex PCR for *femB* and *mecA* PCR on clinical isolates. Lane 1; Negative control, **Lane 2:** Extraction control, **Lane 3-8:** Showing amplified *femB* gene in isolates, **Lane 9 and 10:** Shows absence of *femB* gene in isolate, **Lane 11:** PC *S.aureus* MSSA ATCC 6538, **Lane 12:** MW - Molecular weight 100 bp ladder.

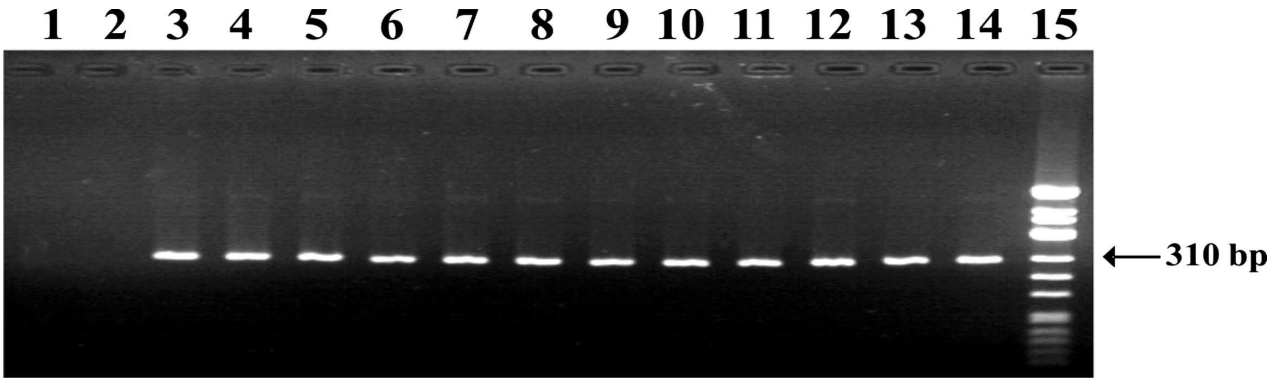


Figure 4l: Agarose gel electrophoretogram showing results of uniplex PCR of *mecA* gene on clinical isolates. Lane 1: Negative control, **Lane 2:** Extraction control, **Lane 3-13:** Showing amplified *mecA* gene in isolates, **Lane 14:** PC *S. aureus* MRSA ATCC 33591, **Lane 15:** MW - Molecular weight 100 bp ladder.

4.5.5. Results of duplex PCR for *femB* and *mecA* gene PCR:

Of the 110 Staphylococcal, isolates 18 (16.4%) was positive both for *mecA* and *femB* gene (MRSA), 52 (47.2%) was *mecA* gene PCR positive and *femB* gene PCR negative (MRCoNS), 5 (4.5%) others was positive for *femB* and negative for *mecA* (MSSA) and remaining 35 (31.8%) was *femB* and *mecA* PC-R negative (MSCoNS) is shown in figure 4m.

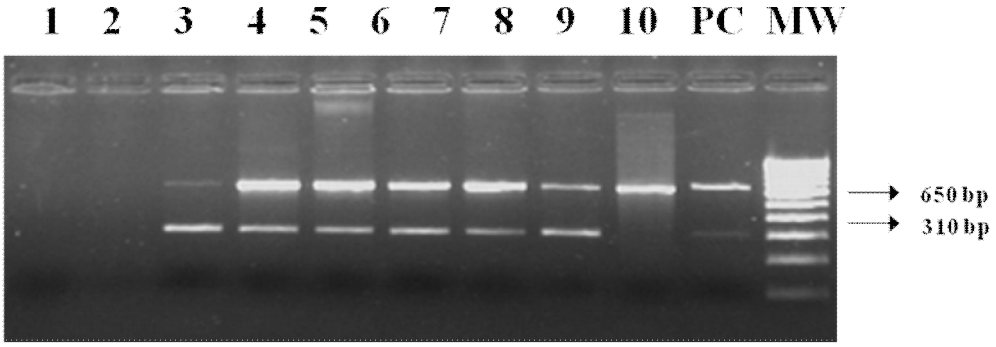


Figure 4m: Agarose gel electrophoretogram showing results of duplex PCR of *femB* and *mecA* on clinical isolates. Lane 1: Negative control, **Lane 2:** Extraction control, **Lane 3-9:** Showing amplification of *mecA* gene and *femB* in clinical isolates, **Lane 10:** *femB* along positive clinical isolate (MSSA), **PC:** Positive control *S.aureus* MRSA ATCC 33591, **MW:** 100bp ladder

4.5.6. Comparison of results of conventional method with molecular method:

Comparing the results of conventional method with molecular method among 110 staphylococci, 18 was tube coagulase, *femB* and *mecA* positive (MRSA), 52 was tube coagulase and *femB* negative and *mecA* positive (MRCoNS). All MRS isolates positive for *mecA* was MR by disc diffusion, and microbroth dilution methods. Five was tube coagulase and *femB* positive and *mecA* negative (MSSA) and 35 was tube coagulase, *femB* and *mecA* negative (MSCoNS). The molecular method has aided in detection of 15 more MRS strains compared to conventional technique.

4.5.5. Results of RFLP – *gap* gene:

Seventy MRS was identified up to species level using PCR-RFLP of *gap* gene, 18 (25.71%) as *S. aureus*, 11 (15.71%) as *S. epidermidis*, 27 (38.57%) *S. haemolyticus*, 6

(8.57%) *S. cohnii* subspp. *urealyticum*, 6 (8.57%) *S. equorum*; 1 (1.42%) *S. xylosus*; and 1 (1.42%) *S. hominis* (Figure 4n). Results of RFLP were confirmed by DNA sequencing and by blast search. There was 100 per cent correlation between RFLP pattern and sequencing results when blast search was carried out (Table 4e).

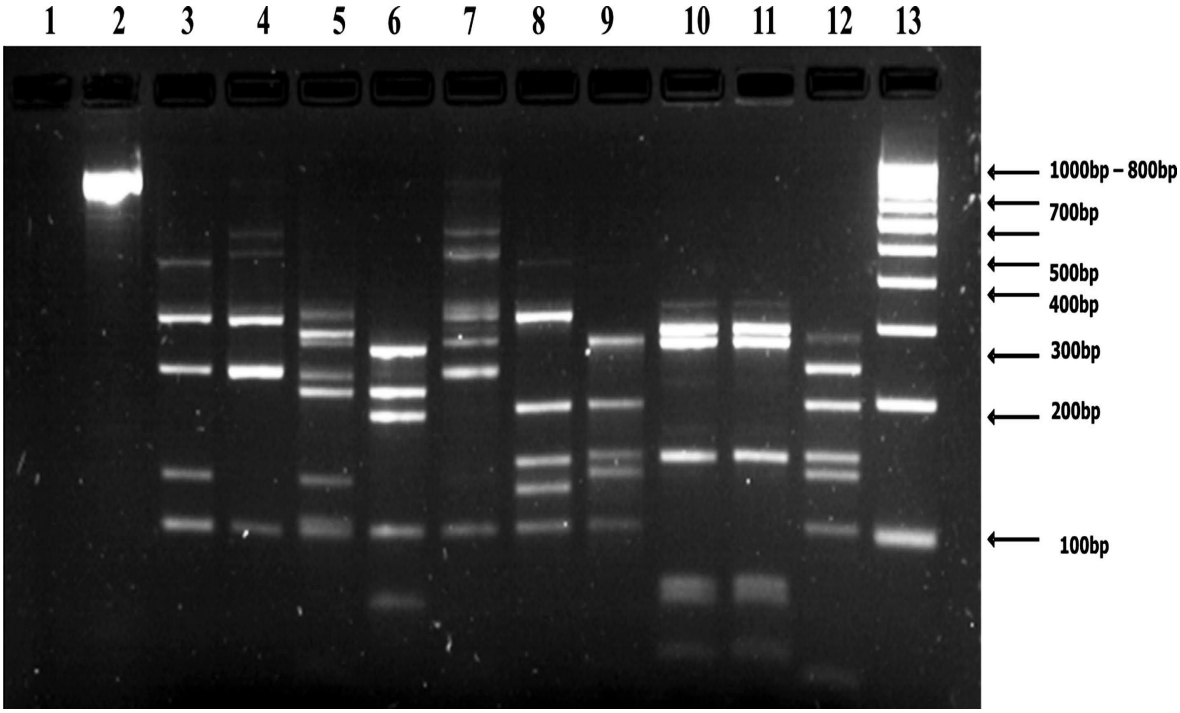


Figure 4n: Agarose gel electrophoresis showing PCR-RFLP *of gap* gene amplified products from *Staphylococcus* spp. Lane 1: Negative control, **Lane 2:** Undigested product (933bp), **Lane 3:** *S. saprophyticus* lab isolate, **Lane 4***:* *S. haemolyticus*, **Lane 5:** *S. xylosus*, **Lane 6:** *S. hominis*, **Lane 7:** *S. cohnii* subspp.*urealyticum*, **Lane 8:** *S. equorum*, **Lane 9:** *S. epidermidis*, **Lane10:** *S.aureus*, **Lane11:** *S. aureus* (ATCC 6538), Lane12**:** *S. epidermidis* (ATCC 12228), **Lane 13:** MW - 100bp ladder

Table 4e: Comparison of results of RFLP and DNA sequencing on identification of *Staphylococcus spp.*

S.No	Spp. identified by RFLP	Total no. strains of this pattern	DNA sequencing results	
			% of homology and spp. identified by Forward primer	% of homology and spp. identified by Reverse primer
1	*S. aureus*	18 (25.71%)	97% *S. aureus*	98% *S. aureus*
2	*S. epidermidis*	11 (15.71%)	96% *S. epidermidis*	100% *S. epidermidis*
3	*S. haemolyticus*	27 (38.57%)	98% *S. haemolyticus*	99% *S. haemolyticus*
4	*S. cohnii* sub species *urealyticum*	6 (8.57%)	96% *S. cohnii* sub species *urealyticum*	96% *S. cohnii* sub species *urealyticum*
5	*S. equorum*	6 (8.57%)	99% *S. equorum*	99 % *S. equorum*
6	*S. xylosus*	1 (1.42%)	97% *S. xylosus*	97% *S. xylosus*
7	*S. hominis*	1 (1.42%)	97% *S. hominis*	98% *S. hominis*
STANDARD STRAINS				
8	*S. aureus* (ATCC 6538)		98% *S. aureus*	98% *S. aureus*
9	*S. epidermidis* (ATCC 12228)		100% *S. epidermidis*	100% *S. epidermidis*
10	*S. saprophyticus*		100% *S. saprophyticus*	100% *S. saprophyticus*

4.6. Discussion

MRSA & MR-CoNS are predominant bacterial pathogens isolated from ocular specimens (Sharma S et al., 2000, Pinna et al., 1999, Sharma S et al., 1999). Conjunctivitis is most commonly reported manifestation (Rajaduraipandi et al., 2006). These conjunctival swabs were taken from outpatients, showing that MRS was not hospital acquired but was of community origin.

In our study, *S. haemolyticus* was the predominant MRCoNS isolated followed by *S. epidermidis.* In a similar study conducted by Pinna et al., 1999, *S. epidermidis* was identified as predominant CoNS, by application of API ID32 system. In many studies, *S.epidermidis* was predominantly isolated from various clinical specimen followed by *S.haemolyticus*. Chaudhury et al., 2007 also reported *S. haemolyticus* as predominant strain. Result of PCR-based RFLP on *gap* gene was 100% in concordance with DNA sequencing results. Maximum intra-sequence variation was observed among *gap* gene of *Staphylococci* spp., hence this region could be used for staphylococci spp. identification as it is cost-effective compared to application of API ID32 system used by others for similar type of study (Pinna et al., 1999).

Present study showed that 24 h incubation period was sufficient for detecting MRS, but for ruling out heterogeneous resistance, additional 24 h of incubation was needed. All MRS isolates in our study was positive for *mecA* gene and all MSS isolates was negative for *mecA* gene. In this study we demonstrated that breakpoint of <0.1µg of oxacillin/ml (instead of <0.5µg of oxacillin/ml) for CoNS (CLSI, M2-A9. 2000 and CLSI, M7-A7.

2000) permitted detection of 10 more CoNS isolates with *mecA*-associated resistance. Kohner et al., 1999 also found more of CoNS resistant strains with breakpoints of <0.1µg/ml of oxacillin.

4.7. Conclusion:

In conclusions, the isolation rate of the MRS was 63.6 per cent among our isolates and MR-CoNS constituted a larger portion of MRS. *S. haemolyticus*, followed by the *S. epidermidis* was predominant CoNS isolated. PCR assay was superior in identifying intermediate and heterogeneous MR in shorter duration of time. PCR-RFLP of *gap* gene was found to be specific for Staphylococcal spp.

4.8. Future prospects:

This PCR technique for simultaneous detection of *femB* and *mecA* gene of will aid in rapid detection of resistance from clinical isolates of Staphylococci. This approach will thus help in both speciation and identification of occurrence of methicillin resistance rapidly.

Chapter - 5

Screening of Enterobacteriaceae strains isolated from ocular clinical specimens for the presence of chromosomal *blaNDM-1* and Extended Spectrum Beta-Lactamases (ESBLs) genes: A two years study at a tertiary eye care centre.

5.1. Aim:

To study the prevalence of *CTX-M*, *OXA*, *TEM*, *SHV* and *blaNDM-1*genes that confer the Extended Spectrum of Beta-Lactamases (ESBLs) production in bacteria belonging to family *Enterobacteriaceae* isolated from ocular clinical specimens from a tertiary care centre Chennai, Tamil Nadu, India along with screening of existence of *blaNDM-1*genes.

5.2. Review of literature:

5.2.1. Beta Lactamases:

One of the main mechanisms by which certain bacteria exhibit resistance to antimicrobial agents is through the production of the enzyme beta-lactamase. Beta-lactamases render resistance to beta-lactam antibiotics, which are characterized by the presence of beta-lactam ring. Beta-lactamases constitute a heterogeneous group of enzymes. The beta-lactam ring is part of the structure of several antibiotic families, principally the penicillins, cephalosporins, carbapenems and monobactams, which are therefore also called beta-lactam antibiotics. These antibiotics work by inhibiting the bacterial cell wall synthesis (http://en.wikipedia.org/wiki/ Beta-lactamase). Beta-lactamase enzymes can destroy beta-lactam ring of the beta-lactam group of antibiotics through

hydrolysis, and without a beta-lactam ring, these antibiotics are ineffective against the bacteria (www.niaid.nih.gov/factsheets/antimicro.html).

5.2.2. Extended-Spectrum Beta Lactamases (ESBLs):

Extended spectrums of beta lactamase are enzymes that mediate resistance to third generation cephalosporins e.g, ceftazidime, cefotaxime, and ceftriaxone and monobactams e.g., aztreonam but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g, meropenem or imipenem). *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca* are the most common ESBL-producing pathogens. These ESBL producing organisms are highly resistant to an array of antibiotics and infections by these strains are difficult to treat (Medeiros, 1984). According to the amino acid sequences extended-spectrum beta- lactamases are typed as TEM (found in a single strain of *Escherichia coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM), SHV (sulphydryl variable), CTX-M (cefotaxime), OXA (oxacillin) (Bradford et al., 2001, Spratt et al., 1988).

Over the last 20 years, many new Beta-lactam antibiotics have been developed that were specifically designed to be resistant to the hydrolytic action of Beta -lactamases. However, with each new class that has been used to treat patients, new Beta –lactamases emerged that caused resistance to that class of drug. Therefore, antibiotic therapy for treating these infections is limited to a small number of expensive drugs which has resulted in more than 200 types of ESBLs in various spp. of the *Enterobacteriaceae* family (Humeniuk et al., 2002).

5.2.2.1. *TEM beta-lactamases* (class A):

TEM-1 is the most commonly encountered beta-lactamase in gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. TEM-1 is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine. TEM-2, the first derivative of TEM-1, had a single amino acid substitution from the original beta-lactamase. TEM-3, originally reported in 1989, was the first TEM-type beta-lactamase that displayed the ESBL phenotype (Patricia, 2001).

5.2.2.2. *SHV beta-lactamases* (class A):

SHV-1 shares 68 percent of its amino acids with TEM-1 and has a similar overall structure. The SHV-1 beta-lactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this spp.. More than 60 SHV varieties are known. They are the predominant ESBL type in Europe and the United States and are found worldwide. SHV-5 and SHV-12 are among the most common (Patricia, 2001).

5.2.2.3. *CTX-M beta-lactamases* (class A):

CTX-M enzymes, mainly produced by *E. coli*, have been predominantly reported in the India and thought the world (Perilli et al., 2002). CTX-M preferentially hydrolyzes cefotaxime (Johann et al., 2004) and they show only 40% identity to TEM or SHV ESBLs, but they are closely related to Beta lactamases of the Khuyvera spp. (Ramazanzadeh et al., 2010) Notably, clinical CTX-M-15- producing *Enterobacteriaceae* has become more and more widespread where as *AmpC* Beta-lactamases are usually chromosomally encoded usually located multidrug resistance plasmids. (Eckert et al., 2006)

5.2.2.4. *OXA beta-lactamases* (class D):

OXA beta-lactamases were long recognized as a less common but also plasmid-mediated beta-lactamase variety that could hydrolyze oxacillin and related anti-staphylococcal penicillins. These beta-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Patricia, 2001).

5.2.2.5. New Delhi metallo-beta-lactamase (*NDM-1*):

Beta-lactams have been widely used as the mainstay of treatment for severe infections due to these bacteria, with carbapenems often representing last-line of defense. (Rolain et al., 2010) Recently, the New Delhi metallo-beta-lactamase (*NDM-1*) a novel type of metallo-beta-lactamase (MBL) conferring resistance to almost all beta-lactam antibiotics, including carbapenems that are used in treatment of antibiotic-resistant bacterial infections have emerged and has therefore alarmed the world. (Diene et al., 2011, Kumarasamy et al., 2010) This worldwide spread of *Enterobacteriaceae* carrying *blaNDM-1*, has thus become a significant threat to human health due to its extensive drug resistance, leaving few or no therapeutic options thus becoming a major public health problem throughout the world, particularly in India. (Zarfel et al., 2011) Many reports on the existence and dissemination of *blaNDM-1* arise in a rapid rate throughout the world and also from India. (Seema et al., 2011, Kumarasamy et al., 2010, Rolain et al., 2012, Deshpande et al., 2012)

5.2.2. 1. CLASSIFICATION OF BETA- LACTAMASES:

Several classification schemes have been proposed according to their hydrolytic spectrum, susceptibility to inhibitors, genetic localization (plasmid or chromosomal), and gene or a protein sequence is shown in table 5a. Bush et al., 1995, Ambler, 1980, http://en.wikipedia.org/wiki/Antibiotic_resistance)

Table 5a: Classification of Beta-lactamases

Bush Group	Sub group	Ambler Molecular Class	Characteristics
1		C	Mainly chromosomal located in Gram-negative bacteria but may be plasmid mediated. Confer resistance to beta-lactams (except carbapenems). Not inhibited by clavulanate.
2		A.D	Most enzymes inhibited by clavulanate (unless otherwise stated).
	2a	A	Penicillinases (narrow hydrolysis spectrum) conferring resistance to penicillins.
	2b	A	Broad-spectrum penicillinases TEM-I & SHV-1 primarily from Gram-negative bacteria.
	2be	A	Extended-spectrum beta-lactamases conferring resistance to oxyimino-cephalosporins and monobactams.
	2br	A	Inhibitor resistant beta-lactamases (mostly TEM-types and to a lesser extent SHV derived enzymes).
	2c	A	Carbenicillinases.
	2d	D	Oxacillinases. modestly inhibited by clavulanate.
	2e	A	Cephalosporinases inhibited by clavulanate.
	2f	A	Serine active site carbapenemases. inhibited by clavulanate.
3	3a. b. c	B	Metallo-beta-lactamases conferring resistance to beta-lactams (except monobactams). not inhibited by clavulanic acid.
4			Miscellaneous unsequenced beta-lactamases that do not conform to other groups.

Functional Classification:

The functional classification scheme of beta-lactamases proposed by Bush Jacoby and Medeiros (1995) defines four groups according to their substrate and inhibitor profiles.

Molecular Classification:

Molecular classification introduced by Ambler (1980) (shown in table 5a) is based on the amino-acid sequence that recognizes four molecular classes designated A to D. Classes A, C, and D posses evolutionarily distinct groups of serine enzymes, whereas the class B are the zinc-dependent ("EDTA-inhibited") enzymes.

5.2.3. Need for the study:

Nosocomial and hospital acquired infections caused by ESBL-producing strains are increasing in higher frequency in India due to increasing population. ESBL-producing strains have been reported by several countries in the world whereas few reports on correlation of antibiotic pattern and their corresponding *CTX-M*, *TEM*, *OXA* and *SHV* genotypes exist in south India. So far *blaNDM-1* has not been screened among the ocular pathogens. *Enterobacteriaceae* group of microorganisms are isolated at significantly higher rates am-ong ocular pathogens. The major antibacterial drugs used in the treatment of ophthalmic bacterial infections include beta-lactams antibiotics such as cefazolin and ceftazidime. Therefore, in this study we aimed to explore ophthalmic clinical isolates belonging to the family of *Enterobacteriaceae* in quest of the existence of ESBLs and *blaNDM-1* at a tertiary eye care centre in South India.

5.3. Objective:

To study the prevalence of *CTX-M, OXA, TEM* and *SHV* that confer the Extended Spectrum of Beta-Lactamases (ESBLs) production in *Enterobacteriaceae* isolated from ocular clinical specimens from a tertiary care centre Chennai, Tamil Nadu, India along with screening of existence of *blaNDM-1*genes.

1. To detect ESBL production by conventional method using double disc diffusion test.
2. To analyze the prevalence of Extended Spectrum of Beta-Lactamases (ESBLs) genes such as *CTX-M, OXA, TEM* and *SHV g*enes among the *Enterobacteriaceae* isolated from ocular clinical specimens.
3. To screen for the presence of virulent gene; *blaNDM-1* among ocular *Enterobacteriaceae* isolates.

5.4. Materials and Methods:

Prospective analysis was carried out on 74 bacteria belonging to *Enterobacteriaceae* isolated from patients with clinical suspicion of bacterial ocular diseases such as conjunctivitis, canaliculitis, keratitis, dacryocystitis, endophthalmitis and panophthalmitis along with screening of Donor corneal rim (DCR) & swab from Multi organ donor (MOD) during the period of 2 years from Jan 2010 – Dec 2011 from a total of 7,598 specimens including both extraocular and intraocular received for microbial culture at L & T Microbiology Research Centre, Kamalnayan Bajaj Research Centre, Sankara Nethralaya, a tertiary eye care centre at Chennai, India. Ocular clinical isolates were screened for ESBL production and presence of *blaNDM-1*gene. Table 5b shows detailed distribution of *Enterobacteriaceae* among ocular specimen.

Table 5b: Distribution of ocular *Enterobacteriaceae* isolates included in the study

Enterobacteriaceae isolates	No of isolates (%)	Intraocular Specimens* (n = 20)	Extraocular Specimens # (n = 34)	Other Specimens @ (n = 20)
K. pneumoniae	16 (21.3%)	8	5	3
K. oxytoca	14 (18.9%)	2	6	6
E. coli	10 (13.5%)	0	7	3
E. aerogenes	9 (12.1%)	2	4	3
C. freundii	8 (10.8%)	2	4	2
P. mirabilis	5 (6.7%)	2	2	1
C. koseri	4 (5.4%)	3	1	0
S. marscensens	3 (4.0%)	0	3	0
P. rettgeri	1 (1.3%)	1	0	0
M. morganii	1 (1.3%)	0	1	0
E. cloacae	1 (1.3%)	0	1	0

NOTE:

*** Intraocular specimens include - Aqueous humor (n=1), Vitreous humor (n=4), Eviscerated material (n=6), Intraocular lens (n=2), Silicon rod (n=3).**

 # Extraocular specimens include - Orbital biopsy (n=2), Canalicular pus (n=2), Contact lens (n=7), Corneal scraping (n=7), Corneal button (n=1), Conjunctival swab (n=14), Scleral tissue (n=2), Lid abscesses (n=3).

@ Other specimens include - Donor corneal rim (n=14), Swab from multiorgan donor (n=6).

Ocular specimens were processed for isolation of pathogenic bacteria by standard conventional bacteriological methods and the bacteria isolated were identified to spp. level using standard biochemical tests (Folkens et al., 1996). A positive culture was defined as growth of the microorganism in two or more media or confluent growth at the site of inoculation were included for the study. Study protocol was approved by the institutional ethics sub-committee (IRB).

5.4.1. Screening for Production of ESBLs by phenotypic methods:

5.4.1.1. Double disc synergy test (DDST):

a. Requirements for DDST:

Muller Hinton agar, Muller Hinton broth, bacterial isolates to be tested (in 0.5 McFarland's standard concentration), quality control strains *Escherichia coli* ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603 (positive control) and *Escherichia coli* ATCC 25922 (negative control) and antibiotic discs ceftazidime (30µg) versus ceftazidime/clavulanic acid (30/10µg) and cefotaxime (30µg) versus cefotaxime/clavulanic acid (30/10µg)

b. Procedure for performing the DDST:

Three to five well-isolated colonies of *Enterobacteriaceae* were selected from an agar plate culture. The colonies were picked with edge of sterile loop and inoculated into a tube containing 4 to 5 ml of Muller Hinton broth. The broth culture was incubated at 37°C until it achieves or exceeds the turbidity of the 0.5 McFarland standards (usually 2 to 6 h). Once the turbidity was adjusted, optimally, within 15 minutes after adjusting turbidity, a sterile cotton swab was dipped into adjusted suspension, rotated several times

and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of a Muller-Hinton agar plate was swab streaked over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to obtain a uniform lawn culture throughout the surface of the agar. Lid was left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow any excess surface moisture to be absorbed before applying drug impregnated disks.

Disks containing the ceftazidime was placed 15 mm apart (edge to edge) from ceftazidime - clavulanic acid disc containing 10 µg of latter compound. Similarly cefotaxime (30µg) and cefotaxime/clavulanic acid (30/10µg) antibiotics were placed parallel to the previous discs. Inoculated plates were incubated at 37^{o}C for 18 – 24 hours after which the plates were read.

c. Reading Plates and Interpreting Results:

Any enhancement of the zone of inhibition between a beta-lactam disk and that containing beta-lactamase inhibitor is indicative of the presence of an ESBL (CLSI, 2006).

5.4.1.2. Determining of Minimum Inhibitory concentration (MIC):

Methodology: MIC determination by agar/ broth micro dilution as per CLSI guidelines: (M7-A7/Jan 2006; M100-S18/Jan 2008)

Forty four out of 74 ocular clinical isolates of *Enterobacteriaceae* were randomly chosen to screen for ESBL and carbapenemases resistance by Minimum Inhibitory concentration **(**MIC) method. The MIC experiments were carried out at Orchid Chemicals and Pharmaceuticals Limited, at Shollinganallur, Chennai, India.

a. Agar dilution:

Mueller-Hinton agar plates containing various concentrations of antibiotics, and combinations with beta lactamase inhibitors (BLIs) were prepared. Bacterial inoculum was seeded onto these plates by a multipoint inoculator with each inoculum spot containing ~$1x10^4$ colony forming units (CFU). Plates were incubated at 37°C in an ambient atmosphere for 18-20 h. After incubation, the Minimum Inhibitory Concentration (MIC) was recorded as the lowest concentration, which showed no growth of inoculated culture.

b. Broth Microdilution:

Microtitre 96 well plates with cation adjusted Mueller Hinton Broth (CaMHB) containing various concentrations of antibiotics and combinations with beta lactamase inhibitors (BLIs) were prepared. CaMHB containing bacterial inocula were added into the wells. The final inoculum was ~$1x10^5$ CFU/well. The inoculated plates were incubated at 37°C in an ambient atmosphere for 18-20 h. The plates, after incubation, were observed with the aid of optical mirror plate reader and Minimum inhibitory concentration (MIC) was recorded as the lowest concentration, which showed no growth of the inoculated culture

c. Antibacterials tested:

Penicillins: Amoxicillin & Piperacillin

3rd Generation Cephalosporins: Ceftriaxone, Cefotaxime & Ceftazidime

4th Generation Cephalosporin: Cefepime

Carbapenems: Imipenem, Meropenem, Ertapenem & Doripenem

BLIs: Tazobactam & Clavulanic acid

5.4.2. Uniplex Polymerase chain reaction (PCR) for detection of ESBL genes:

All the 57 clinical isolates of *Enterobacteriaceae* were subjected for uniplex polymerase chain reaction (PCR) amplification targeting CTX-*M* genes, (Johann et al. 2004) *TEM,* (Eckert et al., 2006) *OXA*, (Féria et al., 2002) *SHV*, (M'zali et al., 1996) and chromosomal mediated *blaNDM-1*gene was carried using the primers and protocol as described by us previously (Sowmiya et al., 2012). Non-ESBL *E. coli* ATCC 25922, and ESBL producing *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 35218 strains were used as controls. The primers used in this study are shown in table 5c.

Specificity of the PCR for all primer was tested with DNA extracted from *Staphylococcus aureus* (ATCC-6538), *Staphylococcus epidermidis* (ATCC-10211), (ATCC-12384), *Streptococcus pneumoniae* (ATCC-6301), *Bacillus cereus* (eviscerated material)., *Mycobacterium tuberculosis H37 RV* , *Nocardia asteroides* (isolated from canalicular pus and DNA sequencing proved), *M. xenopi,* (ATCC-1432), *Haemophilus influenzae* (ATCC-10211), *Pseudomonas aeruginosa* (ATCC 9742), *Propionobacterium acne* (ATCC-11828), *Acinetobacter calcoaceticus* (ATCC-9956), *Serretia marscensens* (isolated from pus and DNA sequencing proved), *Enterobacter aerogenes* (ATCC-13048), *Escherichia coli* (ATCC-4157), *Citrobacter freundii* (isolated from pus DNA sequencing proved), *Proteus mirabilis* (isolated from Donor Corneal Rim and DNA sequencing proved), *Klebsiella pneumoniae* (isolated from Corneal scraping and DNA sequencing proved), *Salmonella typhi, Salmonella paratyphi A and* (all three spp. of *Salmonella* are isolated from Blood culture and DNA sequencing proved), *Herpes Simplex Virus 1* (VR 733), *Candida albicans* (ATCC 90028), Human DNA.

Table 5c: ESBL gene targets, their corresponding primers and thermal profiles.

PRIMERS[33]	PRIMER SEQUENCE (5'---3')	PRODUCT SIZE (bp)	PCR THERMAL PROFILE	Reference
CTX-M group I – F	5' GACGATGTCACTGGCTGAGC 3'	499	Initial denaturation 15 sec at 96°C; 24 cycles with 96°C for 15 sec, 55°C for 15 sec, and 72°C for 2 min; final extension 72°C for 10 min	Johann et al. 2004
CTX-M group I – R	5' AGCCGCCGACGCTAATACA 3'			
CTX-M group II –TOHO – F	5' GCGACCTGGTTAACTACAATCC 3'	351	Initial denaturation 15 sec at 96°C; 24 cycles with 96°C for 15 sec, 55°C for 15 sec, and 72°C for 2 min; final extension 72°C for 10 min	Johann et al., 2004
CTX-M group II –TOHO – R	5' CGGTAGTATTGCCCTTAAGCC 3'			
CTX-M group III – F	5' CGCTTTGCCATGTGCAGCACC 3'	307	Initial denaturation 15 sec at 96°C; 24 cycles with 96°C for 15 sec, 55°C for 15 sec, and 72°C for 2 min; final extension 72°C for 10 min	Johann et al., 2004
CTX-M group III – R	5' GCTCAGTACGATCGAGCC 3'			
CTX-M group IV – F	5' GCTGGAGAAAAGCAGCGGAG 3'	474	Initial denaturation 15 sec at 96°C; 24 cycles with 96°C for 15 sec, 62°C for 15 sec, and 72°C for 2 min; final extension 72°C for 10 min	Johann et al., 2004
CTX-M group IV – R	5' GTAAGCTGACGCAACGTCTG 3'			
TEM FORWARD	5' ATGAGTATTCAACATTTCCG 3'	850	Initial denaturation 5 min at 94°C; 2 cycles with 94°C for 7min, 60°C for 5min and 72°C for 1 min; followed by 30 cycles with 94°C for 1min, 60°C for 2min and 72°C for 1 min final extension 72°C for 10 min	Eckert et al., 2006
TEM REVERSE	5' CCAATGCTTAATCAGTGAGG 3'			
OXA FORWARD	5'TATCTACAGCAGCGCCAGTC-3'	199		Féria et al., 2002
OXA REVERSE	5' CGCATAAATGCCATAAGTG-3'			
SHV FORWARD	5'-TCAGCGAAAAACACCTTG-3'	475		M'zali et al., 1996
SHV REVERSE	5-TCCCGCAGATAAATCACCA-3'			
NDM – 1 – F	5' TCTCGACATGCCGGGTTTCGG 3'	475	Initial denaturation 10 min at 94°C, followed by 30 cycles with 94°C for 1min, 60°C for 2min and 72°C for 1 min final extension 72°C for 10 min	Sowmiya et al., 2012
NDM – 1 – R	5' ACCGAGATTGCCGAGCGACT 3'			

Analytical sensitivity of uniplex PCR was determined using serial ten-fold dilutions of DNA. All PCR products were loaded in 2 per cent agarose gel with ethidium bromide, (50ng/ml from Hi-Media Laboratories Private Limited, Mumbai) and results were documented in gel documentation system (Vilber Lourmat - France).

5.4.3. DNA sequencing reaction:

Cycle sequencing reaction, consisted of 4µl of big dye terminator, 2µl of DNA, 2 picomoles/µl of either forward or reverse primer, 3µl of deionized water. PCR profile consisted of denaturation at 96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min and final hold of 4°C. Products were purified according to standard protocol, loaded onto ABI 3100 Genetic Analyzer (Applied Biosystem, USA) with polymer POP7 and sequenced (Appendix 3). Sequences were then analyzed using BIO EDIT, (*http://www.mbio.ncsu.edu/BioEdit/bioedit.html*), and finally blasted with NCBI Blast website *http://blast.ncbi.nlm.nih.gov/Blast.cgi* to identify spp. and DNA homology.

5.5. Results:

In the present study, a total of 74 *Enterobacteriaceae* isolated from various ocular specimens were included. The highest rate of *Enterobacteriaceae* was isolated was from the male population (n = 44, 59.5%) compared to female population (n = 30, 40.5%). The mean age was 40.1 years for male group (range, 5 months to 78 years) and 52.5 years for female group (range, 12 to 75 years). Of the 74 clinical isolates, maximum number was isolated from DCR (16.2%) and conjunctival swabs (16.2%) specimens. Details on number of *Enterobacteriaceae* isolates recovered from various ocular diseased conditions are shown in table 5d.

Table 5d: Details on number of *Enterobacteriaceae* isolates recovered from various ocular diseased conditions

Organism (N=74)	Conjuntivitis	Canalicularitis	Keratitis	Trauma	Post-PK	Post Suture	Vitrec-tomy	DCR	MOD	Endophthalmitis			Total no of isolates recovered
										Endogenous	Post – operative	Pan-ophthalmitis	
Citrobacter freundii	-	-	3	-	1	-	1	-	1	-	1	1	8
Citrobacter koseri	-	-	1	-	-	-	1	1	1	-	-	-	4
Enterobacter aerogenes	1	-	1	1	1	-	-	-	1	-	2	2	9
Enterobacter clocae	-	-	-	1	-	-	-	-	-	-	-	-	1
Escherichia coli	5	-	-	2	-	-	-	1	1	-	1	-	10
Klebsiella pneumoniae	3	-	1	2	-	-	2	4	2	-	3	1	18
Klebsiella oxytoca	3	1	-	1	1	2	-	3	-	2	1	-	14
Morganella morganii	-	-	1	-	-	-	-	-	-	-	-	-	1
Proteus mirabilis	1	1	-	1	1	-	-	1	-	-	-	-	5
Providencia rettgeri	-	-	1	-	-	-	-	-	-	-	-	-	1
Serratia marscensens	1	-	2	-	-	-	-	-	-	-	-	-	3
Total specimens (N=74)	14 (18.9%)	2 (2.7%)	11 (14.9%)	8 (10.8%)	3 (4.0%)	2 (2.7%)	4 (5.4%)	10 (13.5%)	6 (8.1%)	2 (2.7%)	8 (10.8%)	4 (5.4%)	74

Of the 74 *Enterobacteriaceae* isolates, from 34 (52.7%) specimens *Enterobacteriaceae* were obtained as pure culture and rest 23 (31.1%) were isolated along with other group of bacterial strains other than *Enterobacteriaceae*, 10 (13.5%) strains were isolated along with fungi and 2 (2.7%) strains were isolated along with other group of bacteria and fungus. Data of polymicrobial distribution of isolates along with other bacteria and fungi is shown in table 5e.

Table 5e: Data of polymicrobial distribution of *Enterobacteriaceae* isolates along with other bacteria and fungi.

***Enterobacteriaceae* (N=74)**	**No of Isolates & % isolation**	**Pure culture**	**Polymicrobial – other bacteria isolated**	**Polymicrobial - with fungi**	**Polymicrobial-with bacteria & fungi**
Citrobacter freundii	**8**	**3**	**2 (Bacillus spp. + *P. aeruginosa*)** **1 (MRSA)**	**1 (*A. flavus*)**	**-**
Citrobacter koseri	**4**	**2**	**-**	**1 (*A. flavus*)**	**1 (MRSE + MRSA + Fusarium)**
Enterobacter aerogenes	**9**	**4**	**2 (*P. aeruginosa*)** **3 (MRSE)**	**-**	**-**
Enterobacter clocae	**1**	**-**	**1 (MRSE)**	**-**	**-**
Escherichia coli	**10**	**8**	**2 (MRSE)**		
Klebsiella pneumoniae	**18**	**10**	**2 (MRSE)** **1 (MRSA)** **1 (Bacillus + MRSE +*P.acnes*)** **1 (*P. aeruginosa*)**	**3 (Fusarium)**	**-**
Klebsiella oxytoca	**14**	**5**	**1 (*P. aeruginosa*)** **3 (MRSE)** **2 (MRSA)**	**2 (Fusarium)** **1 (*A. flavus*)**	**-**
Morganella morganii	**1**	**1**	**-**		**-**
Proteus mirabilis	**5**	**3**	**-**	**2 (Fusarium)**	**-**
Providencia rettgeri	**1**	**1**			
Serratia marscensens	**3**	**2**	**1 (*P. aeruginosa*)**		**1 (*P. aeruginosa* + Fusarium)**

5.5.1. Phenotypic results of ESBL detection in *Enterobacteriaceae* isolates:

5.5.1.1. Screening for Production of ESBLs by Double disc synergy test (DDST):

Of the total 74 ocular clinical isolates of *Enterobacteriaceae* tested for the production of ESBLs by double disc diffusion test, 77% (57/74) exhibited ESBL positivity, of which 3.2% (3/57) were *C. freundii*, 14.0% (8/57) *C. koseri*, 12.2% (7/57) *E. aerogenes*, 17.5% (8/57) *E. coli*, 21.0% (12/57) *K. pneumoniae*, 17.5% (10/57) *K. oxytoca*, 10.5% (6/57) *P. miralibilis* and 5.2% (3/57) *S. marscensens* respectively. Results of screening test for ESBLs are shown in table 5f.

Among the 57 *Enterobacteriaceae* isolates that are positive for ESBL production, highest percent ESBL production was exhibited by *K. pneumoniae* (21.0%), followed by *K. oxytoca* (17.5%). Ocular clinical isolates of *Enterobacteriaceae* showed overall 77.0% positivity for ESBL production.

Rate of ESBL isolations from intraocular specimens was 30% (predominant specimen was eviscerated material) and in case of extraocular group of specimens ESBL isolation rate was 41.1% (were predominantly from conjunctival swabs specimens).

The rate of the isolation of ESBL producing *Enterobacteriaceae* isolates recovered from DCR, MOD swab, silicon rod implants, IOL was 31.5% (n=18/57), whereas the isolation rates from rest of the specimens were 68.5% (n=39/57). Result of DDST of a *K. pneumoniae* strain isolated from an eviscerated material along with positive and negative control strains is shown in figure 5a.

Table 5f: Distribution *Enterobacteriaceae* isolates according to clinical source and their results of screening test for ESBL's production.

Ocular specimens	ESBL Screening test results		
	No tested	ESBL POSITIVE (%)	ESBL NEGATIVE (%)
A. Intraocular Specimens	**20**	**16 (80.0)**	**4 (20.0)**
Aqueous humor	1	1 (100.0)	0
Vitreous humor	4	2 (50.0)	2 (50.0)
Eviscerated material	6	5 (83.3)	1 (16.7)
Intraocular lens (IOL)	2	1 (50.0)	1 (50.0)
Orbital biopsy	2	2 (100.0)	0
Silicon rod	3	3 (100.0)	0
Canalicular pus	2	2 (100.0)	0
B. Extraocular Specimens	**34**	**27 (79.5)**	**7 (20.5)**
Contact lens	7	5 (71.4)	2 (28.6)
Corneal scraping	7	6 (85.7)	1 (14.3)
Corneal button	1	1 (100.0)	0
Conjunctival swab	14	12 (85.7)	2 (14.3)
Scleral tissue	2	0	2 (100.0)
Lid abscesses	3	3 (100.0)	0
C. Other Specimens	**20**	**14 (70.0)**	**6 (30.0)**
Donor corneal rim (DCR)	14	12 (85.7)	2 (14.3)
Swab from multiorgan donor (MOD)	6	2 (33.3)	4 (66.7)
TOTAL	**74**	**57 (77.0)**	**17 (23.0)**

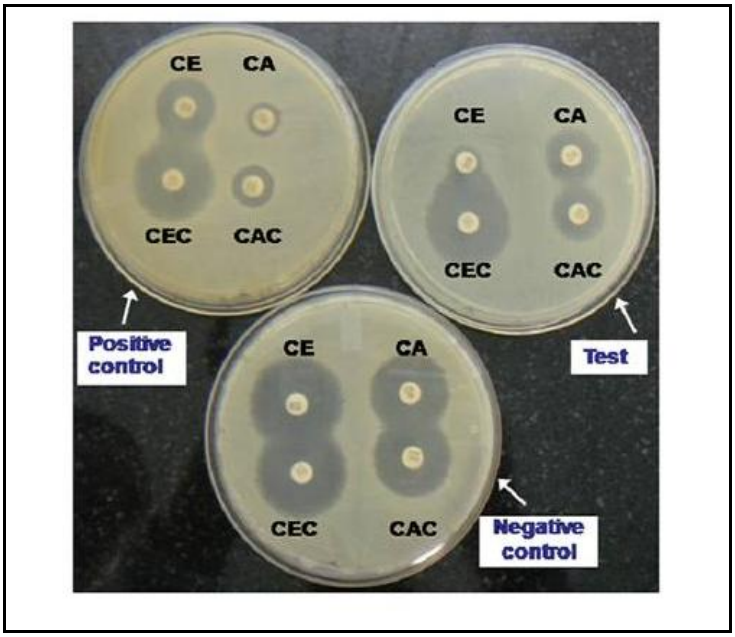


Figure 5a: Result of DDST of a *K. pneumoniae* strain isolated from an eviscerated material along with positive and negative control strains. CE: Cefotaxime (30µg), CA: Ceftazidime (30µg), CEC: Cefotaxime + Clavulanic Acid (30/10µg), CAC: Ceftazidime + Clavulanic Acid (30/10µg)

5.5.1.2. Results of Minimum Inhibitory concentration (MIC):

Among the 44 strains, 19 strains (43.18%) indicated ESBL-antibiotype and showed three fold reduction in the presence of either one of the beta lactamase inhibitor ie. Tazobactam & Clavulanic acid and 18 (40.9%) others were negative for ESBL. Seven strains (15.9%) of *Enterobacteriaceae* showed resistance to most carbapenems tested and one strain showed resistance to imepenum alone. A *Citrobacter spp.* (2.27%) predominantly showed inducible *AmpC* phenotype. Thirteen (68.4%) out of 19 ESBL positive isolates and 2 (28.5%) carbapenamase producing *Enterobacteriaceae* showed resistance to 4^{th} generation cephalosporin ie. cefepime. Suggested phenotype of these strains correlated with their antibiotype. The results of MIC carried on 44 ocular clinical isolates were shown in Table 5g I, II, III.

Table 5g I: The results of MIC carried on 44 ocular clinical isolates of *Enterobacteriaceae*.

S.No	Strain ID	Organism	Phenotype	Amoxicillin	Amoxicillin/ clav (2:1)	Piperacillin	Pip/taz (4 mcg/mL)	Ceftazidime	Ceftazidime /clav (4 mcg/mL)	Ceftriaxone	Ceftriaxone/ clav (4 mcg/mL)	Cefotaxime	Cefotaxime/ clav (4 mcg/mL)	Cefepime	Imipenem	Meropenem	Ertapenem	Doripenem
1	1773/09	*E. coli*	ESBL-ve	>16	>16/8	2	2	0.125	0.5	0.125	0.5	0.25	0.5	0.125	1	0.125	0.06	0.125
2	2916/09	*C. freundii*	ESBL-ve	>16	>16/8	2	2	0.125	0.5	0.125	0.125	0.06	0.125	0.06	0.5	0.06	0.015	0.06
3	4305/11	*K. oxytoca*	ESBL+ve	>16	>16/8	>128	16	32	2	>32	0.25	>32	0.25	16	0.5	0.06	0.125	0.06
4	4296/11	*K. pneumoniae*	ESBL+ve	>16	>16/8	>128	8	16	0.5	>32	0.25	>32	0.25	16	0.25	0.06	0.125	0.06
5	307/09	*C. koseri*	Imipenem - R	>16	>16/8	16	2	2	8	4	8	4	8	0.125	4	0.06	0.03	0.125
6	987/09	*C. freundii*	Carbapenem- R	>16	>16/8	8	8	1	4	>32	>32	32	16	2	8	1	8	0.5
7	103/09	*K. pneumoniae*	Carbapenem- R	>16	>16/8	1	2	0.25	2	8	>32	8	>32	1	16	2	8	2
8	3245/11	*E. coli*	ESBL+ve	>16	>16/8	>128	16	16	2	>32	0.25	>32	0.5	16	0.25	0.06	0.125	0.06
9	555/10	*C. freundii*	ESBL+ve	>16	>16/8	>128	16	2	0.5	>32	0.125	>32	0.25	4	0.25	0.03	0.06	0.06
10	3364/11	*K. oxytoca*	ESBL-ve	>16	>16/8	>128	16	16	2	>32	0.25	>32	0.5	4	0.25	0.06	0.125	0.06
11	4240/11	*K. pneumoniae*	ESBL-ve	>16	2/1	8	2	0.125	0.5	0.03	0.06	0.03	0.06	0.06	0.25	0.06	0.015	0.06
12	4328/11	*K. oxytoca*	AmpC?	16	16/8	16	16	4	16	8	16	16	16	2	0.5	0.25	2	0.25
13	4327/11	*K. pneumoniae*	ESBL-ve	>16	4/2	8	2	0.125	0.25	0.03	0.06	0.03	0.06	0.03	0.5	0.06	0.015	0.06
14	1325/11	*C. koseri*	ESBL-ve	>16	4/2	8	2	0.125	0.5	0.03	0.06	0.03	0.06	0.125	0.25	0.06	0.015	0.06
15	1325/11	*E. aerogenes*	ESBL-ve	>16	>16/8	>128	16	16	2	>32	0.5	>32	4	16	0.25	0.03	0.125	0.06

Table 5g II: The results of MIC carried on 44 ocular clinical isolates of *Enterobacteriaceae*.

S.No	Strain ID	Organism	Phenotype	Amoxicillin	Amoxicillin/ clav (2:1)	Piperacillin	Pip/taz (4 mcg/mL)	Ceftazidime	Ceftazidime /clav (4 mcg/mL)	Ceftriaxone	Ceftriaxone/ clav (4 mcg/mL)	Cefotaxime	Cefotaxime/ clav (4 mcg/mL)	Cefepime	Imipenem	Meropenem	Ertapenem	Doripenem
16	1645/10	*E. coli*	ESBL+ve	>16	>16/8	>128	16	16	2	>32	0.25	>32	0.5	16	0.25	0.06	0.125	0.06
17	925/10	*E. coli*	ESBL+ve	>16	>16/8	>128	16	32	2	>32	0.5	>32	1	>16	0.25	0.03	0.125	0.03
18	883/09	*C. freundii*	ESBL-ve	>16	>16/8	2	2	0.25	2	0.125	1	0.25	1	0.06	0.5	0.06	0.125	0.125
19	881/09	*C. freundii*	ESBL-ve	>16	>16/8	2	2	0.25	2	0.03	0.25	0.06	2	0.06	0.5	0.06	0.125	0.125
20	3685/09	*C. koseri*	ESBL-ve	4	4/2	2	2	0.125	0.5	0.125	0.06	0.03	0.06	0.03	0.25	0.03	0.015	0.03
21	2247/09	*E. coli*	ESBL+ve	>16	>16/8	>128	8	8	1	>32	0.125	>32	0.25	4	0.25	0.03	0.03	0.06
22	2777/09	*K. oxytoca*	Carbapenem- R	>16	>16/8	2	2	0.5	2	16	>32	16	32	1	16	2	>8	2
23	2249	*K. pneumoniae*	ESBL+ve	>16	>16/8	>128	16	>32	2	>32	0.25	>32	0.25	16	0.5	0.06	0.125	0.06
24	1028	*E. cloacae*	ESBL+ve	2	2/1	2	2	8	8	0.5	0.25	0.125	0.125	0.5	0.125	0.25	2	0.125
25	7193	*K. pneumoniae*	ESBL+ve	>16	>16/8	>128	16	16	0.5	>32	0.125	>32	0.125	4	0.5	0.06	0.06	0.06
26	2247/10	*E. coli*	ESBL+ve	>16	>16/8	>128	8	16	0.5	>32	0.125	>32	0.25	4	0.25	0.03	0.06	0.06
27	1439/11	*C. diversus*	ESBL+ve	>16	>16/8	>128	2	0.125	0.5	0.06	0.06	0.03	0.125	0.06	0.25	0.03	0.015	0.03
28	838	*E. coli*	Carbapenem- R	0.5	0.5/0.25	8	8	8	>32	8	16	16	32	>16	0.5	1	4	1
29	2298/08	*C. koseri*	ESBL-ve	>16	>16/8	2	2	0.125	0.125	0.125	0.25	0.125	0.25	0.03	0.5	0.03	0.125	0.125
30	3362/11	*E. aerogenes*	Carbapenem- R	>16	>16/8	>128	>128	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16

Table 5g III: The results of MIC carried on 44 ocular clinical isolates of *Enterobacteriaceae*.

S.No	Strain ID	Organism	Phenotype	Amoxicillin	Amoxicillin/ clav (2:1)	Piperacillin	Pip/taz (4 mcg/mL)	Ceftazidime	Ceftazidime /clav (4 mcg/mL)	Ceftriaxone	Ceftriaxone/ clav (4 mcg/mL)	Cefotaxime	Cefotaxime/ clav (4 mcg/mL)	Cefepime	Imipenem	Meropenem	Ertapenem	Doripenem
31	2946/10	*E. aerogenes*	ESBL-ve	>16	>16/8	2	2	0.5	0.125	0.25	0.06	0.25	0.06	0.03	0.5	0.06	0.125	0.06
32	3829/11	*P. mirabilis*	ESBL+ve	>16	8/4	>128	0.5	>16	0.5	>16	0.008	>16	0.03	>16	2	0.06	0.06	0.125
33	3244/11	*E. coli*	ESBL+ve	>16	>16/8	>128	32	>16	1	>16	0.25	>16	0.25	>16	0.125	0.03	0.25	0.03
34	2916/08	*P. mirabilis*	ESBL-ve	1	1	0.5	0.5	0.06	0.06	0.008	0.008	0.015	0.015	0.03	2	0.06	0.03	0.25
35	1489/08	*P. mirabilis*	ESBL+ve	>16	8/4	>128	0.5	>16	1	4	0.015	>16	0.06	>16	2	0.06	0.06	0.25
36	107/10	*E. aerogenes*	ESBL+ve	>16	8/4	8	0.5	>16	0.5	>16	0.015	>16	0.03	>16	2	0.06	0.06	0.125
37	4025/08	*P. rettgeri*	ESBL-ve	>16	>16/8	8	2	0.5	0.5	0.5	0.25	0.5	0.25	0.125	0.5	0.06	0.06	0.125
38	3246/10	*Serratia*	ESBL-ve	>16	>16/8	4	4	0.5	0.125	0.125	0.125	0.25	0.125	0.06	1	0.06	0.125	0.125
39	3013/11	*M. morganii*	ESBL-ve	>16	16/8	8	1	8	2	0.5	0.125	2	0.5	0.125	4	0.125	0.125	0.5
40	1216/11	*P. mirabilis*	ESBL-ve	1	1/0.5	0.5	0.5	0.125	0.06	0.008	0.008	0.015	0.03	0.06	2	0.06	0.03	0.25
41	3679/11	*K. pneumoniae*	ESBL+ve	>16	>16/8	>128	8	16	0.25	>16	0.06	>16	0.06	16	0.25	0.03	0.06	0.03
42	4216/11	*Serratia*	Imipenem - R	>16	>16/8	2	2	0.25	0.5	0.125	0.25	0.125	0.25	0.06	1	0.06	0.125	0.125
43	342/11	*K. pneumoniae*	ESBL+ve	>16	>16/8	>128	8	>16	0.25	>16	0.25	>16	0.25	>16	0.25	0.03	0.06	0.015
44	3440/11	*K. oxytoca*	ESBL-ve	>16	2/1	8	2	0.25	0.125	0.06	0.06	0.06	0.06	0.03	0.25	0.03	0.03	0.03

5.5.2. Genotypic results of ESBL detection in *Enterobacteriaceae* isolates:

All the 57 ESBL producing isolates were subjected for ESBL detection by the molecular method. Among the 57 ESBL producing isolates, single ESBL gene were present in 20 (35.0%) isolates of which *blaCTX-M* (n= 8, 14.0%) being the most common ESBL gene detected, followed by *blaSHV* (n=5, 8.7%), *blaTEM* (n=4, 7.0%) and *blaOXA* (n=3, 5.2%). Among the 22 (38.5%) isolates harboring two ESBL genes, *blaTEM* + *blaCTX-M* was the most common combination (n=10, 17.5%) followed by *blaSHV* + *blaTEM* (n=6, 10.5%), *blaOXA* + *blaTEM* (n=3, 5.2%), *blaOXA* + *blaSHV* (n=3, 5.2%).

Nine isolates carried three ESBL genes, and the predominant combination was *blaCTX-M* + *blaTEM* + *blaOXA* (n=4, 7.0%), followed by *blaCTX-M* + *blaOXA*+ *blaSHV* (n=2, 3.5%), and rest 3 (1.7%) combinations were *blaCTX-M* + *blaSHV* + *blaTEM*, *blaCTX-M* + *blaTEM* + *blaSHV*¸ *blaOXA*+ *blaTEM* + *blaSHV* each was exhibited. Six (10.5%) isolates had all the 4 gene combinations ie, *blaCTX-M* + *blaOXA* + *blaSHV* + *blaTEM*.

Other CTX-M genes (II, III, and IV) and *blaNDM-1* were not detected in any of the isolates. The *blaNDM-1* gene was absent among all the ESBL producers. Phenotypic and genotypic methods of ESBLs detection showed a 100 % correlation. Sensitivity of uniplex PCR's were of range from 10ng – 50 ng DNA of ESBL producing positive controls. The primers were highly specific and no amplification was observed with fungal, viral and human DNA. Distribution ESBLs *Enterobacteriaceae* isolates according to genotypic combinations is shown in table 5h.

Table 5h: Extended spectrum Beta-lactamase (ESBL) genotypes detected among 57 ocular *Enterobacteriaceae* strains that were tested positive for ESBL production

Positive by PCR for ESBL genes	ESBL producer (N = 57)								
	K. pneumoniae (n =12, 21.0%)	*K. oxytoca* (n=10, 17.5%)	*E. coli* (n=8, 14.0%)	*E. aerogenes* (n=7, 12.2%)	*C. freundii* (n= 3, 5.2%)	*P. mirabilis* (n= 6, 10.5%)	*C. koseri* (n=8, 14.0%)	*S. marscensens* (n = 3, 5.2%)	TOTAL n=57 (%)
A. Single ESBL gene positivity									
***blaCTX-M* only**	2	2	1	2	-	-	1	-	8 (14.0%)
***blaOXA* only**	-	-	3	-	-	-	-	-	3 (5.2%)
***blaSHV* only**	4	1	-	-	-	-	-	-	5 (8.7%)
***blaTEM* only**	1	-	-	-	1	-	2	-	4 (7.0%)
B. Two genes positivity									
blaCTX-M + blaTEM	4	3	-	1	-	1	-	1	10 (17.5%)
blaOXA + blaTEM		1	-	1	-	-	1	-	3 (5.2%)
blaSHV + blaTEM	1	1	1	1	-	1	1	1	6 (10.5%)
blaOXA + blaSHV	-	-	-	-	-	1	1	1	3 (5.2%)
C. Three ESBL genes positivity									
blaCTX-M + blaSHV	-	1	-	-	-	-	-	-	1 (1.7%)
blaCTX-M + blaTEM + blaOXA	-	-	-	1	2	-	1	-	4 (7.0%)
blaCTX-M + blaTEM + blaSHV	-	-	-	-	-	-	1	-	1 (1.7%)
blaCTX-M + blaOXA+ blaSHV	-	1	-	-	1	-	-	-	2 (3.5%)
blaOXA+ blaTEM + blaSHV	-	-		1	-	-	-	-	1 (1.7%)
D. Four ESBL genes positivity									
blaCTX-M + blaOXA+ blaSHV + blaTEM	-	-	3	-	-	3	-	-	6 (10.5%)

Agarose gel electrophoretogram showing the results of uniplex PCR targeting CTX-*M* gene (Figure 5b), *TEM* (Figure 5c), *OXA* (Figure 5d), *SHV* (Figure 5e) and *blaNDM-1*gene (Figure 5f) on ocular clinical isolates are shown below.

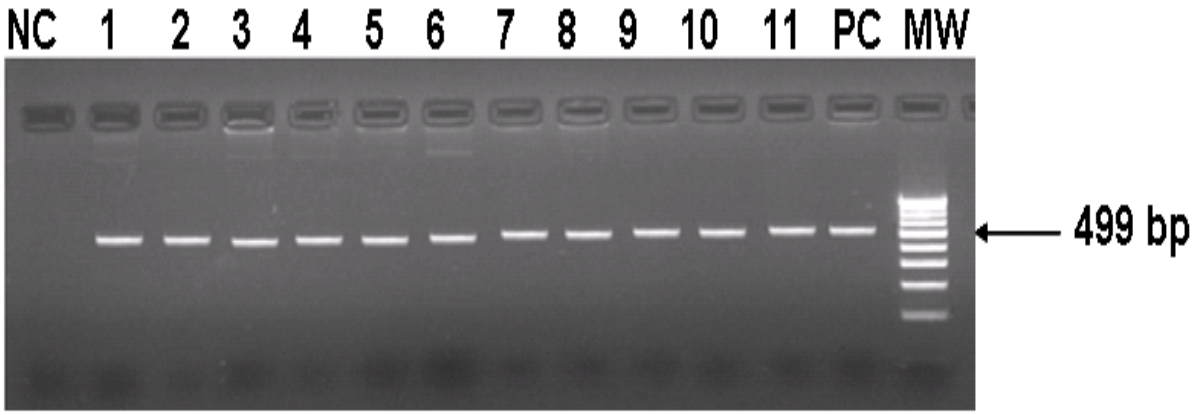


Figure 5b: Agarose gel electrophoretogram showing the results of uniplex PCR targeting *CTX-M* gene on ocular clinical isolates. Lane NC: Negative control, **Lane 1-11:** Showing amplification of *CTX-M* gene among ocular clinical isolates of *Enterobacteriaceae*, **Lane PC:** Positive Control - *Klebsiella pneumoniae* ATCC 700603, **Lane 12:** MW – Molecular weight 100bp ladder.

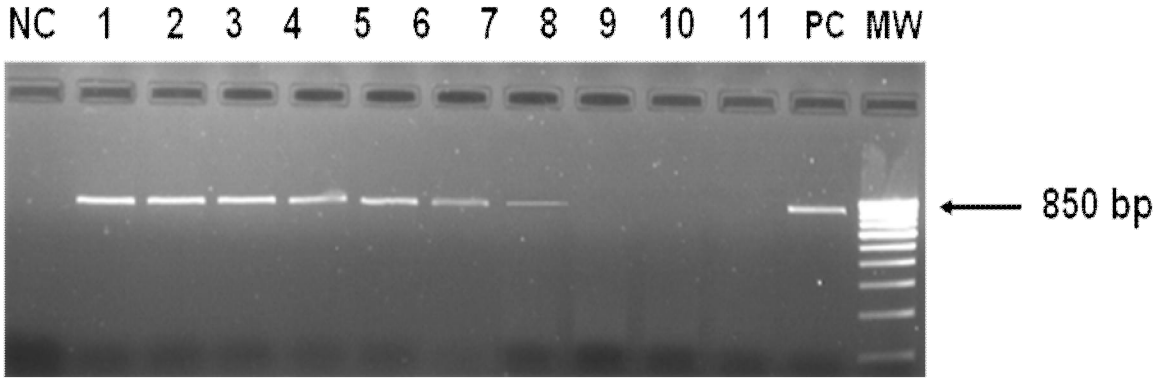


Figure 5c: Agarose gel electrophoretogram showing results of uniplex PCR targeting *TEM* gene on ocular clinical isolates. Lane NC: Negative control, **Lane 1-8:** Showing amplification of *TEM* gene among ocular clinical isolates of *Enterobacteriaceae*, **Lane 9-11:** Showing absence of *TEM* gene amplification in the isolates, **Lane PC:** Positive Control - *Escherichia coli* ATCC 35218, **Lane 12:** MW - Molecular weight 100bp ladder.

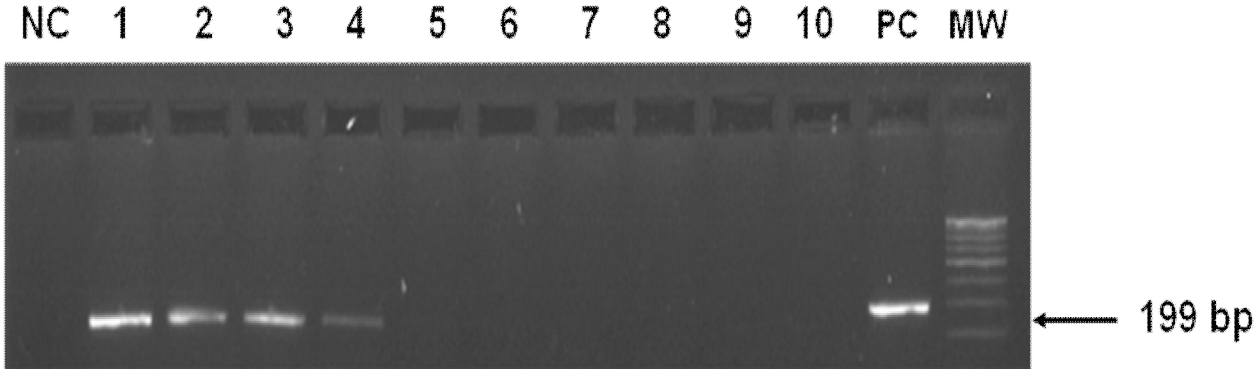


Figure 5d: Agarose gel electrophoretogram showing results of uniplex PCR targeting *OXA* gene on ocular clinical isolates. Lane NC: Negative control, **Lane 1-4:** Showing amplification of *OXA* gene among ocular clinical isolates of *Enterobacteriaceae*, **Lane 5-10:** Showing absence of *OXA* gene amplification in the isolates, **Lane PC:** Positive Control **–** M.No. 3655/11 – *E. coli* (DNA sequencing confimed lab isolate), **Lane 12:** MW - Molecular weight 100bp ladder.

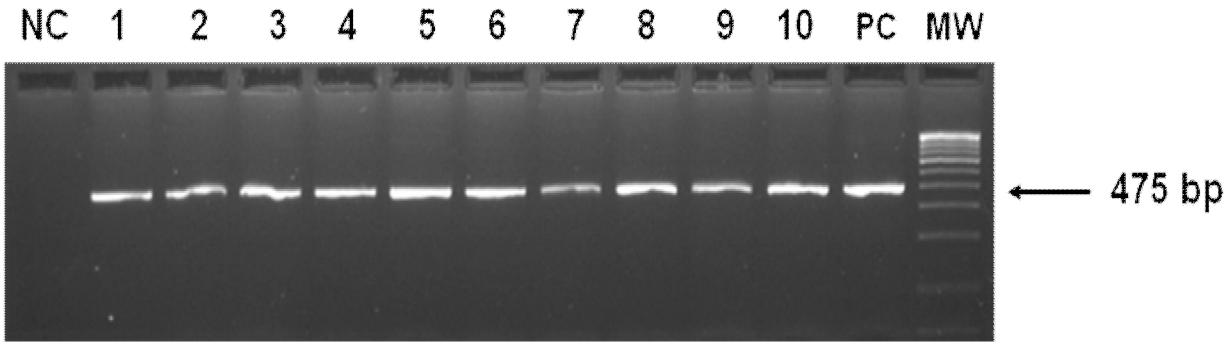


Figure 5e: Agarose gel electrophoretogram showing results of uniplex PCR targeting *SHV* gene on ocular clinical isolates. Lane NC: Negative control, **Lane 1-10:** Showing amplification of *SHV* gene among ocular clinical isolates of *Enterobacteriaceae*, **Lane PC:** Positive Control - *Klebsiella pneumoniae* ATCC 700603, **Lane 12:** MW - Molecular weight 100bp ladder.

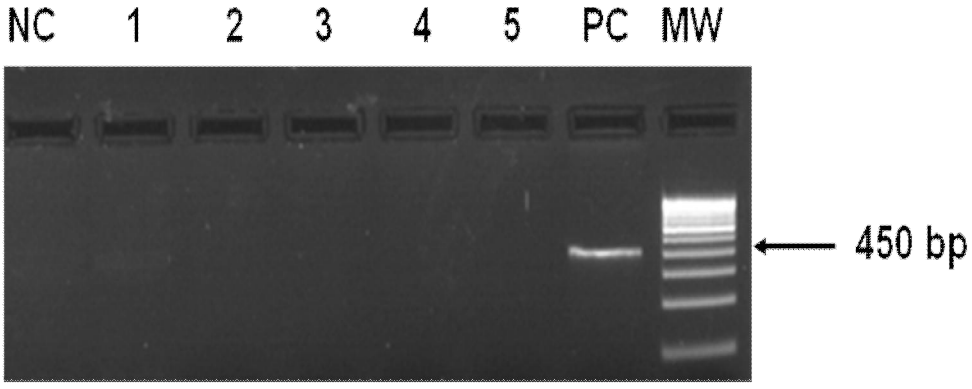


Figure 5f: Agarose gel electrophoretogram showing results of uniplex PCR targeting the *NDM-1* gene on ocular clinical isolates. Lane NC: Negative control, **Lane 1-11:** Showing amplification of *blaNDM-1* gene among ocular clinical isolates of *Enterobacteriaceae*, **Lane PC:** Positive Control **–** *Acinetobacter baumannii* lab isolate, **Lane 12:** MW - Molecular weight 100 bp ladder.

5.5.3. DNA sequencing:

PCR products were subjected to DNA sequencing with forward primer or the reverse primer of the corresponding genes. Nucleotide sequence results were analyzed by **www.ncbi.nlm.nih.gov/blast.** Blast analysis showed that all genotypically *CTX-M* -I positive isolates were found to be of *blaCTX-M-15* beta lactamase type, *OXA* gene belong to class *blaOXA-1, SHV* gene belong to class *blaSHV-1, TEM* gene belong to *blaTEM-1* type.

5.5.4. Nucleotide sequence accession number:

The nucleotide sequence data reported here will appear in the GenBank nucleotide sequence data base under accession number JN019833 - JN019857 (25 strains), JN043372 (1 strain), JN043373 - JN 043374 (2 strains), JN043375 - JN043384 (10 strains).

5.6. Discussion:

The present study clearly showed a high prevalence ESBL producing *Enterobacteriaceae* isolates among ocular clinical isolates. Most of these patients were not staying in the hospital and indicating ESBL genes in these isolates could be community acquired whereas *blaNDM-1* gene was not detected in this present study indicating absence of the same in the community acquired infections. Many studies on existence of *blaNDM-1* gene among *Enterobacteriaceae* isolated from patients who underwent transplantation, chronic disease and accidents has been reported by Liang et al. 2011).

In this study, large numbers of *Enterobacteriaceae* were isolates from conjunctival ocular surface followed by the DCR isolates. Predominant *Enterobacteriaceae* tested, were isolated

from patients suspected of conjunctivitis (18.9%) followed by keratitis (14.9%). In the conjunctivitis cases, the primary pathogens isolated were *E. coli* followed by *K. pneumoniae* where as in case of keratitis the predominant pathogen is *C. freundii*.

Jones et al., (2002) have recorded the incidence of ESBL positivity to be 60–68% in India, from strains isolated from major hospitals. However, the prevalence studies on ocular ESBL producing ocular isolates from India are very few. Bharathi et al., (2012) had reported the incidence of ESBL among the ocular population to be 14 %. In our study the rate of ESBL positivity was 77% by double disc diffusion test. In our previous study conducted by us on DCR and MOD isolates (Sowmiya et al., 2012), ESBL production was 58.3%. *K. oxytoca* was isolated from intraocular specimens of two patients with endogenous endophthalmitis whose urine, blood culture also showed growth of same organism This confirms the endogenous spread of *K. oxytoca* in both the cases and the isolates were tested negative for ESBLs and *blaNDM-1*gene.

In order to prevent external contamination, all donor corneas were procured by whole globe enucleation, followed by corneoscleral rim excision at the host eye bank with aseptic techniques. Under aseptic precautions, they were stored in McCarey and Kaufman medium (M-K medium) containing gentamicin (100µg/mL). At the time of surgery, the cornea was trephined for transplantation, and the remaining rim of corneoscleral tissue, was transferred with M-K medium and sent to the clinical microbiology laboratory for bacterial culture within 15 minutes after of performing penetrating keratoplasty. *Enterobacteriaceae* thus isolated from these donor corneal rims were tested for ESBLs.

Fourteen isolates (n=14/20, 70%) from MOD and DCR specimens were positive for ESBL among *Enterobacteriaceae* tested. The organisms isolated among these MOD and DCR specimens were of nosocomial in origin since all the patients would have had a reasonable period of stay in the hospital before the donation of organs. Similarly isolation of ESBL producing *Enterobacteriaceae* in case of silicon rods and intraocular lenses were also nosocomial as the patients gain infection predominantly during surgery inspite of all standard aseptic precautions such as use of providine-iodine, installation of antibiotics, use of sterilized instruments and IOLs.

ESBL positive isolates showed increased susceptibility to gatifloxacin (74.4%), moxifloxacin (69.5%) and ofloxacin (68.9%). Higher degree of resistance was exhibited towards ceftazidime (79.7%) followed by aminoglycoside group which includes gentamicin (56.7%) and tobramycin (55.4%). Phenotypic and genotypic data showed 100 % correlation. The phenotype data shows an emerging trend of carbapenem resistance among ocular clinical isolates of *Enterobacteriaceae*.

In a study conducted in our hospital by Madhavan *et al.,* in 1999, 62.5% of sensitivity to ceftazidime was reported among Gram negative endopthalmitis isolates. Shift in the resistance rates of ceftazidime (commonly used ocular antibacterial agent) from 37.5% to 79.7% proves the increasing spread of drug resistance among ocular population during this intervening period. The most common type of ESBL enzyme encountered in this study is the CTX-M type; more specifically *CTX-M-15*. Studies conducted by Johann et al., (2004) and Ensor et al., (2006) had showed *CTX-M-15* to be the most widely disseminated ESBL enzymes. Our present study reaffirms these findings. Six (10.5%) isolates had all the 4 gene combinations ie, *blaCTX-M* +

blaOXA + *blaSHV* + *blaTEM* were detected in 3 *E. coli* and 3 *P. mirabilis*. These 6 isolates were from DCR and conjunctival swab indicating the prevalence of the drug resistance. Further studies may be needed to determine the reason for these findings.

Although there are many reports on presence of *blaNDM-1* genes among clinical isolates in India, still a single report stating the absence of *blaNDM-1* genes among faecal flora exists (Deshpande et al., 2012). Our study also showed absence of *blaNDM-1* genes among ocular specimens. This demonstrates that at this time point, *blaNDM-1* genes are not carried among the organisms isolated from both extraocular specimens that are highly exposed to community acquired infections and also from intraocular specimens making them free from increased drug resistance thus making the treatment not a hard task. However the high rates of ESBL producing isolates among ocular strains is an important finding.

Cefazolin and cefotaxime are being routinely used in the treatment of ocular infections. considering the increasing spread of such ESBL genes in the community, all the patients culture positive report should carry a note indicating, whether the isolate is ESBL producer or not. This will help the clinicians to avoid the use of penicillins, aztreonam, and all cephalosporins (except cephamycins), irrespective of their *in vitro* susceptibility.

5.7. Conclusion:

This is the first extensive study to screen to identify the predominant drug resistance encoding ESBL genes among the ocular isolates. We have demonstrated the common ESBL genotypes present among Enterobacteriaceae isolated in our hospital set up. Ours is a first study

carried out for screening *blaNDM-1* genes on the ocular isolates of *Enterobacteriaceae*. The phenotype data shows an emerging trend of carbapenem resistance among ocular clinical isolates of *Enterobacteriaceae*. Overall rate of nosocomial infections were found in 31.5% of hospitalized patients and rest 68.5% were community acquired and these important findings indicate the existence of community associated strains of ESBL producing *Enterobacteriaceae* and a remarkable percentage of such strain do exist as of hospital acquired.

5.8. Future prospects:

This PCR technique for detection of ESBL genes of will aid understanding the spread of drug resistance from ocular clinical isolates of *Enterobacteriaceae*. This approach detection of ESBL genes and *blaNDM-1* gene will be further extended among *Enterobacteriaceae* isolated from extra-ocular specimens and the prevalent genotype pattern will be studied.

Chapter - 6

Screening for the prevalence of drug resistance and existence of *blaNDM-1* among microorganisms isolated from Multi organ donor and Donor corneal rim in a tertiary eye care centre, India

6.1. Aim:

To screen for the prevalence of drug resistance and existence of *blaNDM-1* among microorganisms isolated from Multi organ donor and Donor corneal rim among ocular Indian population.

6.2. Review of literature:

6.2.1. Carbapenemases:

Carbapenemases are diverse enzymes that vary in the ability to hydrolyze carbapenems and other β-lactams. Detection is a crucial infection control issue because (i) they are often associated with extensive, sometimes total, antibiotic resistance and (ii) more-resistant organisms such as strains of *Pseudomonas* and *Acinetobacter* spp., that have acquired a carbapenemase can be vectors responsible for carbapenemase transmission to members of the family *Enterobacteriaceae* in which the resistance mechanism is not recognized. The major concern is with transmissible and not chromosomal carbapenemases. The transmissible enzymes can be acquired unpredictably by important pathogens such as *P. aeruginosa, A. baumannii*, and members of the family *Enterobacteriaceae*. Chromosomal enzymes occur predictably in less common pathogens such as *S. maltophilia, Aeromonas* spp., *Chryseobacterium* spp., and others (Thomson et al., 2010).

Carbapenemases belongs to Ambler classes A, B, and D class of β-lactamases. The class A enzymes (Bush group 2f) are inhibited to various degrees by clavulanate and usually hydrolyze penicillins or cephalosporins more efficiently than carbapenems (Black et al., 2005). For this reason, some carbapenemases such as *KPC* enzymes, have lesser carbapenemase activity and may be considered ESBL that can also hydrolyze carbapenems.

Class A carbapenemases includes the *KPC* (*Klebsiella pneumoniae* Carbapenemases), *IMI* (Imipenem-hydrolyzing β -lactamase), and *SME* (*Serratia marcescens* Enzyme) families, *NMC* (Non-Metalloenzyme Carbapenemase), and some *GES* (Guiana extended spectrum) enzymes. They are most commonly produced by members of the family *Enterobacteriaceae* but have been recently detected in isolates of *P. aeruginosa* (Thomson et al., 2010).

Class B enzymes (Bush group 3) are metallo-β-lactamases (MBLs) which typically hydrolyze carbapenems efficiently and resist currently available β-lactamase inhibitors and aztreonam. They are inhibited by chelating agents such as EDTA. The most important genes include the *VIM* (Verona integron-encoded metallo-Beta-lactamase) and *IMP* (metallo-Beta-lactamase in *Pseudomonas aeruginosa)* families and *SPM-1* (Sao Paulo metallo-Beta-lactamase), which have been detected in strains of *P. aeruginosa*, members of the family *Enterobacteriaceae*, and *A. baumannii*.

Most class D carbapenemases hydrolyze carbapenems weakly and are inhibited poorly by clavulanate. They belong to the OXA family and are most commonly produced by *Acinetobacter* spp. but have also been reported in some *P. aeruginosa*, *K. pneumoniae*, and *E. coli* strains (Thomson et al., 2010).

6.2.2. *NDM -1* GENE:

Increased prevalence on carbapenemases production among clinical isolates of *Enterobacteriaceae* has increased over the past decade (Struelens et al., 2010). Although beta-lactams have been widely used as the mainstay of treatment for severe infections due to these bacteria, carbapenems are often representing last-line of defense (Rolain et al., 2010). Recently, the New Delhi metallo-beta-lactamase (*NDM-1*) a novel type of metallo-beta-lactamase (MBL) conferring resistance to almost all beta-lactam antibiotics, including carbapenems that are used in treatment of antibiotic-resistant bacterial infections have emerged and has therefore alarmed the world (Lascols et al., 2011, Zarfel et al., 2011, Seema et al., 2011, Kumarasamy and Kalyanasundaram, 2012, Deshpande et al., 2012). Like other acquired MBLs, *NDM-1* hydrolyses all beta-lactam antibiotics except aztreonam, which is usually inactivated by co-produced extended-spectrum or *AmpC* beta-lactamases. An association with other resistance mechanisms makes a majority of *Enterobacteriaceae* with *blaNDM-1* extensively resistant to antibiotics and susceptible only to colistin, less consistently, tigecycline (Struelens et al., 2010, Deshpande et al., 2012).

An extensive survey performed in the UK, India and Pakistan identified NDM-1 producing *Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii, Morganella morganii, Providencia* spp., and *Enterobacter cloacae* isolates. This worldwide spread of *Enterobacteriaceae* carrying *blaNDM-1*, has thus become a significant threat to human health due to its extensive drug resistance, leaving few or no therapeutic options thus becoming a major public health problem throughout the world, particularly in India (Poirel et al., 2010, Kaase et al., 2012). Spread of this novel carbapenemases gene is considered as a serious threat since the

reservoir of *NDM-1* producers is at least in part related to the Indian subcontinent that is inhabited by the second largest population in the world and where *NDM-1* producers are reported also in community-acquired infections The worldwide spread of *Enterobacteriaceae* that carry carbapenemase-producing genes, including *blaNDM-1*, is a significant threat to human health: Firstly, the production of carbapenemase, in association with other resistance determinants, confers extensive drug resistance, leaving few or no therapeutic options. Secondly, the association with travel underscores the risk of healthcare in countries where antibiotic-resistant bacteria are endemic. Lastly, studies of patients infected with *Enterobacteriaceae* producing KPC are at increased risk of complications and death (Struelens et al., 2010, Poirel et al., 2010).

6.2.3 *NDM-2* Gene*:*

NDM-2 is a variant of *blaNDM-1* that has a C to G substitution at position "82" resulting in an amino acid substitution of proline to alanine at position "28". In strains with this *NDM-2* positivity genes encoding extended-spectrum β-lactamases or *16S RNA* methylase were not detected. The strain also lacked detectable plasmids and *blaNDM-2* was not transferred by conjugation. (Kaase et al., 2012, Ghazawi et al., 2012)

6.2.4. Corneal transplantation:

Annually more than 100,000 corneal transplants are performed worldwide (Ehler, 1997). Donor-to-host transmission of bacteria and fungi is a well-recognized complication leading to keratitis and endophthalmitis have been reported (Ritterband et al., 2007). Endophthalmitis after penetrating keratoplasty (PK) is usually rare ranging from 0.05% to 0.4%. Contamination of donor cornea rims due to bacteria is relatively common (12%–39%) (Ritterband et al., 2007,

Leveille et al., 1983, Antonios et al., 1991, Rehany et al., 2004). However, the outcome of endophthalmitis may be devastating: some patients require evisceration or enucleation (Everts et al., 2001).

According to WHO, currently 45 million individuals worldwide are bilaterally blind; of which 6-8 million are blind due to corneal diseases. Approximately 3 million eyes need cornea transplantation. Corneal transplantation has become a frequently performed procedure which has been made a successful procedure by advances in eye banking, corneal surgery and postoperative treatment availability (Lohiya et al., 2011). The rationale of performing rim cultures may be either for quality control in the eye bank or for providing information about microbial contaminants that may cause endophthalmitis (Wiffen et al., 1997).

6.2.5. Microbiological spectrum of Donor corneal rim and Multi organ donor swabs:

The spectrum of microbial organisms contaminating Donor corneal rim (DCR) and Multi organ donor (MOD) swabs has been previously reported. This microbiological spectrum most likely represents the normal flora of the donor ocular surface (Ritterband et al., 2007). It has been postulated that the organisms responsible for endophthalmitis after corneal transplantation derive from the donor tissue itself in approximately half of all cases (Everts et al., 2001). Eye banks routinely take tremendous precautions to reduce and to prevent the microbial contamination of donor eyes, (Ritterband et al., 2007) still rare prevalence of endophthalmitis due to penetrating keratoplasty (PK) exists.

At the time of corneal transplantation, the residual rim of peripheral corneal and scleral tissue (corneoscleral rim) is submitted for culture, primarily to study the contamination rates. The main objective of performing rim cultures may be either for quality control in the eye bank or for providing information about microbial contaminants that may cause endophthalmitis. Because endophthalmitis typically occurs within 48 to 72 hours postoperatively and progresses rapidly, there is little opportunity for the results of directed diagnostic microbiologic procedures to influence therapy during the critical period, to use antibiotic results of cultures to guide immediate, empirical antimicrobial therapy in the recipient earlier than would otherwise be possible if endophthalmitis should occur (Everts et al., 2001, Lohiya et al., 2011).

Emergence and evolution of antibiotic resistance among bacterial pathogens has a global impact on public health. Antibiotic use in clinical practices has been a major source of emergence and spread of resistant strains carrying drug resistance genes in the community (Thaller et al., 2010). This emergence of resistance amongst pathogens generates visions of the potential post-antibiotic era threatening present and future medical advances (Raghunath et al., 2008).

Donor corneal transplants carrying these pathogenic organisms both resistant and sensitive strains poses a source of transmission of these bacteria via corneal transplants by PK and it results in increased difficulties in the treatment of endophthalmitis caused by them. The rate of bacterial isolates from DCR, resistant to varied drugs has poorly been studied which paved the path for the initiation of this analysis.

6.2.4. Need for this study:

Since August 2010, the spread and dissemination of *NDM-1* positive strain has occurred, with cases being globally reported by medias from countries including United States, Canada, Sweden, United Kingdom, Austria, Belgium, France, Netherlands, Germany, Africa, Oman, Australia, Japan and China (Liang et al., 2011). Very recently, *NDM-1* producers were found in seepage samples and public tap water in New Delhi, India, showing that the dissemination of these multidrug-resistant bacteria might be higher than expected (Poirel et al., 2010).

Till date presence of *blaNDM-1* gene among ocular *Enterobacteriaceae* isolates was unanswered. So we screened 74 ocular *Enterobacteriaceae* isolates received during the period of two years from January 2010 – December 2011 for the presence of *blaNDM-1* gene.

Many studies on existence of *blaNDM-1* among *Enterobacteriaceae* isolated from patients who underwent renal or bone marrow transplantation, dialysis, cerebral infarction, chronic obstructive pulmonary disease, pregnancy, burns, road traffic accidents, and cosmetic surgery was reported by Liang et al., 2011. Studies on screening ocular isolates of *Enterobacteriaceae* for the existence of showed absence of *blaNDM-1* genes.

Therefore we in this study, we screened all the extra ocular specimens such as DCR and conjunctival swab from MOD who died in road traffic accident for understanding the drug resistance pattern among them and Gram negative bacilli subject for the detection of *blaNDM–1* gene.

6.3. Objective:

To screen for the prevalence of drug resistance and existence of *blaNDM-1* among microorganisms isolated from Multi organ donor and Donor corneal rim among ocular Indian population.

a. To study the microbial spectrum of DCR and MOD.
b. To understand antibiotic susceptibility pattern of isolates recovered from DCR and MOD.
c. To analyze the prevalence of Extended Spectrum of Beta-Lactamases (ESBLs) genes such as *CTX-M, AmpC, TEM* and *SHV g*enes among the *Enterobacteriaceae* isolated from DCR and MOD by PCR based DNA sequencing method.
d. To screen for the presence of virulent MBL gene; *blaNDM-1* among ocular *Enterobacteriaceae* isolates and to confirm the same by PCR based DNA sequencing method.

6.4. Materials and Methods:

A total of 118 prospective specimens of 76 Donor corneal rim (DCR) after the completion of corneal transplantation and 42 conjunctival swabs from multiorgan donors was collected before the eyes were removed for the corneal transplant by the Eye bank of Sankara Nethralaya ophthalmic hospital, Chennai during a period of 6 months (from January 2011 – June 2011), were included in this study. Consent was obtained for donating the organs. These specimens were received for microbial culture at L & T Microbiology Laboratory, Sankara Nethralaya, a tertiary eye care centre at Chennai, India. Study protocol was approved by the institutional ethics sub-committee (IRB). Following information about donors was collected as

follows: (a) Name of donor (b) Age / Sex (c) Time since death (d) Cause of death. The name was coded at its receipt in the eye bank.

6.4.1. Exclusion criteria:

The exclusion criteria for the collection of the donor corneal tissue were:

a. Systemic Criteria:

The systemic exclusion criteria include death of unknown cause, death following central nervous system disease of unknown etiology, Septicemia, Leukemia, Hodgkin's disease. Patient detected with Hepatitis B, C virus, Human Immunodefiency Virus (HIV), Cruetzfeld – Jakob disease, Rabies were also excluded.

b. Ocular Criteria include:

Corneal opacity, Infective eye disorders & Ocular tumors; the eyes from these donors were excluded for transplant purposes and therefore were not subjected for the present study.

6.4.2. Procedure for enucleation of the cadaveric corneas:

Before enucleation of the cadaveric corneas, the hands were washed and using sterile gloves eyes were examined with torch light to detect any anterior segment problems or any evidence of infection, in which case the eyes were not used. Previous history of any eye disease or surgery was enquired to the patient's relatives. The periorbital skin around the eye was cleaned with 70% isopropyl alcohol using sterile cotton and then with povidone-iodine, antiseptic solution. A sterile eye drape was placed and 2 – 3 drops of a povidone-iodine eye drop of concentration 0.5w/v% (Apidine – 5), was applied onto the conjunctiva and left for 3 – 5

minutes and excess of them was wiped off using sterile gauze by closing the cadaver eyes once the exposure time is over. After application of povidone-iodine, 2 – 3 drops of ciprofloxacin antibiotic solution of concentration 0.3% w/v was applied left for 3 – 5 minutes. A speculum was applied; peritomy close to the limbus was made and undermined towards the fornices. The rectus muscles are hooked and cut close to their insertion. A 2 – 3mm of lateral rectus stump is left for holding the eyes. The eyeball was then lifted up and the optic nerve is cut with the help of enucleation scissors. The eye was then placed in a eye holder with the cornea facing-up. The stump of the optic nerve should fix into the slot meant for the same. Eye ball after enucleation were transported on ice to the eye bank. Eye balls were irrigated by 2 – 3 drops of sterile saline and ciprofloxacin antibiotic solution of concentration, 0.3% w/v in case of delay of more than an hour in transport of eye ball to the eye bank.

6.4.3. Collection of donor corneas:

Donor corneas were procured by whole globe enucleation, followed by corneoscleral rim excision at the host eye bank under a horizontal laminar flow hood with aseptic techniques. This was followed by application of 2 – 3 drops of ciprofloxacin eye drops 0.3% w/v and left for 5 minutes. Under all aseptic precautions, they were transferred and stored in McCarey and Kaufman (developed at L.V. Prasad Eye Institute, Hyderabad) medium containing gentamicin (100µg/mL).

At the time of surgery, the cornea was trephined for transplantation, and the remaining rim of corneoscleral tissue, along with M-K medium, was sent to the clinical microbiology within 15 minutes after the transplantation was over. DCR's cultures of all Penetrating

keratoplasty carried out at a single ophthalmic hospital are included for analysis. All the DCR's are received at the laboratory within 10-20 minutes from the operation theater to prevent the influence of delay in transport on isolation of bacteria /fungi. Donor corneal rims are maintained in the M-K medium till is received at the clinical microbiology laboratory for the bacterial and fungal culture. Preoperative antibiotics include ciprofloxacin and albicid were used onto the patient's eye before transplantation.

6.4.4. Collection of conjunctival swabs from Multi organ donor:

Before enucleation of eye balls from multi organ donor, periorbital skin around the eye was cleaned with 70% isopropyl alcohol using sterile cotton and then with povidone-iodine, antiseptic solution. Following this a sterile eye drape was placed and 2 – 3 drops of povidone-iodine eye drop of concentration 0.5 w/v % (Apidine – 5), was applied onto the conjunctiva. The eye was left unopened for 3 – 5 minutes; excess of drop was wiped off using sterile gauze by closing the cadaver eyes once the exposure time was over.

Then the conjunctival swab were collected from the conjunctiva using sterile swab stick for bacterial and fungal culture and inoculated onto Brain Heart Infusion Broth (BHIB) medium provided by the eye bank at the bedside of the patient itself. All enucleation were done within 1 – 2 hours of natural death / brain death and all the cadaver eyes were transferred to eye bank within 2 – 4 hour of enucleation. Once enucleated cadaver eyes were received in the eye bank, the corneas were separated and transferred to the M- K medium 1 – 2 hours. The eyeball were usually collected from the donor within 6 hours after death and processed immediately as soon as it is received at clinical microbiology laboratory.

6.4.5. Processing of donor corneas and conjunctival swabs from Multi organ donor for bacterial and fungal culture:

The DCR and the conjunctival swabs were processed for bacterial and fungal culture by inoculation onto Brain Heart Infusion agar and a subsequent subculture onto Blood agar. The isolated organism was identified using standard biochemical tests (Folkens et al., 1996). A single colony of the bacterial isolates after completion of antibiotic susceptibility testing was picked up from 24 h old plate and stab cultured onto stock agar (Hyer's and Johnson agar), which was made in sterile cryovials and maintained at 4^0C. The organism were retrieved from the stock culture by sub culturing onto blood agar and incubated at 37^0C for 18-24 h. The antibiotic sensitivity was performed by Kirby-Bauer method as per the Clinical Laboratory Standard Institute (CLSI) guidelines on Mueller Hinton agar plates (CLSI 2006).

6.4.6. Antibiotic susceptibility testing:

Antibiotic susceptibility testing was carried against the following antibiotics for all the organisms isolated in donor cornea subjected for culture. They were: cefazolin, ciprofloxacin, moxifloxacin, norfloxacin, gentamicin, tobramycin, ofloxacin, gatifloxacin to all the isolates. All Gram positive bacteria were tested for vancomycin, penicillin. Along with that *Staphylococcus spp.* were screened for methicillin and oxacillin. Gram negative isolates were subjected for double disk synergy method of ESBL's detection by CLSI guidelines (CLSI 2006). Multidrug resistant isolates (i.e. Resistant to more than 5 antibiotics tested) alone were further subjected for MIC detection by Vitek 2- Biomerieux. Antibiotic discs were obtained from Hi-Media Laboratories Private Limited, India. The drugs were decided based on the general susceptibility of group of bacteria likely to be recovered from patients with

endophthalmitis following PK surgeries and as per the antibiotic policy of our hospital. The donors are not from diverse geographic area and they are only from Chennai, Tamil Nadu, India, therefore the same antibiotic spectrum is applied.

6.4.7. Genotypic analysis:

All gram negative bacterial isolates were subjected to uniplex PCR's for *AmpC* (Feria et al., 2002) *SHV* (M'Zali et al., 1996), *TEM* (Eckert et al., 2004), *OXA* (Feria et al., 2002), CTX-*M* genes (Johann et al., 2004) and Class I Integrons (Levesque et al., 1995). Non-ESBL *E. coli* ATCC 25922, *E. coli* ATCC 35218 strains and ESBL producing *K. pneumoniae* ATCC 700603 were used as controls. PCR targeting NDM-1 was designed using primer premier software5 and primegens with the consensus sequences of *blaNDM-1*. Primers were *NDM-1*-F 5'-TCTCGACATGCCGGGTTTCGG-3' & *NDM-1*-R 5'- ACCGAGATTGCCGAGC GACT-3' resulting in an amplified product of 475bp.

PCR's was standardized with DNA which was extracted using Bioneer kit, USA (Appendix 2). From the extracted DNA, 5µl was added to 45µl of PCR mixture consisting of 5µl buffer (10X buffer containing 15mM $Mgcl_2$) 200µM dNTPs, 20 picomoles of primers for their respective PCR, 30µl deionized water and 1.25U *Taq* polymerase. Additionally 1µl of 1:10 diluted formamide is added per reaction for *NDM* PCR. PCR thermal profile for *NDM* comprised of initial denaturation at 94°C for 10 min followed by 30 cycles of 1min denaturation at 94°C, 1min annealing at 60°C and 1min extension at 72°C, a final extension step of 10 min at 72°C and hold at 25°C in thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, Massachusetts, USA).

Specificity for all individual uniplex PCR's were tested with DNA extracted from bacterial, fungal, viral and human DNA. Analytical sensitivity of uniplex PCRs was determined using serial ten-fold dilutions of DNA.

6.4.8. DNA sequencing reaction:

Cycle sequencing reaction, consisted of 4µl of big dye terminator, 2 µl of DNA, 2 picomoles/µl of forward reverse primer, 3µl of deionized water. PCR profile consisted of denaturation at 96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min and final hold at 4°C. Products were purified according to standard protocol (Malathi et al., 2009), loaded onto ABI 3100 Genetic Analyzer (Applied Biosystems, USA.) with polymer POP7 and sequenced (Appendix 3). The sequences were analyzed using BIOEDIT, (downloaded from *http://www.mbio.ncsu.edu/BioEdit/bioedit.html*), and finally blasted with NCBI Blast website *http://blast.ncbi.nlm.nih.gov/Blast.cgi* to identify spp. and their corresponding homology.

6.5. Results:

Out of 76 DCR processed, in 30 (39.5%) specimens there was no bacterial and fungal growth was observed. In 46 (60.5%) specimens showed positive bacterial growth (Gram positive cocci – 36, Gram positive bacilli – 4, Enterobacteriaceae – 5 and Non-fermentors 1). In case of 42 conjunctival swabs collected from multi organ donors, all the 42 (100%) specimens showed positivity for bacterial culture (Gram positive cocci – 20, Gram positive bacilli – 4, *Enterobacteriaceae* – 17 and Non-fermentors 1). These conventional bacterial culture results are shown in table 6a.

Table 6a: The results of bacterial culture of microorganisms isolated from Donor Rim and Swabs from multi organ donor culture (n=118)

Types of bacteria	No. of DCR (%) received in 6 months	No. of Swabs from MOD (%) received in 6 months
No growth	30 (39.5%)	Sterile
Growth positive	**46/76 (60.5%)**	**42/42 (100%)**
Gram positive cocci	**N = 36 (47.3%)**	**N= 20 (47.6%)**
Coagulase Negative Staphylococci	32 (42.1%)	10 (23.8%)
Coagulase Positive Staphylococci	2 (2.6%)	0
Streptococcus spp.	2 (2.6%)	10 (23.8%)
Gram positive bacilli	**N= 4 (5.2%)**	**N= 4 (9.5%)**
Bacillus spp.	0	4 (9.5%)
Diptheroids	4 (5.2%)	0
Gram negative bacilli		
Enterobacteriaceae	5 (6.5%)	17 (40.4%)
Non fermentors	1 [#] (1.3%)	1 * (2.3%)
TOTAL	**76**	**42**

* *A. baumannii blaNDM-1* isolate, [#] *P. aeruginosa***;** Both are multidrug resistant strains.

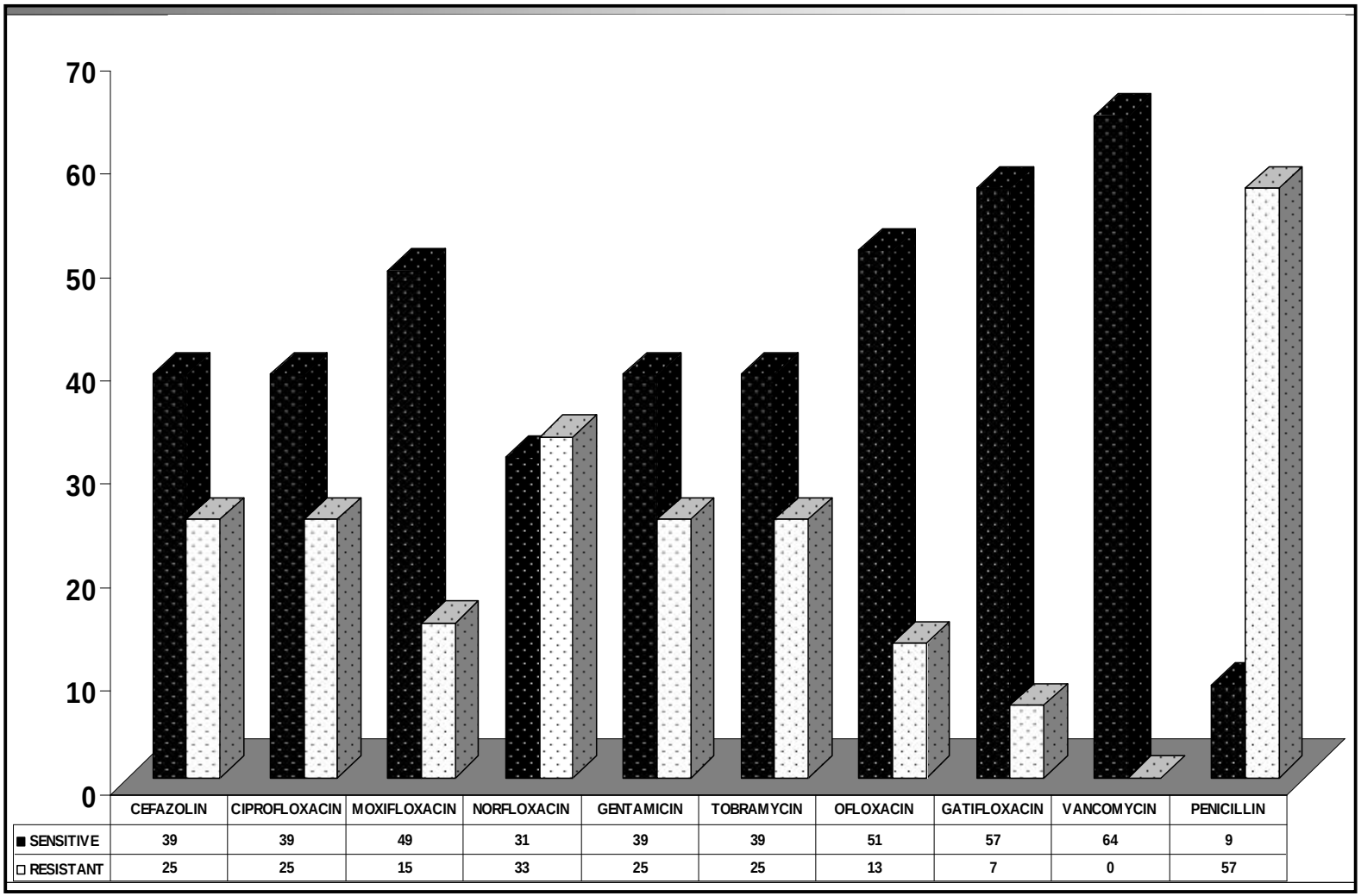
The rate of isolation of Gram positive and Gram negative bacteria among DCR and multiorgan donors group were compared. Maximum drug resistance was seen among MOD and the difference was found statistically significant (p=0.002). Similarly the rate of prevalence of

multidrug resistant isolates (i.e. resistant to more than 5 drugs) with that of sensitive isolates among DCR and multiorgan donors group were compared and was found statistically significant (p=0.002).

6.5.1. Results of antibiotic susceptibility testing:

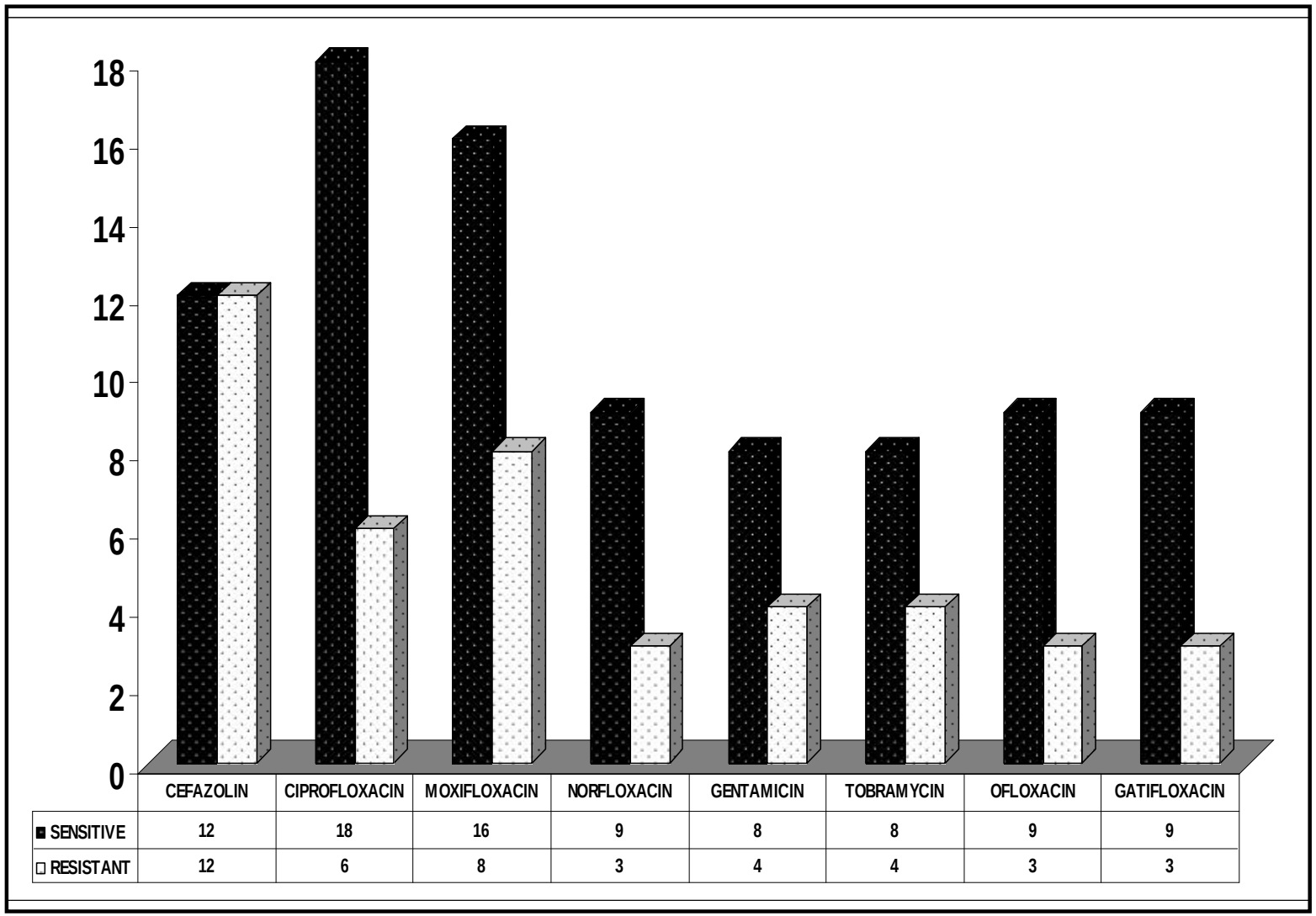
Eighty eight (74.5%) bacteria isolated from 118 specimens were included in this study. Antibiotic resistance pattern of 64 Gram positive organisms and 24 Gram negative bacteria is shown in the figure 6a and 6b. Among 64 Gram positive organisms, 57 (89.1%) were resistant to penicillin followed by 33 (51.5%) resistant to norfloxacin and 25 (30.1%) isolates were resistant to each cefazolin, ciprofloxacin, gentamicin and tobramycin is shown in figure 6a. All the 64 isolates were sensitive to vancomycin. Among 44 *Staphylococcus spp.* tested for methicillin and oxacillin resistance, 20 (45.4%) isolates were sensitive and 24 isolates showed resistance as shown in figure 6b.

In case of 24 Gram negative bacteria isolated in culture, 12 were resistant to cefazolin, followed by 8 isolates resistant to moxifloxacin. Seven (58.3%) out of 12 isolates were ESBL producer as detected by double disk synergy method of ESBL detection. Among the 2 multi drug resistant non-fermentor isolates studied, one was *Pseudomonas aeruginosa* isolated from DCR and another strain was *Acinetobacter baumannii* obtained from conjuntival swab of multi organ donor. We could not revive *P. aeruginosa* from stock culture. *A. baumannii* was subjected for the Vitek 2- Biomerieux method of antibiotic susceptibility testing for MIC determination and results are shown in the table 6b.



	CEFAZOLIN	CIPROFLOXACIN	MOXIFLOXACIN	NORFLOXACIN	GENTAMICIN	TOBRAMYCIN	OFLOXACIN	GATIFLOXACIN	VANCOMYCIN	PENICILLIN
■ SENSITIVE	39	39	49	31	39	39	51	57	64	9
□ RESISTANT	25	25	15	33	25	25	13	7	0	57

Figure 6a: Graph showing results of prevalence of drug resistance among Donor corneal rim (DCR)



	CEFAZOLIN	CIPROFLOXACIN	MOXIFLOXACIN	NORFLOXACIN	GENTAMICIN	TOBRAMYCIN	OFLOXACIN	GATIFLOXACIN
■ SENSITIVE	12	18	16	9	8	8	9	9
□ RESISTANT	12	6	8	3	4	4	3	3

Figure 6b: Graph showing results of prevalence of drug resistance among swab collected from Multi organ donor (MOD)

Table 6b: MIC results of as *Acinetobacter baumannii* by Vitek 2- Biomerieux method

Results of antimicrobial susceptibility tests of *A. baumannii blaNDM-1* isolate described in this report		
	A. baumannii blaNDM-1	
Antimicrobial agent	**MIC*, mg/L**	**Interpretation**
Ampicillin/sulbactum	>= 32	R
Ticarcillin	64	I
Piperacillin	>= 128	R
Piperacillin/Tazobactum	>= 128	R
Ceftazidime	>= 64	R
Ceftriaxone	>= 64	R
Cefoperazone/Sulbactum	32	I
Cefepime	>= 64	R
Imipenum	>= 16	R
Meropenum	>= 16	R
Gentamicin	2	S
Tobramycin	<= 1	S
Ciprofloxacin	<= 0.25	S
Levofloxacin	<= 0.12	S
Tetracycline	<= 1	S
Tigecycline	<= 0.5	S
Colistin	2	S
Trimethoprim/Sulfamethoxazole	<= 20	S

Note:

MIC = minimum inhibitory concentration, NDM-1 = New Delhi metallo-β-lactamase-1,

R = resistant, S= susceptible

* MICs results are based on antimicrobial susceptibility pattern by Vitek-2 automated susceptibility testing system (bioMérieux, France).

6.5.2. Genotypic analysis results:

Out of 7 ESBL producers, 4 strains that tested were positive for *CTX-M* was typed as *CTX-M -15* type, one *AmpC* positive isolate as *blaAmpc-EC7* type, 5 *OXA* positive isolates were typed as *blaOXA-1,* 4 *SHV* positive isolates were of *blaSHV-1* type and all seven positive *TEM* were of *blaTEM-1* by DNA sequencing. Integrons were found in 7 isolates out of 24 with amplicon sizes ranging from 1200- 2000 bp. Blast results of isolates showed 99 – 100 % homology. Genotypic results of *A. baumannii* showed the positivity for presence of *blaNDM-1* and *AmpC* rest genes were absent. Figure 6c shows the results of *NDM* PCR for *A. baumannii*. Results of PCR's were confirmed by DNA sequencing. Homology was found to be 100% when blasted and *blaNDM-1* sequence obtained was submitted to Genbank under accession no JF836807.

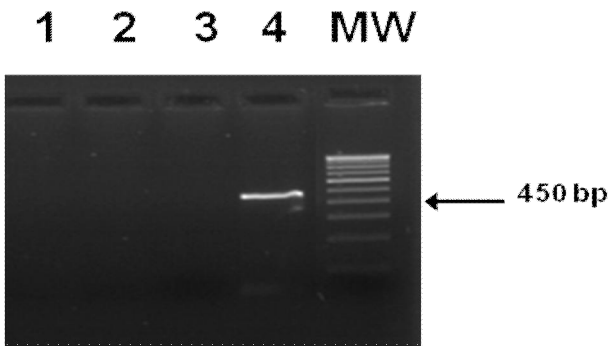


Figure 6c: Agarose gel electrophoretogram showing results of Uniplex PCR targeting NDM-1 gene on the *Acinetobacter* spp.. Lane 1: Negative Control**, Lane 2:** *Acinetobacter calcoaceticus* ATCC 9956**, Lane 3:** *Acinetobacter baumannii (lab isolate)* **Lane 4:** M/1326/2011 – *Acinetobacter baumannii***, MW:** Molecular weight marker (100bp ladder).

6.6. Discussion:

Exiguous amount data exists on resistance encountered in DCR isolates. Understanding the difference between the normal microbial flora and the resistant pathogen is imperative to prevent the emergence of endophthalmitis caused by these resistant bacteria due to PK.

There are few reports exist wherein the post-keratoplasty endophthalmitis was proven to be caused by resistant organisms from DCR. In our study conducted by Malathi et al., 2010, one case of post - keratoplasty endophthalmitis due to Methicillin resistant *Staphylococcus aureus* was reported. Another study by Lam et al., (1998) proved the Post - keratoplasty endophthalmitis by the organisms isolated from DCR and Oguido et al., 2011 proved a multidrug-resistant *Pseudomonas aeruginosa* causing endophthalmitis was identical with that isolated in culture.

Highest rate of isolation of microbes from the multi organ donor is due to their prolonged stay in the hospital before being reported brain dead for the donation of organs. These nosocomial infections are found in 5 to 15% (two million cases are estimated annually) of hospitalized patients and can lead to complication in 25 to 33% of those admitted in Intensive Care Unit (ICU). Studies conducted in hospitals in Delhi and Mumbai report figures as high as 30% (Syed et al., from The Hindu, 2007). Also the prevalence of resistance among the drugs were also high among multi organ donor compared to donor corneal rim cultures

In this study, the rate of isolation of microbes was 60.5% from DCR where as in case of multiorgan donor it was 100%. Majority of the microorganisms isolated in culture from multi organ donor eyes were resistant (90.4%) to more than five drugs compared to what was isolated from DCR (60.8%). Among bacteria isolated from multiorgan donors, highest rate of multi drug resistance were observed among Gram negative organisms (100%).

In a study on the value of routine donor corneal rim cultures in penetrating keratoplasty conducted by Wiffen *et al.,* 1997, the prevalence rate of *Staphylococcus* isolation was 12.1% and *Streptococcus* isolation was 2.1 %. In our study, rate of occurrence of *Staphylococcus* isolation and *Streptococcus* isolation in DCR is 44.7% and 2.6% respectively and in case of multi organ donor the isolation rate of *Staphylococcus* and *Streptococcus* are 23.8% and 23.8% respectively. Increased isolation of Gram positive cocci is followed by the Gram negative bacilli belonging to *Enterobacteriaceae* family, followed by Gram positive bacillus group and Non-fermentors group. *Bacillus,* which are known for its virulence properties were isolated from multi organ group (9.5%) and not from DCR specimens. Increased resistance was exhibited by Gram positive organisms than Gram negative isolates.

In case of conjunctival swabs collected from multiorgan donor, the cornea were used only if the culture was sterile or if the microorganism isolated from the swab were susceptible to minimum of four antibiotics (cefazolin, ciprofloxacin, moxifloxacin, norfloxacin, gentamicin, tobramycin, ofloxacin, and gatifloxacin) tested. All the

multiorgan donors were admitted in the hospital, almost in all cases due to road accident and the patients were kept ventilator before reporting as Brain death. The procedure of culturing swabs was followed for the all the multiorgan donor samples since the nosocomial infections are mostly hospital acquired. In order to prevent the spread of nosocomial infection in the hospitals culturing of swabs are practiced. Donor eyes with multidrug resistant isolates (i.e. Resistant to more than 5 antibiotics tested) were not used for transplantation. But in case of DCR, pre-excision culture was not followed.

In this study the multi-organ donor carrying *Acinetobacter baumannii* was on the ventilator in ICU, which is one of the most predominant ways of transfer of resistance (Abbo et al., 2005, Abbo et al., 2007). Here it was the hospital rather than the community which play leading role in transmission of these multidrug resistant genes. The first clue to the presence of a carbapenemase comes from the increased minimum inhibitory concentration (MIC) especially to ertapenem, imipenem, or meropenem. Treatment of infections caused by pathogens producing carbapenemases, including *blaNDM-1*, poses a serious challenge as these infections are resistant to all commonly used antibiotics (Krishna et al., 2010).

Culturing of DCR was mainly performed to correlate the results of microbiological investigations in case of occurrence of the post PK endophthalmitis. Also antibiotic susceptibility results are available within 48 – 72 hours, will further aid is detecting the most appropriate antibiotic agent that can be used if post PK endophthalmitis develops. Thus there is no delay in the availability of tissue by adding

several days by waiting for culture results. All the corneas are maintained in M-K medium and they are usually used within 4 – 5 days. The bacterial culture of DCR is reported within 48 hours from the time of processing and the DCR will be further incubated for a total of 12 days before reporting fungal culture negative. In our previous study conducted by Malathi *et al.,* 2010, in a total period of 8 years, 10 postoperative endophthalmitis (0.5%) has occurred after penetrating keratoplasty (PK). One out of 10 postoperative endophthalmitis was due to fungus. Fungus causing postoperative endophthalmitis is were rare in our step and the cultivation of DCR and the conjunctival swabs were processed for bacterial and fungal culture by inoculation onto Brain Heart Infusion agar and a subsequent subculture onto Blood agar is sufficient for the isolation of both fungus and bacteria.

In our 8 year study conducted by Malathi *et al*., 2010, 10 postoperative endophthalmitis (0.5%) occurred after penetrating keratoplasty (PK). The infective agents isolated from 10 patients who had postoperative infections following PK included *Enterococcus faecalis*-3, *Pseudomonas aeruginosa*-2, Methicillin-resistant *Staphylococcus aureus* (MRSA)-1, *Alkaligenes faecalis*-1, *Aspergillus flavus*-1, *Klebsiella pneumoniae*-1 and *Pseudomonas stutzeri*-1. Among these 10, the source of infection could be established as donor corneal scleral rims in six (60%) patients since they showed growth of *E. faecalis*-2, *P. aeruginosa*-2, MRSA-1 and *K. pneumoniae*-1, with the phenotypic characteristics identical with the corresponding bacterium isolated from the diagnostic intra-ocular specimens collected from the respective patients.

Aaberg et al., (1998) has showed a higher incidence of infection in patients with penetrating keratoplasty may be caused by contaminated donor corneal buttons, a risk factor unique to penetrating keratoplasty. If in all these cases the antibiotics susceptibility results were similar to the antibiotics susceptibility results of organism grown DCR and therefore the probability of transmission of the resistant pathogens could have been avoided.

In our study the use of povidone – iodine and ciprofloxacin has reduce the development of post PK endophthalmitis among all the cases out of 46 DCR specimens that were positive for the presence of bacteria except in 1 case (2.1%) were post PK endophthalmitis has occurred due to corneal transplant. In case of multiorgan donor out of 42 cases that were positive for the presence of bacteria only 4 corneas that had *Coagulase Negative Staphylococci* which was resistant to methicillin and sensitive to rest of the drug used were only used from the corneal transplant and rest of the cornea containing multi drug resistant bacteria were not used for the transplant. Similarly if the organism is going to be multi drug resistant according different group of antibiotics needs to be selected.

Jeremy et al., (1995) has reported that ciprofloxacin eye drops could be used as an empirical treatment for suspected bacterial keratitis since it has higher degree of potency and superior penetrating capacity. Therefore ciprofloxacin is chosen to reduce the microbial load over the donor eye ball from the time of enucleation till the eye ball is transferred to the M-K medium. Kill times are likely too long to be of much use with a

single application of antibiotic, ciprofloxacin. But we still practice the use of single antibiotic to prevent the development and spread of resistance genes. Another reason behind the use of single antibiotic is as per the suggestions of our hospital policy in the transplantation of cornea.

The most widely reported normal flora of the eye in the Indian scenario is Gram positive bacteria which includes Coagulase *negative Staphylococci*, *Streptococcus pneumonia* followed by equal proportion of Gram negative bacilli predominantly non-fermentor group i.e. *P. aeruginosa*, *P. stutzeri*, *Alkaligenes faecalis* and *Acinetobacter* spp and *Enterobacteriaceae* family predominantly *Klebsiella* spp. Except with the genus *Streptococci,* for other group of bacteria ciprofloxacin had wide range of activity and hence used as broad spectrum antibiotic.

Panda et al., 1988 have reported 23 years back about the microbial contamination of DCR. In the present scenario, the eye balls are being treated with povidone-iodine, ciprofloxacin and transported in sterile container to the eye bank. Further the eye is preserved in MK medium which also contains antibiotics. Therefore the organisms isolated may not be called as contaminants in case of DCRs. Rather they are the resident flora of the conjunctiva of the donors. Also the microbial contamination is not possible as the swabs from multiorgan eye are collected under dedicated safety cabin and immediately processed for isolation of bacteria and fungi. The DCR removed during PK procedure is placed back inside the MK medium without touching the rim of the bottle and transported within 15 hours to the laboratory.

Wilhelmus et al., (2007), have reported that the risk of endophthalmitis after keratoplasty has previously averaged 0.2%, 1 a positive donor rim would raise the probability of endophthalmitis to 1%. Thus endophthalmitis after penetrating keratoplasty is more likely with a culture-positive donor rim.

This is the first study where NDM-1 carrying *A. baumannii* was detected from ocular specimen (DCR) among Indian population. Cornea from which the NDM-1 carrying *A. baumannii* was isolated was not used for the transplant which was possible only due to pre-excision culture. However, one case of endophthalmitis developed on transplantation of a positive donor rim culture. The organisms isolated from the donor culture and from the endophthalmitis case are same. The most important fact is the bacterium was multidrug resistant. Prior culture of corneal specimens would have prevented from causing endophthalmitis.

Thus proper and rapid screening of these resistant bacteria by routine conventional method of antibiotic susceptibility testing by Kirby Bauer method against drug which are used in the treatment of ocular infections and proper intimation to the surgeon who performed the transplant will aid is rapid use of appropriate antibiotic and thus will play a important role in prevention of infectious endophthalmitis in patients.

6.7. Conclusion:

To conclude thus positive pre-excision culture of multiorgan donor will help in prevention of development of infection. Therefore our contention of culturing preoperatively the conjunctival swabs from multi organ donor and DCR will be helpful in saving eye sight even in a single patient is still useful technique. This is the first time

molecular typing has been carried out to know the most prevalent class of drug resistance present among eye donors. Though this may not be possible to carry out in a routine clinical set up, part of the present work carried out as research showed that prevalent genotypes detected among eye donors were similar to what was reported among other clinical isolates of ESBLs from other sites. Molecular detection of susceptibility testing will aid in significant increase in turnaround time for the detection and identification of drug resistance pathogens and control the spread of these bacteria.

6.8. Future prospects:

The points of practice given below may be followed in present and future PK for a better eye care.

i. Povidone-iodine usage prior removing the donor eye.

ii. Better storage media with antibiotic coverage will reduce the microbial contamination of the donor eye.

iii. Use of fluroquinolones pre and post operatively will contribute significantly in decreasing the infection rate.

iv. However, in case of an infection following PK, donor rim cultures results and antibiotic sensitivity will enable accurate identification of the cause of infection and to initiate treatment.

v. Besides the usual protocol for asepsis, in these recipient eyes, there is a need for closer and frequent follow-ups postoperatively.

Chapter - 7

Analysis New Delhi Metallo-Beta-Lactamse-1 structure producing *Acinetobacter baumannii* isolated from Donor Swab in a tertiary eye care centre, India and structural analysis of its antibiotic binding interactions

7.1. Aim:

To analyze the *insilico* drug binding interactions and structural analysis of the New Delhi Metallo-Beta-Lactamse-1 producing *Acinetobacter baumannii* isolated from Donor Swab in a tertiary eye care centre, India.

7.2. Review of literature:

7.6.2.1. New Delhi Metallo-Beta-Lactamases (NDM):

Antibiotic-resistant Gram-negative bacilli belonging to *Enterobacteriaceae* and *Non-fermentors* are the major cause of concern in clinical problems in current human healthcare (Féria et al., 2002, Yong et al., 2009). Beta-lactams have been the mainstay of treatment for serious infections in humans (Paterson et al., 2006). Among Gram-negative bacteria, the most common beta-lactam resistance mechanism involves beta-lactamase mediated hydrolysis which results in inactivation of antibiotics which are used in the treatment of infection caused by these organisms (Sekizuka et al., 2006).

One of the last lines of treatment against high level drug resistant infections is the carbapenems and are metallo-beta-lactams (MBL) class of antibiotics, which was developed to resist the beta-lactamase mediated resistance posed by infection causing

microbes (Kim et al., 2011, Wang et al., 2011). Recent studies has proved the inactivation of these carbapenams (meropenem, imipenem, doripenem, ertapenem) by a number of metallo-beta-lactamases which poses an extended spectrum activity against all beta-lactam antibiotics, including carbapenems (Kim et al., 2011, Wang et al., 2011, Struelens et al., 2010).

One such highly emerging dreadful MBL's is New Delhi metallo-beta-lactamase (*blaNDM-1*). This *blaNDM-1* is a novel MBL's conferring resistance to almost all beta-lactam antibiotics, including carbapenem (Rolain et al., 2010, Diene et al., 2011, Poirel et al., 2010, Kumarasamy et al., 2011). Many reports are emerging on the existence and prevalence of *blaNDM-1* throughout the world (Paterson et al., 2006, Sekizuka et al., 2006, Struelens et al., 2010, Chen et al., 2011). Microorganisms carrying *blaNDM-1* gene are *E. coli, K. pneumoniae, K. oxytoca, Enterobacter cloacae, Proteus spp., Citrobacter freundii, Morganella morganii, Providencia spp. Pseudomonas aeroginosa* (Yong et al., 2009, Paterson et al., 2006, Rolain et al., 2010) and also in *Acinetobacter baumannii* (Chen et al., 2011, Nemec et al., 2008).

Presence of *blaNDM-1* gene was also detected among bacteria isolated from seepage samples and public tap waters in New Delhi, India (Poirel et al., 2011). Many reports have revealed the spread of *blaNDM-1* through renal or bone marrow transplantation, dialysis, cerebral infarction, chronic obstructive pulmonary disease, pregnancy, burns, road traffic accidents, and cosmetic surgery (Paterson et al., 2006) Hence, the microorganism carrying the *blaNDM-1* gene will be extensively resistant to

antibiotics and susceptible only to colistin and, less consistently, tigecycline, leading to scarcity of antibiotics for treatment (Rolain et al., 2010, Kumarasamy et al., 2011).

7.2.2. *Acinetobacter baumannii* and NDM:

Acinetobacter baumannii (*A. baumannii*) is a common nosocomial pathogen reported worldwide (Maragakis et al., 2008). Infections caused by *A. baumannii* are associated with adverse clinical outcomes, including high rates of morbidity and mortality, prolonged hospital stay, and substantial health care expenses. Many reports on emergence of *blaNDM-1* among *A. baumannii* have been reported (Nemec et al., 2008, Murali et al., 2008). In our previous study, we have reported the isolation of *blaNDM -1* carrying *A. baumannii* from a multiorgan donor with high level drug resistance (Kaase et al., 2011).

7.2.3. Need of the study:

Though reports on the three dimensional structures of *blaNDM-1*, active site regions, molecular docking and ligands complex molecular dynamic simulation studies in different microbial pathogenic organisms synthesis different protein length of *blaNDM-1* exist, in this study we aimed to correlate the microbiological investigations carried out to screen its level of drug resistance with that of *in silico* studies of drug interactions using bioinformatics tools.

7.3. Objective:

To analyze the *in silico* drug binding interactions and structural analysis of the New Delhi Metallo-Beta-Lactamse-1 producing *Acinetobacter baumannii* isolated from Donor Swab in a tertiary eye care centre, India.

1. To analyze biofilm producing ability of a New Delhi Metallo-Beta-Lactamse-1 gene positive *A. baumannii* isolated from Donor Swab.
2. To study the viability of the biofilm producing bacteria after antibacterial treatment by MTT ((3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.
3. To detect the transfer of drug resistance via conjugation experiments
4. To *in silico* characterize the NDM structure by molecular dynamic studies and by docking tests.
5. To correlate the microbiological investigations carried out to screen its level of drug resistance with that of *in silico* studies of drug interactions using bioinformatics tools.

7.4. Materials and Methods:

A. baumanii, isolated from a conjunctival swab collected from a 23 years old multi organ donor, died in a road traffic accident was utilized for this study, after validation through documented bacteriological methods (Folkens et al., 1996). Further, the isolate was screened for the presence of *blaNDM-1* at clinical microbiology laboratory of L & T Microbiology Research Centre from Sankara Nethralaya, Chennai.

The bacterium was found to harbor the *blaNDM-1* by PCR based DNA technique and was further sequenced for confirmation. The sequence of *blaNDM-1* gene obtained was submitted to Genbank and the accession no is JF836807.

7.4.1. Biofilm production detection:

7.4.1.1. Detection of Biofilm production by Tissue culture plate method:

Biofilm production of *A. baumanii* isolate was carried out aerobically in 96 well round bottom tissue culture plate as described earlier (Mathur et al., 2006, Coenye et al., 2007) with slight modifications. The adherent biofilm producing cells was released by adding 160 ml 33% acetic acid (SRL, India). The sterile uninoculated brain heart infusion broth media served as medium control to check sterility. Carbapenem susceptible *K.pneumoniae* ATCC BAA-1706 and carbapenem resistant *K. pneumoniae* ATCC BAA-1705 served as the inoculum control.

Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader (ELX808, Biotek, India) at a wavelength of 520 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

The experiment was performed in triplicate and the average was derived. To compensate the background absorbance, OD readings from sterile medium, fixative, dye were averaged and subtracted from all the test values. The mean OD value obtained from media control well was deducted from all the test OD values.

7.4.1.2. Interpretation of bacterial adherence:

Bacterial adherence was interpreted based on OD values as obtained for *A.baumanii* isolate (Mathur et al., 2006). Mean OD values derived is shown in table 7a

Table 7a: The interpretation of bacterial adherence

Mean OD values	Adherence	Biofilm formation
<0.120	**Non**	**Non / weak**
0.120-0.240	**Moderate**	**Moderate**
>0.240	**Strong**	**High**

7.4.2. Detection of viability of biofilm producing bacteria after antibacterial treatment:

a. Production of biofilm:

The bacterial isolate was allowed to produce biofilm by inoculating 200µl of 1:10 diluted *A. baumanii* isolate onto three 96 well round bottom tissue culture plate and incubated aerobically at 37^0C for 24 h as described earlier (Mathur et al., 2006, Coenye et al., 2007). After 24 h of growth, the biofilm production was checked out in one of the plate by procedure described above.

b. Antibacterial treatment of Biofilm:

After confirming the production of biofilm, the remaining plates were subjected for antimicrobial susceptibility testing by microbroth dilution method. The medium was first removed by aspiration from the wells and subjected for antibiotic treatment with ceftriaxone (Alkem, India), cefepime (Zuventus, India), cefoperazone (Pfizer, India), imepenum (Ranbaxy, India) and meropenum (Blue cross, India). The bacteria were then

treated with various concentrations of the antibiotics ranging from 1280mg/L – 10mg/L and incubated for 24 hours at 37°C (carried out in duplicates). After overnight incubation, the medium containing the antimicrobial agent was gently aspirated out.

c. Assessment of cell morphology by pseudo- confocal microscope:

The reduction in the amount of bacterial cell count was observed under Axio Observer fluorescent microscope (Carl Zeiss, Berlin, Germany). The images were processed using Axio Vision 4.7 software (Carl Zeiss, Bangalore, India).

d. MTT assay:

Following this, the viability of the bacteria in each of the antibiotic concentration was tested by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Invitrogen, India) assay according to the method described by Kairo et al., 1999. Briefly, after 24 h lasting antibiotic treatment, the wells of micro plate were aspirated out and filled with 150 µl of PBS per well, then 50µl of MTT solution (0.3% in PBS) was added. Plates were incubated for 2 h at 37°C. At the end of incubation period, MTT was replaced with 150 µl of DMSO (Merck, India). To enable complete dissolving of formed purple formazan crystals, the plates were incubated for 15 minutes at room temperature followed by a gentle agitation for 5 min. The optical density of the wells was calculated through micro ELISA auto reader at wavelength of 550 nm.

Cell viability (or cell survival) was calculated as: (Test OD/Control OD) ×100. The 50% inhibitory concentration (IC50) of the five drugs (ceftriaxone, cefepime,

cefoperazone, imepenum and meropenum) was computed using polynomial regression analysis using Microsoft Excel.

7.4.3. Conjugation experiment:

To obtain the transconjugant, 0.2 ml of an exponentially grown culture of the test strain *A. baumanii* (donor cells) was mixed with 1.8 ml of an overnight culture of *E. coli* XL-1 Blue (recipient cells) in Luria Bertani broth (LB broth) (Hi media, India). Mixtures were incubated without shaking at 37°C for 18 h. Transconjugants were selected by their ability to resist streptomycin 65µg/ml (Oxoid), tetracycline 100µg/ml (Merck, India) and cefotaxime 2µg/ml (Himedia, India) incorporated in Luria Bertani agar (LB agar) (Cartelle et al., 2006, Is Hamad et al., 2008). The transconjugants were preceded further for the antibiotic susceptibility testing with the same antibiotic which was used for testing parental isolates.

7.4.4. Sequence Retrieval:

The Protein sequence for *blaNDM-1* in *Acinetobacter baumannii* (UniProtKB accession number: F8UNN7) was retrieved from UniProtKB (http://www.uniprot.org/). BLASTP search was carried out for this protein against the Protein Data Bank (PDB) (http://www.rscb.org/pdb/) to detect the most suitable template for modeling, wherein, *E. coli blaNDM-1* crystal structure (PDB id: 3S0Z) was found to have 100% identity. So, the PDB structure 3S0Z was retrieved from the Protein Data Bank and the same was used for structural studies. However, the crystal structure had a missing loop region (167-170) which was modeled and validated by different structural assessment studies (Kiefer et al., 2009).

7.4.5. Structure validation:-

The crystal Structure (3SOZ) was fixed for missing loop, and was examined by using Q-MEAN SERVER, which estimates quality of a model, whereby, it gives two different scores: Global score, which includes Q-Mean Score which is a global score of the whole model reflecting predicted model reliability ranging from 0 to 1, Z-Score which is calculated by relating Q-Mean score to scores of a non-redundant set of high-resolution x-rays structures of similar size. Local Score includes Residue Error which was estimated per residue (Benkert et al., 2009). Ramachandran plot validation was also performed using Structure Analysis & Verification (SAVES) server (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/refs.html).

7.4.6. Molecular Dynamics (MD) Studies of *blaNDM-1* protein:-

MD Simulation was performed for the modeled protein on OPEN DISCOVERY Linux Platform with pre-installed GROMACS software (Umashankar et al., 2008) to analyze the stability of the protein. GROMOS96 43al (Hess et al., 2008) force field was used. Periodic boundary conditions were applied with 0.9nm of cubic box. The protein was solvated with SPC water model which added 11619 SOL molecules to the system. System was neutralized by replacing water molecules with 10 NA+ counter ions. Steepest Descent algorithm was used for energy minimization which converged in 283 steps. Equilibration of the NVT and NPT ensembles was conducted separately each with 100ps and with 2 fs of integration time steps. Final production run was performed for 5ns. Finally, the decoy with least potential energy was chosen for further studies.

7.4.7. Prediction of Active site:-

Binding pocket for target protein was predicted using computed atlas of surface topography of proteins (CASTP) (Liang et al., 1998). Further, all the binding pockets residues were compared with documented active site residues protein (Guo et al., 2012). Binding Pocket from CASTP was selected which covers all the active site residues of the protein. Finally, this binding pocket was used for further docking studies.

7.4.8. Retrieval of Ligands:-

The structural coordinates of the ligands were retrieved from Ligand Depot, Pubchem and Drug Bank based on availability. The ligands are Ceftriaxone (Pub Chem: CID 5479530); Cefepime (Pub Chem: CID 5479537); and Cefoperazone (Pub Chem: CID 44185) Carbapenems which includes Imipenum (Drug Bank: DB01598) and Meropenum (PDB ID: 3q82); Optimisation of all the ligands were done in ProDrg server (Schuttelkopf et al., 2004).

7.4.9. Molecular Docking Studies:-

Auto Dock 4.0 (Huey et al., 2007) was used for carrying out docking studies of *blaNDM-1* protein with all the five different types of ligands. Semi-flexible docking was done in which protein was kept as rigid and the ligands as flexible (Deepa et al., 2010). For all the ligands, the possible torsion angles were set to rotate freely. In the preparation of protein, polar hydrogens were added and kollman united partial charges were also assigned. Gasteiger charges were also assigned to the ligands. Auto Dock 4.0 was compiled and run under Linux operating system. Auto Dock requires pre assigned grid

maps, and grid must surround the region of interest in the protein. In this study, binding site residues were generated by CASTp server, which includes all the active site residues were used for setting the grid. The grid box size was set at 100, 100 and 100 Å (x, y and z) and grid center 3.0, 3.0, 3.0 for x, y and z- coordinates for all ligand molecules. The spacing between grid point was 0.375 Å for active site in such a way to surround the entire given binding site residues.

The Lamarckian Genetic Algorithm parameter was used for the best docking conformers. The following parameters were followed for all the compounds. A maximum of 10 conformers were considered for each compound, the population size was 150, maximum number of evaluation was set to 2,500,000, maximum number of generations was 27,000, rate of gene mutation was 0.02, rate of crossover was 0.80, GA crossover mode two pt (two point), mean of Cauchy distribution for gene mutation was 0.0, variance of Cauchy distribution for gene mutation was 10.0, and number of generation for picking worst individual was set to 10.

7.5. Results:

7.5.1. Results of *in vitro* biofilm production:

The isolate from the donor corneal rim specimen produced high level of biofilm with an OD of 0.361 at an absorbance of 520nm. Biofilm produced by the *A. baumannii* isolate is shown in figure 7a.

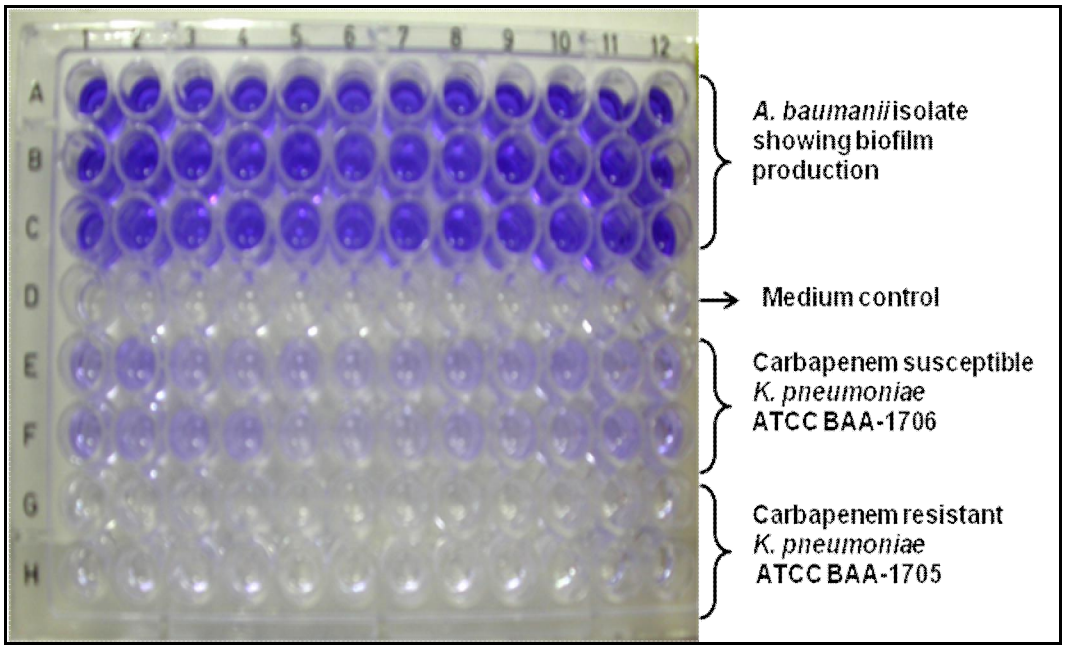


Figure 7a: 96 well plate showing Biofilm production by *A. baumanii* isolate. Crystal violet staining results of *blaNDM-1* producing *A. baumannii* isolate along with the control strains and medium controls are shown.

7.5.2. Results of viability of biofilm producing bacteria after antibacterial treatment:

The viability of biofilm producing *A. baumannii* isolate reduced on exposure to antibiotics and was inferred by reduction in the bacterial cell count after antibiotic treatment. (Observed through Axio Observer fluorescent microscope (Carl Zeiss, Berlin, Germany). The degree of biofilm formation and percent of viability of bacterial cells after antibiotic treatment were analyzed by MTT staining. Higher degree of resistance was observed for all 5 drugs (ceftriaxone, cefepime, cefoperazone, imepenum and meropenum) after 24 hours of antibiotic treatment. The IC_{50} of ceftriaxone, cefepime, cefoperazone, imepenum and meropenum to be 80 mg/L, 80 mg/L, 80 mg/L, 80 mg/L,

and 80 mg/L, respectively. Effect of imepenum on *A. baumannii* at various concentrations is shown in figure 7b.

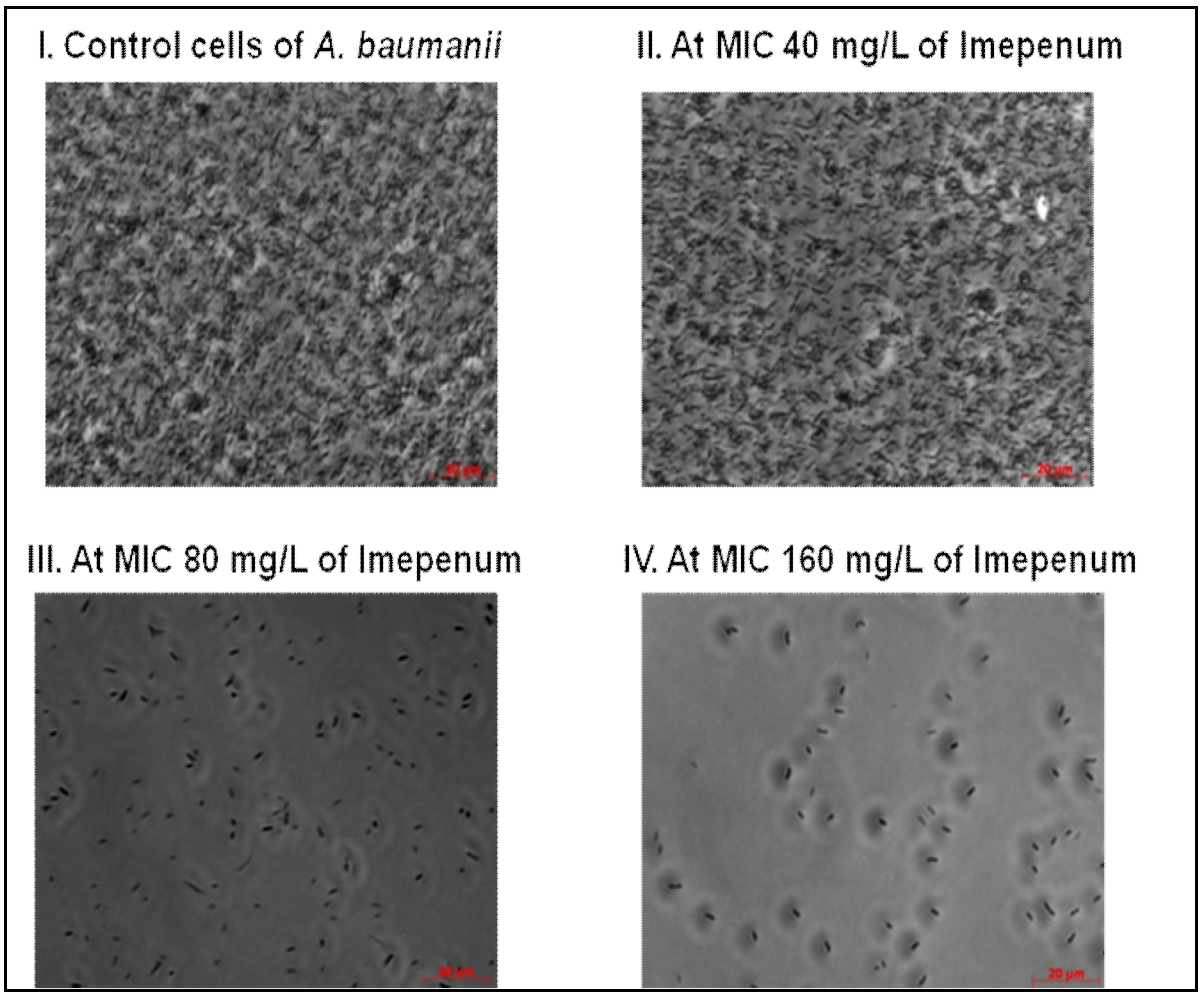


Figure 7b: Morphology of *A. baumannii* cells at various concentrations of imepenum are seen under Axio Observer fluorescent microscope.

I. Control,

II. At an imepenum concentration of 40 mg/L,

III. At an imepenum concentration of 80 mg/L

IV. At an imepenum concentration of 160 mg/L.

7.5.3. Sensitivity of *A. baumannii* cells to increasing concentrations of antibiotics:

Figure 7c presents the percentage viability of *A. baumannii* at various concentrations of antibiotics i.e. ceftriaxone, cefepime, cefoperazone, imepenum and meropenum at increasing dosages higher than their respective IC_{50} at 24 h of treatment. However, at highest dosage, of all five antibiotics at 1280mg/L significant decrease in the cell viability was not observed.

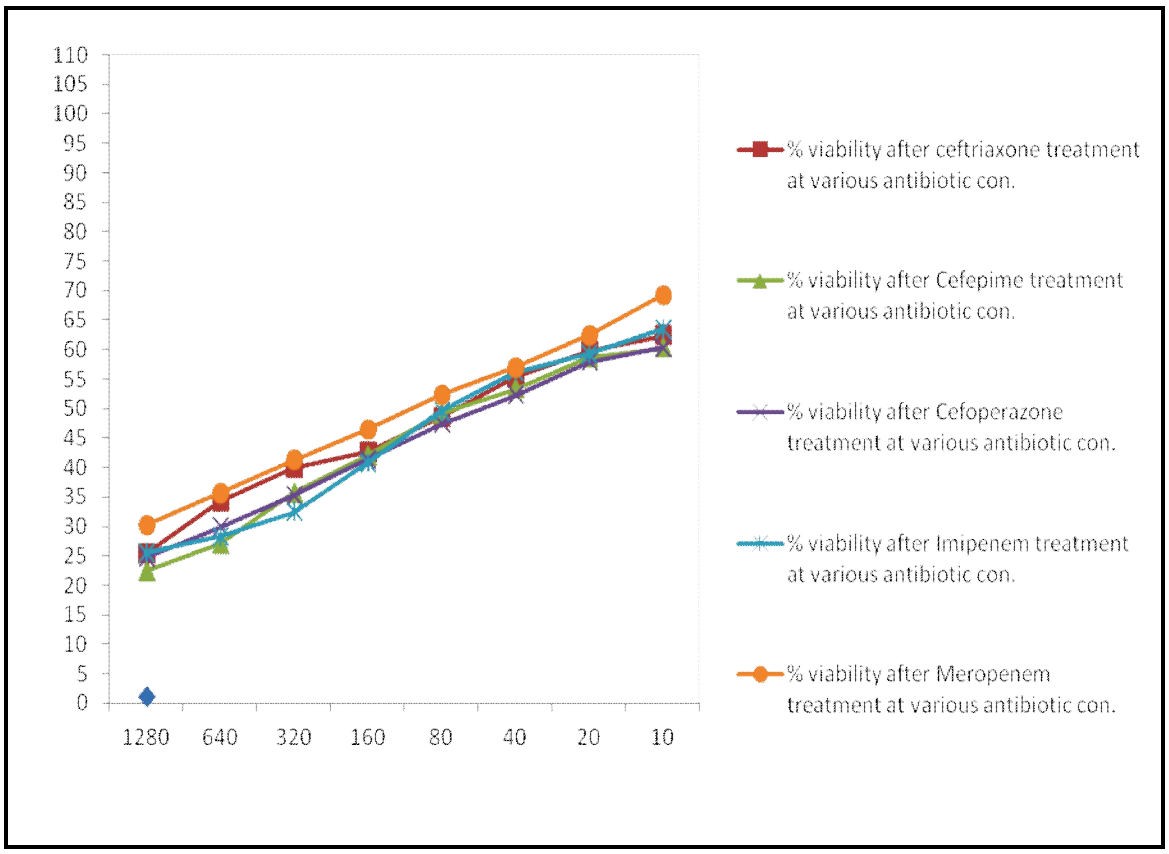


Figure 7c: Line graph representing percentage of viability of *A. baumannii* cells to increasing concentrations of antibiotics.

7.5.4. Results of conjugation experiment:

Successful transconjugation was noted with the *A. baumannii* isolate that harbored *blaNDM-1* gene. The transfer of *blaNDM-1* gene to the *E. coli* XL-1 Blue was confirmed by both PCR and MIC testing. PCR targeting *blaNDM-1* proved that the transconjugants carried the *blaNDM-1* gene and MIC results showed similar antibiotic results when compared to the parental donor strains tested. Transconjugants grown in LB medium incorporated with antibiotics is shown in figure 7d.

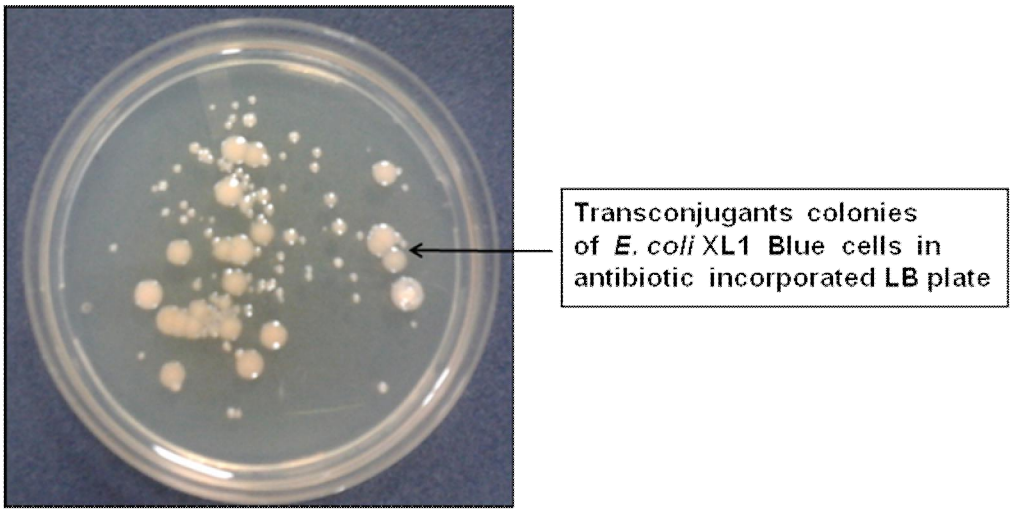


Figure 7d: Results of Conjugation experiment. LB agar containing antibiotics showing the growth of transconjugants confirming the ability of transferring genetic material carrying drug resistance genes to competent cells.

7.5.5. Molecular modeling:

As discussed earlier, the missing residues (167-170) (Figure 7e. I) of 3SOZ_A (Young et al., 2007) was modeled using Swiss model beta server with advanced modeling option with 3SOZ_A as template. The modeled structure was refined, validated and was utilized for further docking studies.

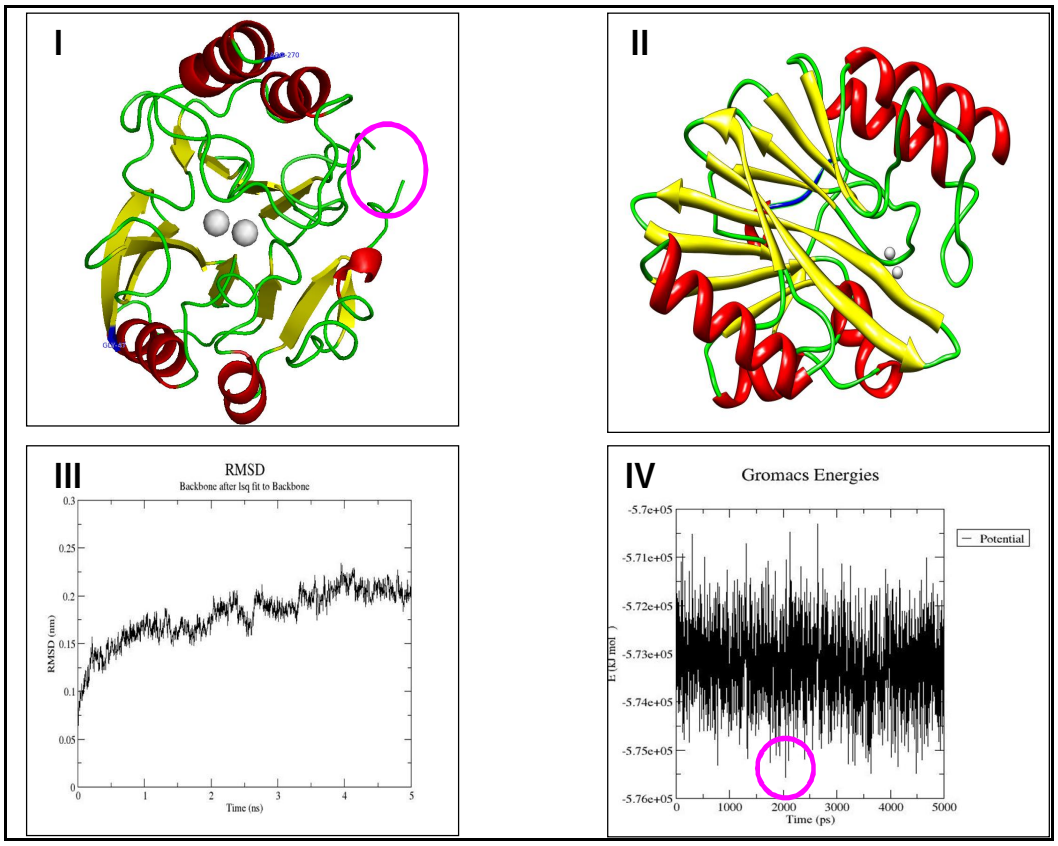


Figure 7e:

- Figure 7e. I: Crystallized 3D structure of *blaNDM-1* in *E. coli*. Magenta color circle show missing loop region in this structure.

- Figure 7e. II: 3D Structure of *blaNDM-1*. In this figure red color indicate helix, yellow color indicate beta strand, green color indicate coil, blue color indicate missing loop region of template structure using USCF - CHIMERA.

- Figure7e. III: C-alpha Backbone residue-wise root mean square deviation (RMSD) of *blaNDM-1* at 5ns.

- Figure 7e. IV: Potential energy graph over 5ns. The lowest potential energy was noted at 2000ps, marked with a Magenta circle.

7.5.6. Structure validation:

The loop fixed model of *blaNDM-1* was subjected to further structural validation through Q-mean server. This server predicted a structure score of 0.68. The PROCHECK analysis shows 92.1% residues in favored regions, 7.9% in allowed region and 0% in disallowed region. The final refined structure of *blaNDM-1* was used for further molecular dynamics simulation studies. (Figure 7e. II).

7.5.7. Molecular dynamics studies:

Molecular Dynamics simulation for 5 nanoseconds revealed the conformational changes and stability of the protein in response to optimal ensemble conditions as shown in Figure 7e. III. The Potential energy traces a reliable drop in value over 5ns of MD simulation, with the lowest potential energy structure observed at 2000ps as shown in Figure 7e. IV.

7.5.8. Active site prediction:

Active site pocket identification was performed using CASTP Server, which predicted 26 different pockets with varied Area and Volume. Each pocket conformation presents residues that matched with the structure of *blaNDM-1* in *E. coli* (PDB id: 3SOZ_A) (Guo et al., 2012). Overall documented active site residues of *blaNDM-1* were corresponding to the 26^{th} pocket (Met21, Phe24, Val27, Ala28, Trp47, His74, His76, Asp78, Leu102, Glu106, His143, Thr146, Gly161, Cys162, Ile164, Lys165, Asp166, Lys168, Ala169, Lys170, Ser171, Leu172, Asp177, Tyr183, Met202, Ser203, His 204, and Ser205) as shown in figure 7f.

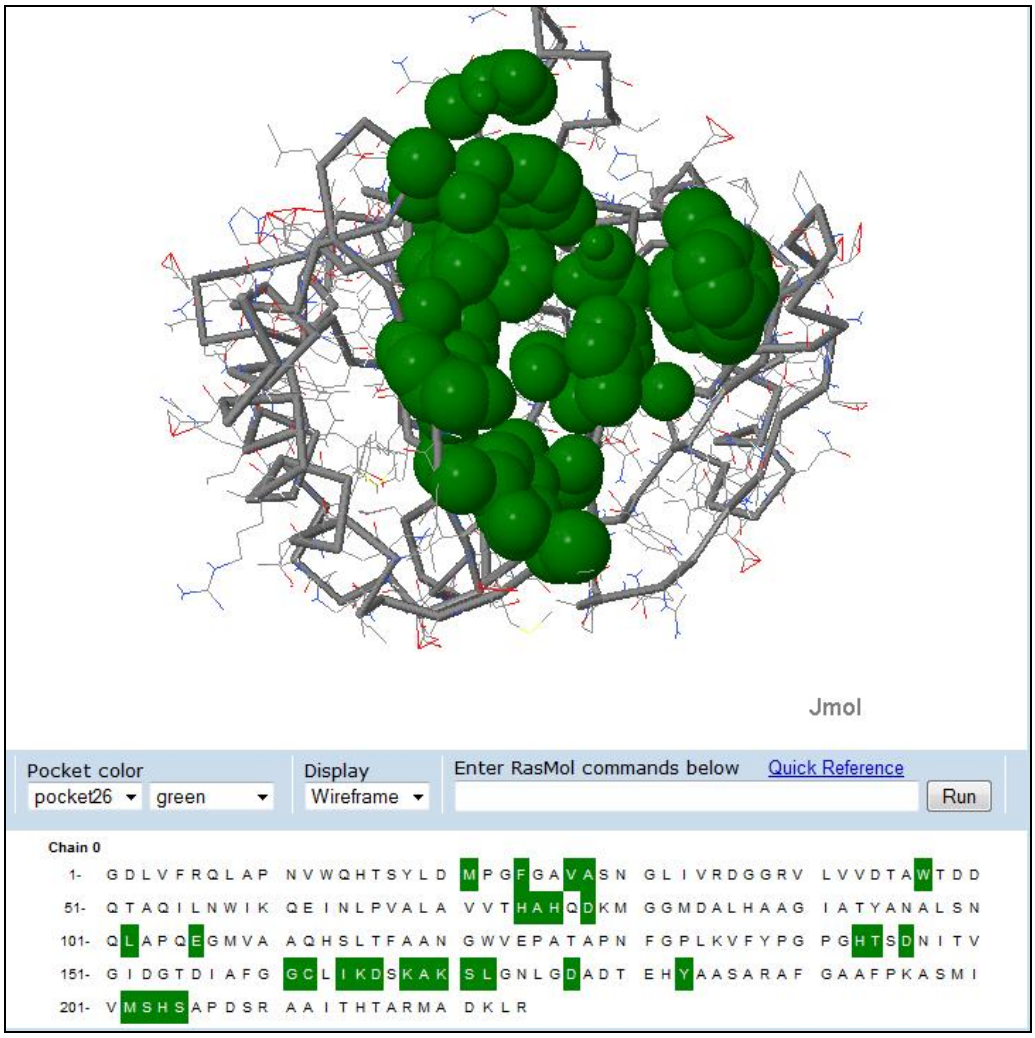


Figure 7f: Active site pocket of *blaNDM-1*. Wire frame illustrated in overall protein and Green color ball illustrated in active site residues.

7.5.9. Ligand optimization:

All the ligands were retrieved from Ligand Depot, Pubchem and Drug Bank based on availability. These ligands were minimization using for using GROMOS forcefield option of PRODRG Server and was further used of docking studies.

7.5.10. Docking studies of *blaNDM-1*:

Docking studies of the chosen ligands were performed on the predicted active site region of *blaNDM-1*. The binding energy of all the interactions reveal that, among all the ligands, the highest binding affinity of (-6.17Kcal/mol) was observed for Cefoperazone. The detailed hydrogen bonding interactions and inhibitory constant value of *blaNDM-1* with all the ligands are shown in Table 7b and Figure 7g, respectively. The docking studies show hydrophobic interactions, and hydrogen bonds, which very well correlated with the documented studies.

Table 7b: The docking results of docking interactions and IC50 calculation.

Ligands	Binding Energy (Kcal/mol)	No. of H - bonds	H- BONDS	IC_{50} of Biofilm MTT Assay (mg/L)	Experimental IC_{50} (µM)
Ceftriaxone	**-5.68**	**3**	**LYS 165, SER 203, ASP 177**	**80**	**144.25**
Cefepime	**-5.48**	**3**	**LYS 165, SER 203, ALA 28**	**80**	**166.47**
Cefoperazone	**-6.17**	**3**	**SER 205, LYS 165, HIS 204**	**80**	**123.90**
Imipenem	**-5.61**	**5**	**LYS 165, SER 203, GLY 161, ASP 78, SER 205**	**80**	**267.24**
Meropenem	**-4.74**	**6**	**LYS 165, SER 203, GLY 161, ASP 78, ILE 164, HIS 204**	**80**	**208.62**

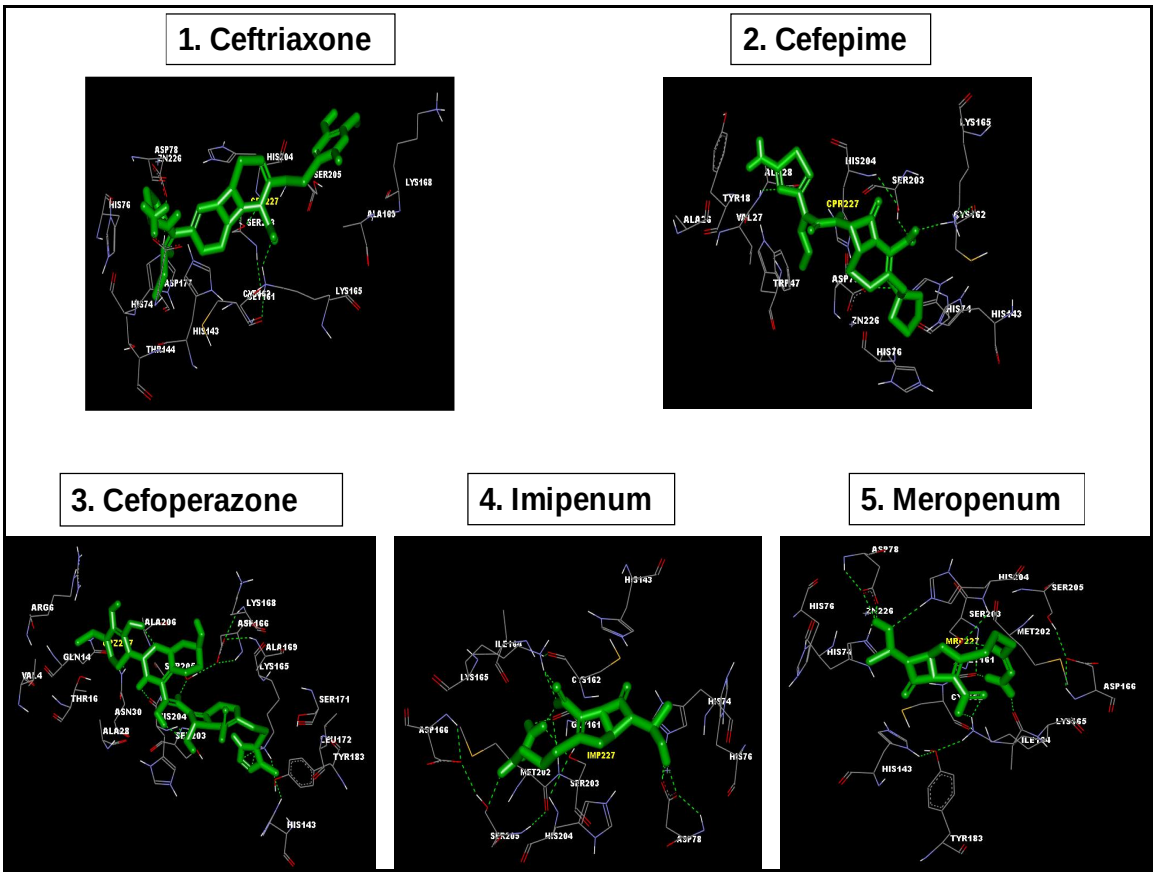


Figure 7g: Docking studies of *blaNDM-1* protein with all the inhibitors. Green color sticks indicates ligand, Green color doted lines hydrogen bond.

i. Ceftriaxone:

Lys 165, Ser 203 and Asp 177 were found to form 3 hydrogen bonds with that of ceftriaxone (Fig 7g. 1). These hydrogen bonds were found to stabilize the binding of ceftriaxone. The IC_{50} of the drug was found to be 144.25µM.

ii. Cefepime:

Result of cefepime docking into the reported active site of *blaNDM-1* is shown in Figure 7g. 2. Under careful observation three hydrogen bonds were found to be formed with the following residues: Lys 165, Ser 203 and Ala 28. The IC_{50} of the drug was found to be 166.4µM.

iii. Cefoperazone:

Cefoperazone was found to dock into the reported active site of *blaNDM-1* is shown in Figure 7g. 3. A thorough examination of the binding pocket indicated that cefoperazone formed three hydrogen bonds with the residue of Ser 205, Lys 165 and His 204. The IC_{50} of the drug was found to be 123.90μM.

iv. Imepenum:

Lys 165, Ser 203, Gly 161, Asp 78 and Ser 205 were found to form 5 hydrogen bonds with that of Imepenum are shown in figure 7g. 4. These hydrogen bonds were found to stabilize the binding of imepenum. The IC_{50} of the drug was found to be 267.24μM.

v. Meropenum:

The result of meropenum docking into the reported active site of *blaNDM-1* is shown in Figure 7g. 5. Under careful observation six hydrogen bonds were found to be formed with the following residues: Lys 165, Ser 203, Gly 161, Asp 78, Ile 164 and His 204. The IC_{50} of the drug was found to be 208.62μM.

7.6. Discussion:

Over the past decade, increased prevalence of multidrug-resistant strains of *A. baumannii* (MDR-AB) has been reported. The prevalence of strains resistant to the usually potent and safe β-lactam antibiotics, such as ampicillin-sulbactam and carbapenems, had increased substantially (Young et al., 2007, Rezin et al., 2008, Reddy et al., 2010, Bassetti et al., 2008). Since the new antimicrobial agents for Gram-negative pathogens shrinks, the longevity of existing compounds becomes a matter of primary concern (Eagye et al., 2007).

Many *Acinetobacter* are now resistant to commonly used antibacterial drugs, including penicillins, expanded-spectrum cephalosporins (Reddy et al., 2010, Morohoshi et al., 1977) cephamycins, aminoglycosides, chloramphenicol, and tetracyclines (Rezin et al., 2008). Carbapenems have become the drugs of choice against *Acinetobacter* infections in many centers but are slowly being compromised by the emergence of carbapenem-hydrolyzing ß-lactamases (Dalla-Costa et al., 2003). Most potent emerging carbapenemase gene is New Delhi metallo-beta-lactamases (*blaNDM-1*).

The first clue for the presence of a carbapenemase was inferred from the increased minimum inhibitory concentration (MIC) especially to imipenem, or meropenem. In our previous study, MIC results of *A. baumannii* isolate showed resistance to Ceftazidime, Ceftriaxone, Cefoperazone/Sulbactum, Cefepime, Imipenum and Meropenum (Data not shown for reference). In the current study, the MIC data and the docking results were in concordance and resistance could be attributed to the biofilm production. The reproducibility of obtained experimental results confirmed the predictive accuracy.

Yong *et al.,* 2009 documented cephalosporins group of antibiotics as most potential inhibitors of *bla*NDM-1, which was observed in our study in terms of Biofilm production and inhibitory mode of interactions. However, carbapenem group of antibiotics studied were proven to be more significant when compared to cephalosporins. Liang Z, *et al.* 2011 has reported *blaNDM-1* contains an additional insert between residues 162 and 166, which is not present in other MBLs. Since the insert is in the opposite side of the active site, with a distance of around 20 Å, its role in the hydrolysis reaction is still unknown. Interestingly, in our study we found both cephalosporins to

interact with LYS 165, whist, carbapenem group was found to interact with GLY161 and LYS165. However, this prediction needs to be confirmed by other structural biology methods.

7.7. Conclusion:

By this study, we have demonstrated the correlation between *blaNDM–1* positivity, and biofilm production with molecular interactions inferred through *in silico* docking studies which have attributed to high level resistance. Further by this study, we also highlight the importance of 162-166 region of *blaNDM-1* as a potential site for drug targeting.

7.8. Future prospects:

Further extended *in silico* studies will aid in understanding its role in drug resistance in *blaNDM–1* producing strains.

Chapter - 8

A study on *Propionibacterium acnes* isolated from ocular infection their clinical correlation with phenotypic and genotypic characteristics, its epidemiological analysis and antibiotic susceptibility profiling.

8.1. Aim:

To identify the *Propionibacterium acnes* (*P. acnes*) isolates from ocular specimens infection and to study their clinical correlation with phenotypic and genotypic characteristics, analysis of epidemiological pattern and antibiotic susceptibility profiling.

8.2. Review of literature:

8.2.1. *Propionibacterium acnes*:

Propionibacterium acnes (*P. acnes*) is a major inhabitant of human skin (Furukawa et al., 2009). *P. acnes*, an anaerobic to aerotolerant, non-spore-forming, pleomorphic gram-positive bacilli that shows extremely slow growth characteristics on culture media (Suzuki et al., 2002) and it is found predominantly in the sebaceous gland-rich areas of the skin (Bruggemann et al., 2004). Apart from causing skin infections, *P. acnes* is also responsible for wide-range of ocular infections such as dry eye, (Graham et al., 2007) lacrimal sac and/or nasolacrimal duct obstruction (Hartikainen et al., 1997), infections of prosthetic implants, including intraocular lenses (IOLs) (Semel et al., 1989, Lai et al., 2006), keratitis (Brook et al., 2001 , Shalchi et al., 2011), blepharitis (Dougherty and Mcculley, 1984), dacryocystitis (Brook and Frazier et al., 1998, Chaudhry et al., 2005), mebiomitis (Suzuki et al., 2005), canaliculitis (Zaldívar and

Bradley, 2009), contamination of the contact lens related microbial keratitis (Szczotka-Flynn et al., 2010). *P. acnes*, is responsible for devastating ocular complications of infectious endophthalmitis both endogenous endophthalmitis (Wong et al., 2000), and exogenous endophthalmitis such as acute (Winward et al., 1993), chronic (Davis et al., 2005), delayed (Lohmann et al., 2000), and post operative endophthalmitis including post traumatic endophthalmitis (Essex et al., 2004). At present, the clinical importance of the *P. acnes* infections is underestimated in the routine diagnostic practices due to inefficient detection and isolation procedures, along with the traditionally held view that, the organism has low virulence and its presence in clinical samples is often considered a contaminant (McDowell et al., 2005).

8.2.2. Genotypic analysis of *P. acnes*:

P. acnes is isolated more frequently — up to 8 times more — than any other spp. of P*ropionibacterium* (Sampedro et al., 2009). According to the literature, 2 distinct phenotypes of *P. acnes* (types I and II), distinguished by their serological agglutination tests and cell-wall sugar analysis exists (Johnson and Cummins, 1972). DNA sequence analysis of *P. acnes*, *recA* gene has revealed presence of type I and II genotypes which are phylogenetically distinct clusters or lineages. A new phylogenetic type, type III that displays differences in their cell surface antigen and the cellular morphology is also reported (McDowell et al., 2008).

Genetic analysis of *P. acnes* is very important to understand the phylogeny of the organism to help us to detect the frequency of a genotypic prevalence in a population. Correlation between various genotypes of *P. acnes* recovered from failed prosthetic hip-

associated bone, tissue samples and dental infections has been analyzed with clinical findings (McDowell et al., 2005). The association between *P. acnes* genotypic patterns and their role in ocular clinical infections has not yet studied and a planned study is needed to determine the same.

8.2.3. Random amplification of polymorphic DNA (RAPD) analysis of *P. acnes*:

Very few studies have been published on the epidemiology of *P. acnes* isolates. Random amplification of polymorphic DNA (RAPD) technique, a modification of Polymerase Chain Reaction (PCR), in which a single primer is able to anneal and prime at multiple locations in a genome, can be of great epidemiological value (Hilton et al., 1997). The success of this method is due to the fact that no prior information about the target sequence is needed. RAPD primers detect polymorphisms in the absence of variations and may work as genetic markers that are used in epidemiologic studies.

8.2.4. Prevalence of antimicrobial resistance among *P. acnes:*

Increasing prevalence of antimicrobial resistance among *P. acnes* has been documented in the last 20 years (Stubbs et al., 2000, Gübelin et al., 2006). Despite *P.acnes* susceptibility to various antibiotics, it is difficult to eradicate, and long courses of treatment are often recommended for biofilm producing isolates (Coenye et al., 2007). Biofilm have been observed on contact lenses, where they are believed to contribute to the development of microbial keratitis. Cataract surgeries with intraocular lens (IOL) placement or the introduction of intraocular infusion pumps, glaucoma tubes, stents, keratoplasties, or other ocular prostheses create opportunities for the development of infections involving microbial biofilms (Behlau et al., 2008).

8.2.4. Need for study:

P. acnes is the most predominant anaerobe isolated from ocular specimens in our hospital set up. Detailed studies have not been carried out so far with *P. acnes* isolated from wide range of ocular clinical specimens, pathogenicity of the bacterium, co-existent site of infection and clinical correlation with the phenotypic and genotypic results of the *P. acnes*, drug resistance and its relationship with biofilm production. Also there is a need to understand the epidemiological pattern of ocular *P. acnes* and their correlation with the antibiotic susceptibility results. Therefore, the current study was undertaken to study the different genotypic prevalence of *P. acnes* recovered from ocular isolates, their epidemiology profiling and antibiotic susceptibility pattern.

8.3. Objectives:

To determine the genotypic prevalence of *Propionibacterium acnes* (*P. acnes*) isolated from ocular specimens and to study their clinical correlation with phenotypic and genotypic characteristics, analysis of epidemiological pattern and also to determine antibiotic susceptibility profiling.

- **a.** To detect *P. acnes* by conventional and molecular techniques.
- **b.** To compare the results of conventional and molecular techniques in detection of *P.acnes.*
- **c.** To standardize PCR targeting *16S rRNA* gene and *recA* gene to understand the prevalent genotypes of *P. acnes* causing infections.
- **d.** To understand the epidemiology of *P. acnes* by RAPD technique.
- **e.** To determine the antibiotic susceptibility pattern of ocular *P. acnes.*
- **f.** To detect the biofilm producing ability of ocular *P. acnes.*

8.4. Materials and methods:

8.4.1. Bacterial strains: One hundred *P. acnes* isolates recovered from ocular clinical disease conditions were included into the study. Details of *P. acnes* isolates recovered from various ocular diseased conditions are shown in table 8a.

Table 8a: Details of *P. acnes* isolates recovered from various ocular diseased conditions

Clinical condition	Not a Pathogen	Primary Pathogen	Secondary Pathogen	No of Specimens
Bacterial Conjunctivitis	6	2	13	21
Viral Conjunctivitis	2	0	0	2
Allergic Conjunctivitis	2	0	1	3
Blepharitis	4	4	3	11
Dacryocystitis	10	0	0	10
Bacterial Keratitis	4	2	8	14
Fungal Keratitis	3	2	6	11
Trauma induced Keratitis	7	1	2	10
Orbital cellulitis	0	0	1	1
Orbital abscesses	1	1	1	3
Lacrimal sac abscesses	2	0	0	2
Scleritis	0	1	1	2
Graft infection	1	1	1	3
Socket implant exposure	1	0	0	1
Endophthalmitis	4	1	1	6
TOTAL	**47**	**15**	**38**	**100**

Eight *P. acnes* isolates were recovered from conjunctival swabs of both eyes of 4 patients (3 males and 1 female) with bacterial conjunctivitis. The isolates were obtained during the period of two years from January 2010 – December 2011 from a total of 7,598 specimens including both extraocular and intraocular received for microbial culture at L & T Microbiology Research Centre, Sankara Nethralaya, a tertiary eye care centre at Chennai, India. Three reference anaerobic American Type Culture Collection strains (ATCC; Manassas, Va): *Propionibacterium acnes* ATCC 11828, *Clostridium sporogenes* ATCC 11437 *Bacteroides fragilis* ATCC 23745 were also included for study. The study protocol was approved by the institutional ethics sub-committee (IRB). Clinical specimens were processed as described elsewhere (Folkens et al., 1996).

8.4.2. Anaerobic culture:

Culture isolates were identified as *P. acnes* using conventional methods namely Gram staining, catalase, indole positivity, and growth characteristics in Brucella blood agar enriched with 5% sheep blood, 5 mg/L haemin and 1 mg/L vitamin K1, (Cat No.M1039, Hi-Media Laboratories Private Limited, Mumbai) by incubating in an anaerobic work station (Don Whitley Scientific Limited, Shipley, West Yorkshire, UK) (85% N_2, 10% H_2, 5% CO_2) until growth is seen (Folkens et al., 1996).

8.4.3. Fermentation tests for the differentiating the Types of *P. acnes*:

Fermentation reactions of *P. acnes* were studied on modified protease peptone yeast agar plates containing 40 mg of bromocresol purple indicator/liter and 1% (wt/vol) sorbitol (Hi-Media Laboratories Private Limited, Mumbai). Organisms were grown

anaerobically, as described above, and a positive fermentation reaction was noted if the agar plates turned yellow due to acid production (McDowell et al., 1998).

8.4.4. Molecular analysis of the *P. acnes*:

All the 100 isolates of *P. acnes* were subjected for the following molecular investigations.

a. Uniplex PCR for identification of the *P. acnes*:

Species-specific uniplex PCR amplification was done using the primer sequences as described by Therese, *et al*. (1998)

b. Amplification of *16S rRNA* and *recA* genes:

DNA was extracted from a single colony isolated from Brucella sheep blood agar using the (Appendix 2) DNA Mini kit (Qiagen, Hilden, Germany) as per the manufacturer instructions. The extracted genomic DNA was stored at -20°C until further subjected for PCR analysis. The *16S rRNA* gene (1532 bp) of *P. acnes* was amplified using the primers described by *Stubbs et al*. (2000) and *recA* gene were amplified using method distributed by McDowell *et al*. 2005. PCR amplifications were carried out using thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, Massachusetts, USA). From the extracted DNA, 5µl was added on to 45µl of PCR mixture consisting of 5µl buffer (10X buffer containing 15mM $Mgcl_2$) 200µM dNTPs, 25 picomoles of primer, 30µl deionized water and 1.25U *Taq* polymerase (Merck, Darmstadt, Germany). PCRs were carried out with the DNA extracts of the positive control strain *P. acnes* (ATCC 11828). A negative control (Milli Q water was added instead of DNA) was included in all PCR based DNA amplification experiments. Following DNA amplification, PCR

products were analyzed by electrophoresis on 2% agarose gels (SRL, India) containing 1x Tris-acetate-EDTA buffer. Molecular size markers (100bp DNA ladder; (Merck, Darmstadt, Germany)) were run in parallel on all gels. Resolved DNA products were stained with ethidium bromide, 50ng/ml (Hi-Media Laboratories Private Limited, Mumbai.) and viewed under UV light using gel documentation system (VILBER LOURMAT – FRANCE).

c. Nucleotide sequence analysis:

Products were loaded onto ABI PRISM 3130 DNA sequencer (Perkin-Elmer Applied Biosystems) with polymer POP7 and sequenced. Sequences were analyzed using BIO EDIT, (downloaded from *http://www.mbio.ncsu.edu/BioEdit/bioedit.html*), and finally analyzed using NCBI Blast website *http://blast.ncbi.nlm.nih.gov/Blast.cgi* to identify spp. and DNA homology.

d. Phylogenetic analysis:

The phylogenetic relationships of *16S rRNA* and *recA* genes were determined by using the Data Analysis in Molecular Biology and Evolution (DAMBE) software (http://web.hku.hk/_xxia/software/ software.htm) and MEGA (Molecular Evolutionary Genetics Analysis at http://www.megasoftware.net/index.html). Multiple sequence alignments were performed by using the CLUSTAL W algorithm and were exported into the DAMBE program. Phylogenetic trees were constructed by the maximum-parsimony method and the neighbor-joining method. The bootstrapping resampling statistics were performed using 100 data sets for each analysis.

e. PCR-RFLP analysis:

(i) Designing of restriction enzyme:

For the genotyping of *P. acnes* isolates, an appropriate restriction enzyme is necessary. The appropriate restriction enzyme were found by subjecting the known nucleotide sequence of *16S rRNA* gene of *P. acnes* NCTC 737 (GenBank accession no.AY642055) and NCTC 10390 (GenBank accession no.AY642061) to Restrictionmapper.3 software (http://www.restrictionmapper.org/) which would identify the specific restriction enzyme in a total panel of 200 enzymes to cleave the amplified product. It was observed that the restriction enzyme "*Hin6I* (HinP1I-G/CGC)" possessed the ability to fragment the 16S *rRNA* nucleotide sequences that helped in determination of the genotype.

(ii) Optimization of RFLP:

RFLP optimization conditions were standardized and applied to amplified PCR products targeting the *16S rRNA* gene. The reaction mixture contained sterile Milli Q water -15µl, Buffer - 4µl, Amplified product - 10µl, and Enzyme - 1µl (1U/µL) and was incubated for 2 hours at 37ºC after which the entire digested sample was viewed in 3% agarose gel containing ethidium bromide, 50ng/ml.

8.4.5. RAPD technique for epidemiological analysis:

a. Optimization of RAPD technique:

To optimize the RAPD fingerprinting technique, the optimal concentrations of primers, DNA template, and $MgCl_2$ used in PCR were first determined. Trying different concentrations ranging from 15, 30, 60 and 100ng/µl the primer concentration was

subjected for optimization. Primer dimer formation was seen with the concentrations of 60 and 100ng/µl. With 15 ng/µl primer concentration the bands were very faint. Primer concentration of 30ng/µl yielded clear banding patterns. Concentration of $Mgcl_2$ ranging from 1 to 4mM $MgCl_2$ produced identical banding pattern, higher concentrations of $MgCl_2$ yielded some artificial background, and lower concentrations of $MgCl_2$ resulted in poor amplification. A concentration of 2mM $MgCl_2$ was chosen for the RAPD reaction. Likewise, low levels of DNA template (5, 10 ng) were found to result in relatively poor amplification while higher level yielded smearing patterns. RAPD patterns, however, standardized with 20ng/µl of total DNA were used. Consequently, a slight difference in DNA concentration obtained from preparation to preparation did not affect RAPD patterns (Ruiz et al., 2000, Maripandi et al., 2007).

To select suitable RAPD primers for subtyping of 100 *P. acnes* isolates, 10 arbitrary primers were first tested with 10 isolates of *P. acnes* and one primer, 7^{th} primer (Accession no for 7^{th} primer is AM773318, Merck, Darmstadt, Germany) was found to produce three or more polymorphic patterns when tested, was taken for the RAPD determination (Sambrook et al., 1989, Austin et al., 1988).

b. PCR-RAPD procedure:

DNA was extracted from a single colony isolated from Brucella blood agar using the Qiagen DNA Mini kit (Qiagen, Hilden, Germany) as per the instructions provided in the manual (Appendix 2). The resulting genomic DNA was stored at -20°C until further subjected for PCR-RAPD analysis. PCR-RAPD was carried out for all *P. acnes* isolates by using 20 ng/µl of genomic DNA added to 20µl of PCR mixture consisting of 5µl 10X

PCR buffer containing 15mM $MgCl_2$, 200µM dNTPs, 100ng/µl of primer, 10µl deionized water of and 1.0 U of *Taq* polymerase (Merck, Darmstadt, Germany). PCR amplifications were carried out using thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, Massachusetts, USA). PCR thermal profile consisted of initial denaturation at 94°C for 5 min, followed by 8 cycles consisting of denaturation at 94°C for 45sec, annealing at 35°C for 1 min, and extension at 72°C for 1.5 min, followed by 35 cycles consisting of denaturation at 94°C for 45sec, annealing at 38°C for 1 min, and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

Entire RAPD products were loaded into 1% agarose gel (SRL, India) containing 1x Tris-acetate-EDTA buffer. Molecular size markers [100bp and 500 bp DNA ladder; (Merck, Darmstadt, Germany)] were run in parallel on all gels. Resolved DNA products were stained with ethidium bromide, 50ng/ml (Hi-Media Laboratories Private Limited, Mumbai.) and viewed under UV light using gel documentation system (VILBER LOURMAT – FRANCE) (Sambrook et al., 1989 , Austin et al., 1988).

8.4.6. Antibiotic Susceptibility Testing:

The following antibiotics powders were used and obtained from the sources indicated: ciprofloxacin, norfloxacin, nalidixic acid, clindamycin, penicillin G, vancomycin and metronidazole (Sigma, USA), cefotaxime and imepenum (Ranbaxy, India) were used to make stock solutions that were filter sterilized and then stored at 4ºC. Final working solutions tested for ciprofloxacin, norfloxacin, nalidixic acid and vancomycin were of 0.125, 0.25, 0.5, 1, 2 and 4 mg/L respectively. For clindamycin, penicillin G, cefotaxime and imipenum, the final concentrations tested were 0.06, 0.125,

0.25, 0.5, 1 and 2 mg/L respectively. Metronidazole final concentrations were 8, 16, 32, 64, 128 and 256 mg/L respectively (Clayton et al., 2006, Mory et al., 2005, Oprica et al., 2005, Smith et al., 2005).

Determining of Minimum Inhibitory Concentration (MIC):

MICs of the nine antimicrobial agents were determined by agar dilution technique as described by the Clinical Laboratory Standard Institute (CLSI) standard with 10^5 CFU/ spot and Brucella base sheep blood agar (McDowell et al., 2005, Sampedro et al., 2009). The plates were incubated in Don Whitley anaerobic work station (Shipley, West Yorkshire, UK) for 48 h at 37°C. The MIC was defined as the lowest concentration of antimicrobial agent that resulted in a marked change in the appearance of growth in comparison with the control plate, as described in the NCCLS protocol. *P. acnes* ATCC 11828 was used as control strain.

8.4.7. Detection of Biofilm production by Tissue culture plate method:

All the 100 isolates from fresh Brucella sheep blood agar plates were inoculated into Thioglycollate media and incubated anaerobically for 24 hours at 37^0C in stationary condition and were diluted 1 in 10 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom culture plates (Tarson, India) wells were filled with 200µl aliquots of the diluted cultures and sterile uninoculated Thioglycollate media served as medium control to check sterility and *P. acnes* ATCC 11828, a non-biofilm producer served as the inoculum control and negative control and a *P. acnes* lab isolate with high drug resistance isolated from a eviscerated material was used as positive control. The plate was incubated anaerobically for 18 to for 24 hours at 37°C.

After incubation content of each well was gently removed by tapping the plates inverted into the discard jar containing 0.25% lysoformin. The wells were washed four times with 200µl of phosphate buffer saline (PBS, pH 7.2, Laboratories Private Limited, Mumbai) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with a modification by addition of 200µl sodium acetate (2%) instead of methanol and incubating at room temperature (RT) for 20 minutes (Coenye et al., 2007). Sodium acetate was gently removed by tapping the plates by inverting them onto the discard jar containing 0.25% lysoformin and 200µl crystal violet (0.1% w/v) was added and the plates were incubated for 20 minutes at RT. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Subsequently, the crystal violet dye bound to adherent cells was released by adding 160 ml 33% acetic acid (SRL, India). Optical density (OD) of stained adherent bacteria has to be determined with a micro ELISA auto reader at wavelength of 520 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiment was performed in triplicate and data was then averaged and standard deviation was calculated. To compensate for background absorbance, OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values. Mean OD value obtained from media control well was deducted from all the test OD values.

Interpretation of bacterial adherence:

For the purpose of data calculation, interpretation based on OD values obtained for individual strains of *P. acnes* (Mathur, et al., 2006). The mean OD values derived as shown below.

Classification of bacterial adherence by TCP method

Mean OD values	Adherence	Biofilm formation
<0.120	Non	Non / weak
0.120-0.240	Moderate	Moderate
>0.240	Strong	High

8.5. RESULTS:

In the present study, a total of 100 *P. acnes* isolates from various ocular specimens were included. The highest rate of *P. acnes* isolation was from the male population (59 male and 41 females). The mean age was 54.3 years for male group (range, 1 to 76 years) and 51.4 ± 18years for female group (range, 8 to 75 years). The age wise distribution of *P. acnes* isolates are shown in figure 8a. Of the 100 clinical anaerobic isolates, maximum number of anaerobe was isolated from the conjunctival swabs (59%).

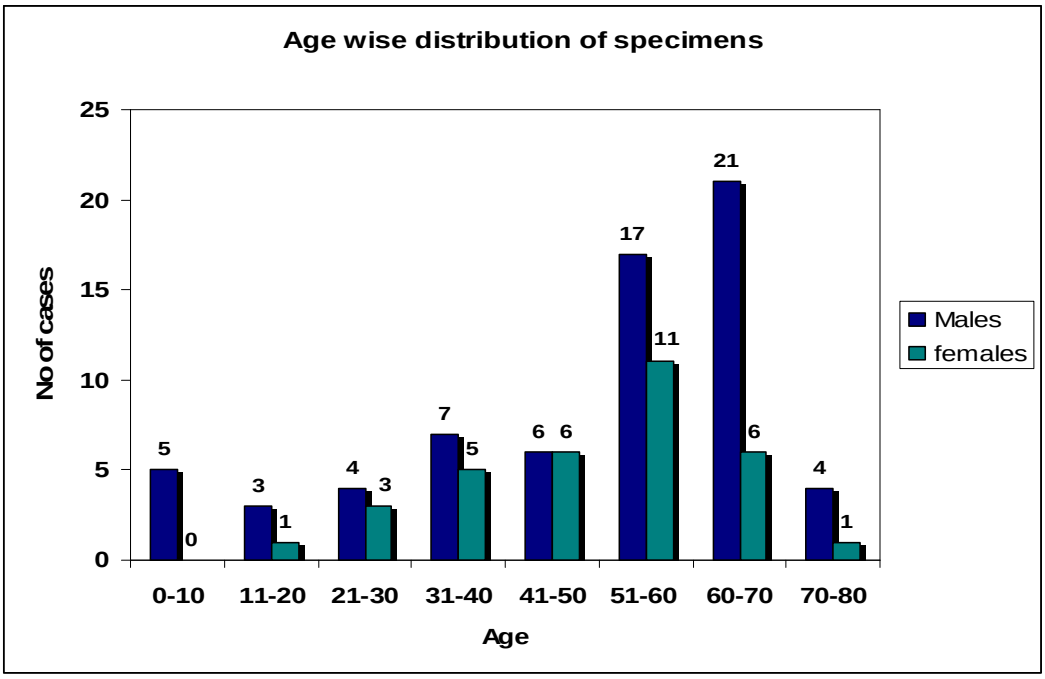


Figure 8a: Graphical representation of age wise distribution of *P. acnes* isolated from ocular clinical specimen

8.5.1. Microbial distribution of isolates:

Among these 100 isolates, *P. acnes* isolates were obtained as pure culture from 38 (38.0%) specimens, 47 (47.0%) strains along with single or multiple bacteria belonging to other than *P. acnes,* 13 (13.0%) strains along with fungi and 2 (2.0%) isolates with both bacteria other than *P. acnes* and fungus were isolated. Microbial distributions of isolates are shown in table 8b.

Table 8b: Microbial distribution of isolates

S. No.	Clinical condition	No of Specimens
1	No of pure cultures	38
2	Polymicrobial - other bacteria isolated	47
3	Polymicrobial -with fungi	13
4	Polymicrobial - with bacteria and fungi	2
TOTAL		100

8.5.2. Association with clinical condition:

Among 100 isolates of *P. acnes* included in the study, in 15 cases (15.0%) *P. acnes* was isolated as the primary pathogen responsible, predominantly from corneal ulcers (n=9/15 cases), in 38 cases (38.0%) *P. acnes* was isolated as secondary pathogen from notably again from corneal ulcers (n=16/38), other frequently encountered underlying factors included a history of trauma (n=5/38) and in remaining 47 cases *P.acnes* is the commensal flora of eye which was isolated during anaerobic culture of conjunctival swabs that is routinely being carried out as pre operative screening for cataract, trabeculectomy surgeries (n=39/47)

8.5.3. Results of genotypic analysis:

a. Uniplex PCR for detecting *P. acnes*: One hundred isolates primarily identified by staining, biochemical and growth characteristics as *P. acnes* gave specific amplified products with the spp. specific uniplex PCR primers for *P. acnes.* Figure 8b shows the amplification of *P. acnes* from various specimens.

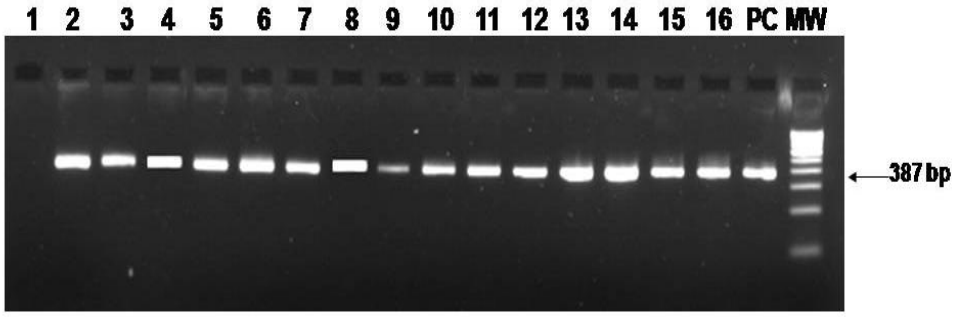


Figure 8b: Agarose gel electrophoretogram showing results of spp. specific Uniplex PCR targeting *16S rRNA* gene of *P. acnes*. Lane 1: Negative control; **Lanes 2-16:** Showing positivity for *P. acnes*; **Lane PC:** Positive control *P. acnes* (ATCC 11828); **Lane MW**: 100 bp molecular weight ladder.

b. Uniplex PCR for Amplification of *16S rRNA* of the *P. acnes*:

All 100 isolates were subjected for amplification with *16S rRNA* gene with *Stubbs et al.* primers (2006). Figure 8c shows the amplification of *16S rRNA* gene of *P. acnes* from various specimens. Amplified products were then subjected for both RFLP and remaining products were used for nucleotide sequence analysis to understand their phylogenetic origin.

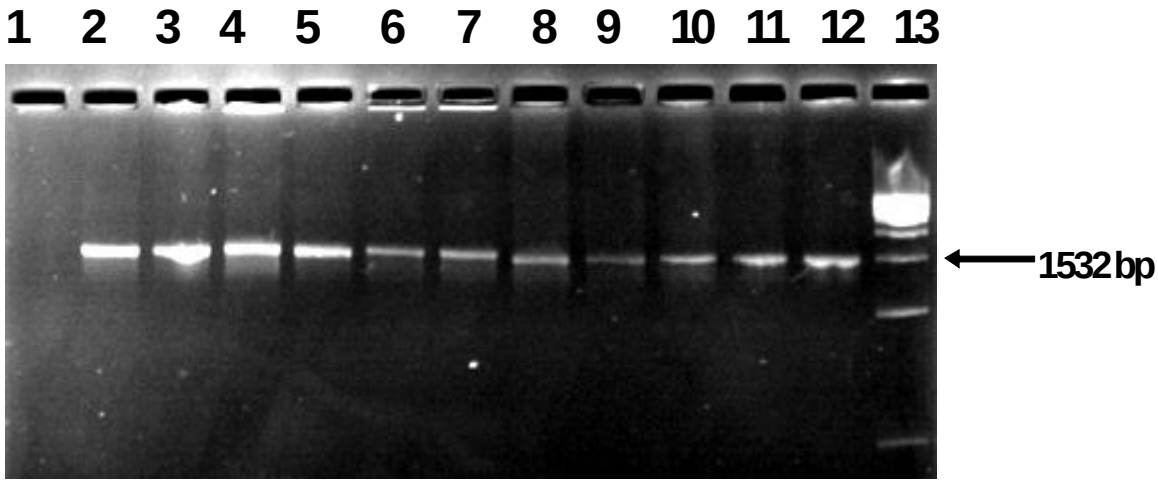


Figure 8c: Agarose gel electrophoretogram showing results of Uniplex PCR targeting *16S rRNA* gene of *P. acnes*. Lane 1: Negative Control, **Lane 2-11:** *P. acnes* isolates showing uniplex PCR positive for *16S rRNA* gene of *P. acnes*, **Lane 12:** PC – *P.acnes* ATCC 11828, **Lane 13:** Molecular Weight 500 bp ladder.

c. Phylogenetic analysis:

i. DNA sequence analysis of *16S rRNA*:

Amplified product of *16S rRNA* gene of all the isolates along with the *P. acnes* ATCC 11828 were subjected for DNA sequence analysis along with the reference strains *P. acnes* NCTC 737 representing genotype I (GenBank accession no.AB042288), and NCTC 10390 representing genotype II (GenBank accession no.AY642044) to Multalin software (http://multalin.toulouse. inra.fr/multalin/ multalin.html).

ii. Nucleotide sequence submission of *16S rRNA* gene sequences:

Genbank accession numbers of *16S rRNA* gene sequences of *P. acnes* are JF277163- JF277165, JF289271- JF289273, JF430007- JF430010, JN700213- JN700225, JN700227- JN700231, JN700233- JN700236, JN714989 - JN714997 (41 sequences).

iii. PCR-RFLP analysis:

PCR-RFLP protocol was standardized with the restriction enzyme *Hin6I*. *P. acnes* type specific polymorphism at 827th nucleotide position that corresponded to the nucleotide T in type I strains and C in type II strains (numbering corresponds to GenBank accession no. AB042288) have been already been reported by McDowell *et al*., 2005.

The amplified products of *16S rRNA* gene (*Stubbs et al*., 2006) of the *P. acnes* when subjected for RFLP analysis yielded a total of four different RFLP patterns (TYPE IA, B, C and TYPE II). Based on type specific polymorphism at 827th; the patterns were divided into type I and type II. Type I pattern were further subdivided into type IA, IB, IC based on restriction digestion profile.

Restriction digestion profiles of the sub-genotypes were as follows:

- Type IA restriction pattern - 1085, 320 & 105bp in size.
- Type IB restriction pattern - 670, 424 & 416bp in size.
- Type IC restriction pattern - 670, 424, 301 and 115bp.
- Type II restriction pattern - 670, 424, 256 & 160bp.

Distributions of this RFLP pattern are shown in the table 8c.

Table 8c: Results of PCR - RFLP on the amplified *16S rRNA* gene PCR products of *P. acnes* isolates

SPECIMENS	No of Strains	TYPE I A	TYPE I B	TYPE I C	TYPE II
Extra ocular specimens					
Conjunctival swab	58	5	25	14	14
Conjunctival scraping	1	-	1	-	-
Corneal scraping	17	-	13	3	1
Corneal button	7	-	-	7	-
Scleral tissue	1	-	1	-	-
Intra ocular specimens					
Canalicular pus	1	-	-	1	-
Lacrimal Pus	2	-	-	2	-
Iris tissue	2	-	-	2	-
Orbital Implant	1	-	-	1	-
Perforated Scleral Nodule	1	-	-	1	-
Aqueous Humor	2	-	-	2	-
Vitreous Humor	1	-	-	-	1
Eviscerated material	6	-	3	3	-
TOTAL	**100**	**5**	**43**	**36**	**16**

These different RFLP patterns were grouped based on band position in the 3% agarose gel and were also verified by molecular weight analysis using the BioID software (Vilber Lourmat). The agarose gel electrophoretogram showing the results of RFLP were displayed in figure 8d.

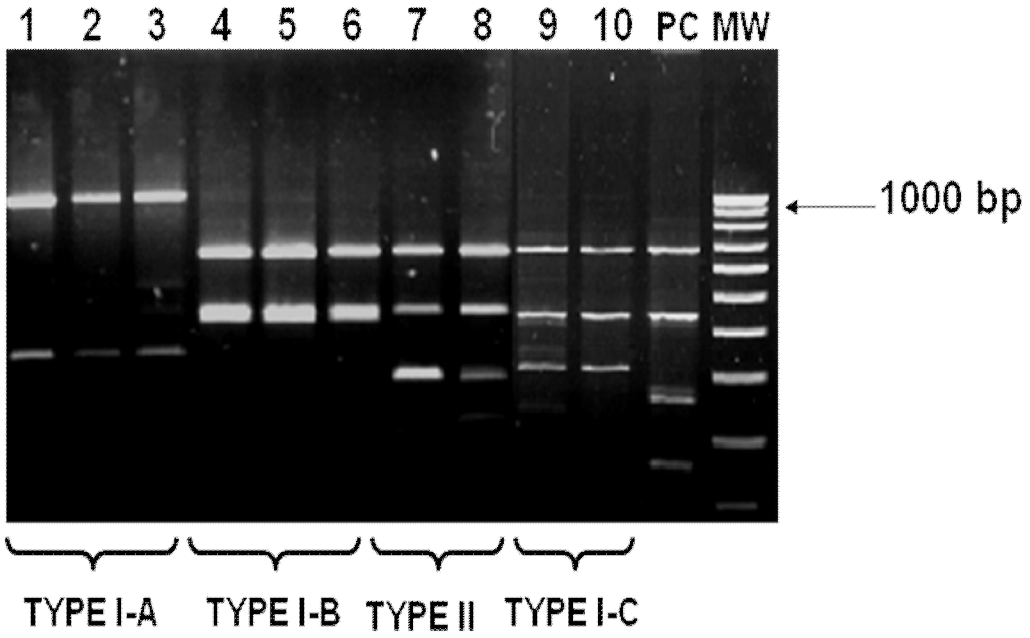


Figure 8d: Agarose gel electrophoresis of fragments of*16S rRNA* gene PCR products of *P. acnes* isolates.

Lane 1-3: Type I A : 1085, 320 & 105 bp

Lane 4-6: Type I B : 670, 424 & 416 bp

Lane 7-8: Type II : 670, 424, 256 & 160

Lane 9-10: Type I C : 670, 424, 301 and 115

PC: Positive control - *P. acnes* ATCC 11828

MW: Molecular Weight 100 bp ladder.

The predominant RFLP pattern detected was Type IB (46.0%) followed by Type IC (36.0%). Conjunctival swabs exhibited majority of Type II RFLP pattern along with 1 corneal scraping and 1 vitreous humour positive for *P. acnes*. Type IA (5.0%) pattern were prevalent among the conjunctival swabs isolates. The results of multalin of the 4 patterns along with their restriction sites were given in figure 8e I and II.

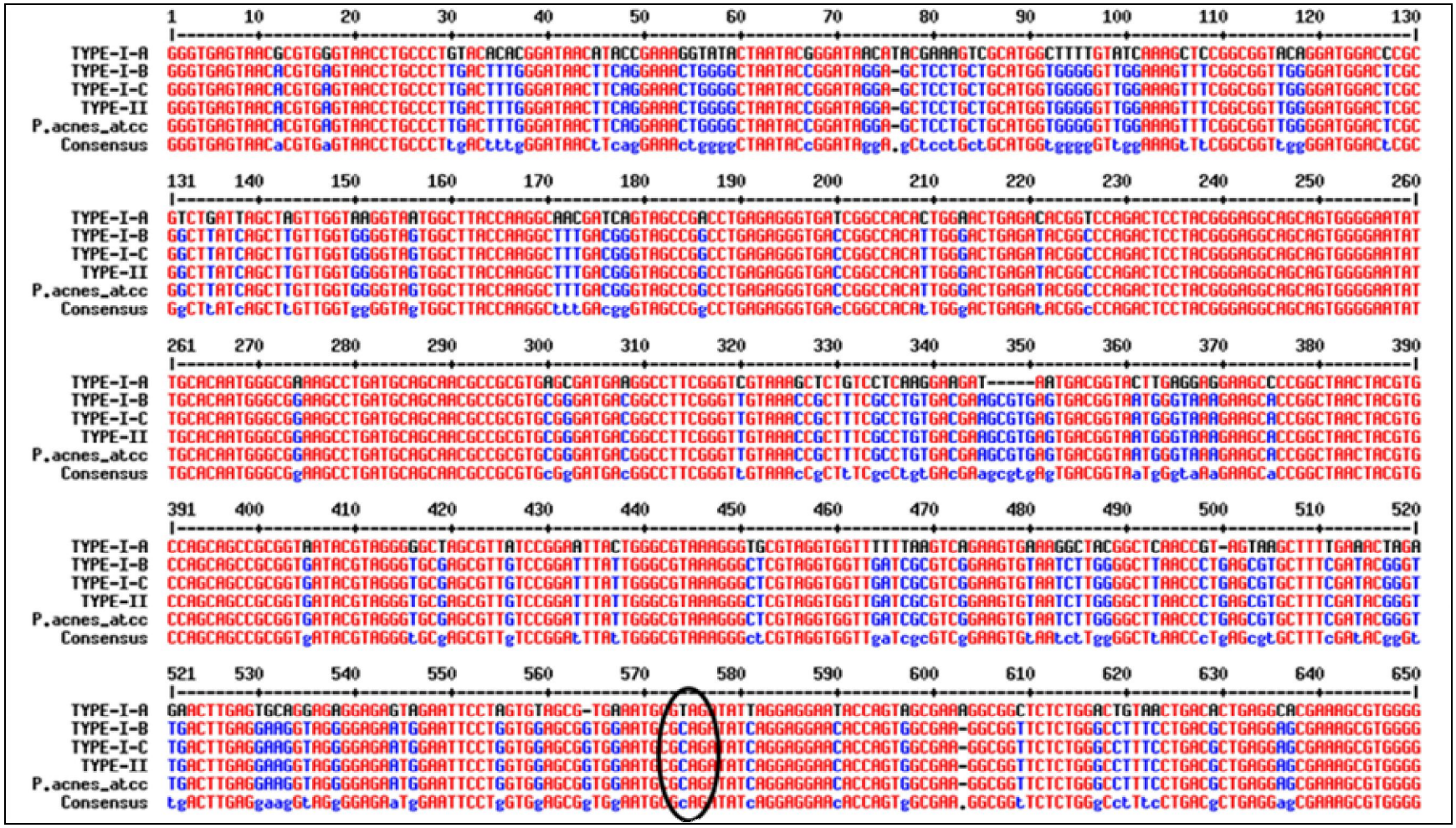


Figure 8e.I: Multalin Results of *16S rRNA* gene showing restriction sites.

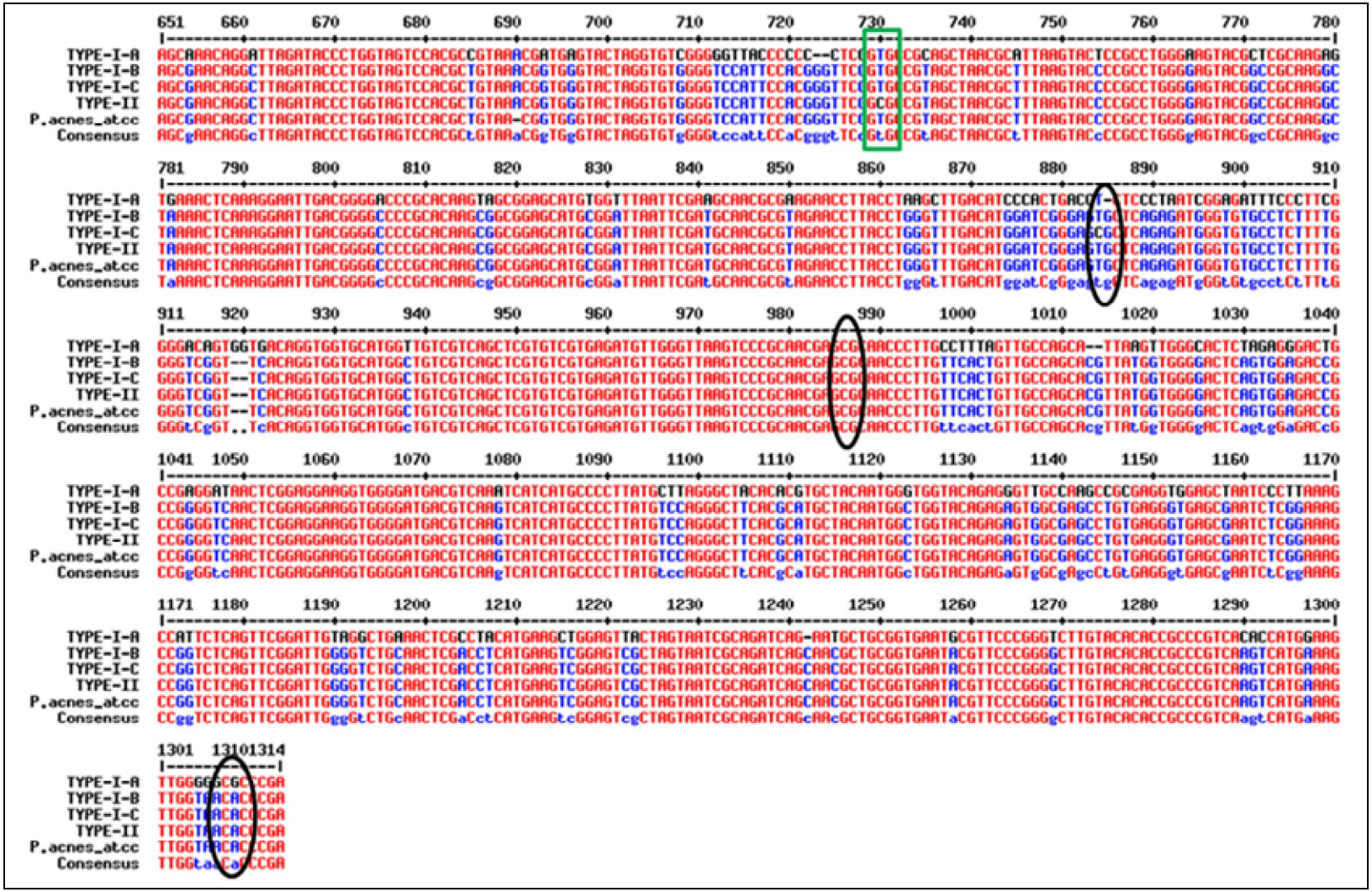


Figure 8e.II: Multalin Results of *16S rRNA* gene showing restriction sites.

iv. DNA sequence analysis of *recA* genes:

Sequenced data of all the *P. acnes* isolates of *recA* gene were subjected for the DNA nucleotide sequence alignment using Multalin software (http://multalin.toulouse.inra.fr/multalin/ multalin.html) along with the *P. acnes* ATCC 11828 and with reference strains *P. acnes* NCTC 737, (GenBank accession no.AY642055), NCTC 10390 (GenBank accession no.AY642061). Results of PCR based sequencing of amplified *recA* gene products of *P. acnes* isolates are shown in table 8d.

Table 8d: Results of PCR based sequencing of amplified *recA* gene PCR products of *P. acnes* isolates

SPECIMENS	No of Strains	TYPE I B	TYPE II
Extra ocular specimens			
Conjunctival swab	58	48	10
Conjunctival scraping	1	1	-
Corneal scraping	17	16	1
Corneal button	7	6	1
Scleral tissue	1	1	-
Intra ocular specimens			
Canalicular pus	1	1	-
Lacrimal Pus	2	1	-
Iris tissue	2	2	-
Orbital Implant	1	1	-
Perforated Scleral Nodule	1	1	-
Aqueous Humor	2	2	-
Vitreous Humor	1	1	1
Eviscerated material	6	6	-
TOTAL	**100**	**87**	**13**

v. **Nucleotide sequence submission of *recA* gene sequences:**

Genbank accession numbers of *recA* gene sequences of *P. acnes* isolates are JF836806, JF430011, JN864886, JN864888 - JN864912, JN864914, JN864915, JN864917 - JN864924, JN864926 - JN864935 were obtained in this study (48 sequences).

vi. Phylogenetic tree:

Phylogenetic trees were constructed using DNA amplified products sequences of *16S rRNA* and *recA* gene of *P. acnes* isolates that showed 99 – 100% identity with gene bank sequences. Nucleotide sequences of both *16S rRNA* and *recA* gene obtained from *Helicobacter pylori* (GenBank accession no U13756 and AE000615) and *Streptococcus agalactiae* (GenBank accession no AF326345 and NC004116) were used as out groups for our phylogenetic analysis, which was performed using the maximum-parsimony and neighbor-joining methods after 100 bootstrapping replications. Consensus tree obtained by using the maximum-parsimony method for *16S rRNA* genome is shown in figure 8f and figure 8g shows the result of phylogenetic analysis for housekeeping gene *recA*. Both *16S rRNA* and *recA* phylogenies of *P. acnes* were highly distinct from unrelated spp. selected as out groups for the trees (bootstrap values, 100%). Phylogenetic trees of *16S rRNA* and *recA* based on protein translation of each nucleotide sequence revealed the similar clustering of type I and II as distinct phylogenetic groups. Among the 100 isolates, 13 strains belonging to type II joined to form a clade along with type II reference strain sequence, and rest 87 isolates clustered along with type I *P. acnes* reference strains.

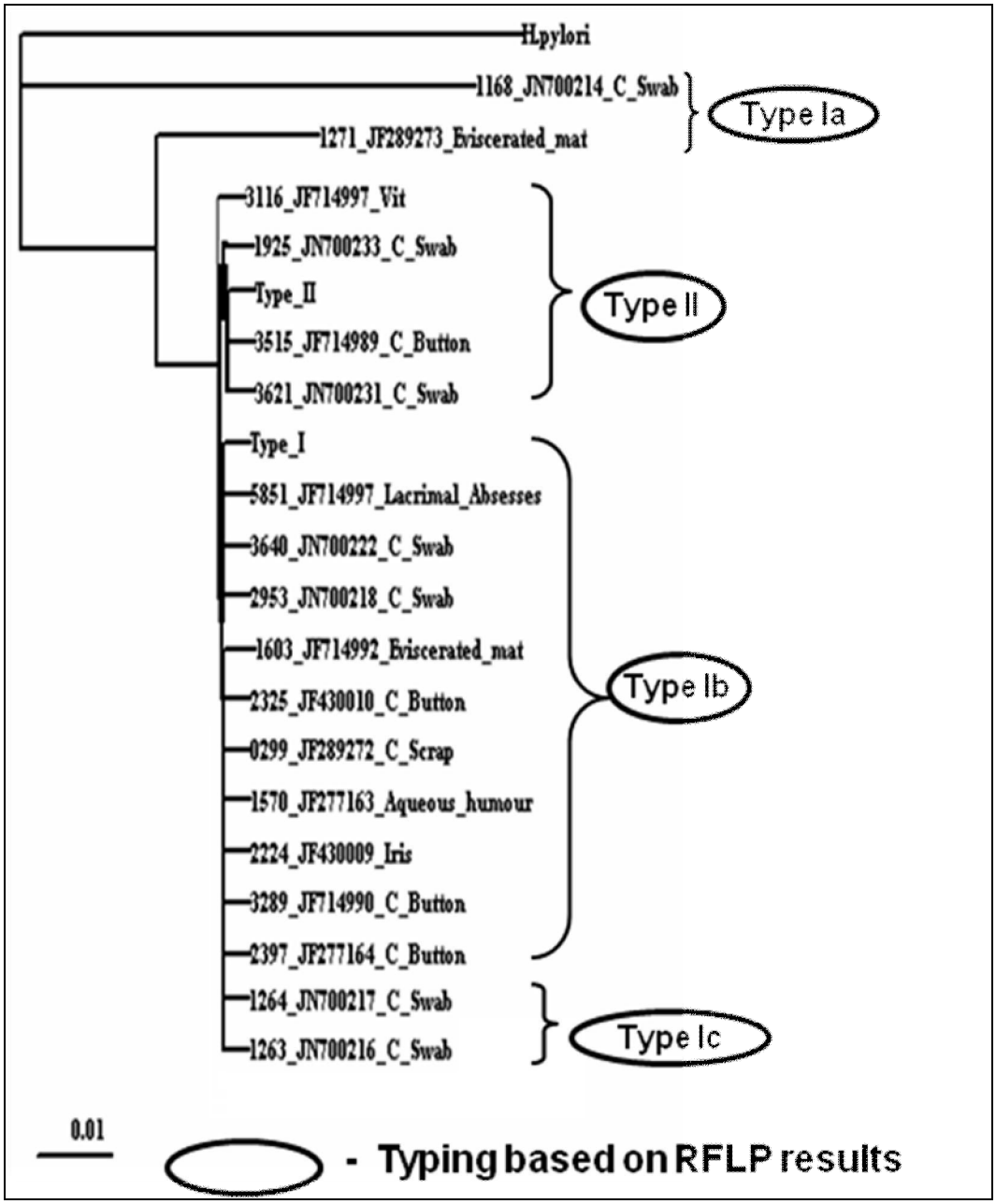


Figure 8f: Phylogenetic trees of *P. acnes* based on the *16s RNA* gene sequences by Maximum-Parsimony method.

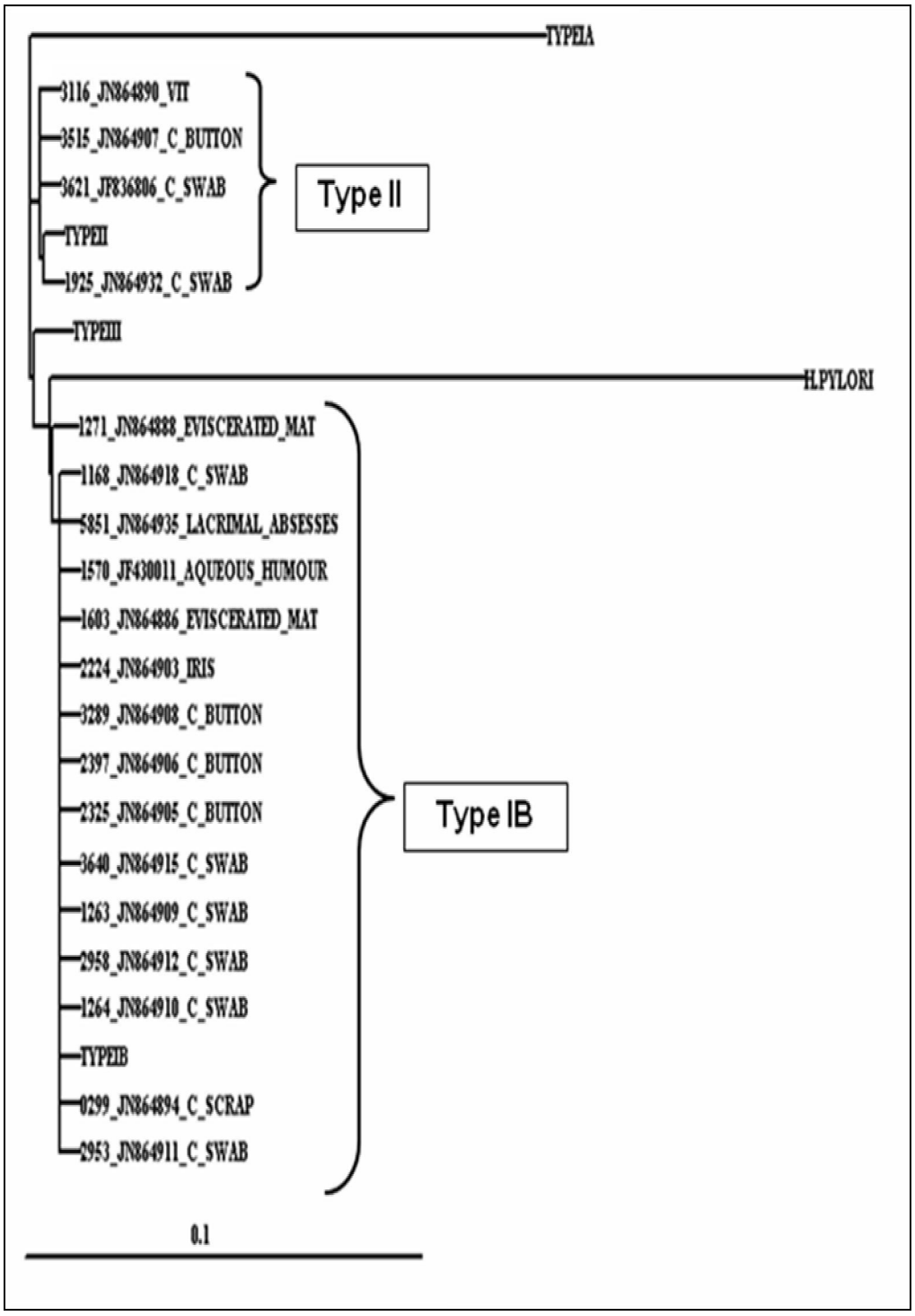


Figure 8g: Phylogenetic trees of *P. acnes* based on the *recA* gene sequences by Maximum-Parsimony method.

8.5.4. PCR-RAPD pattern:

PCR-RAPD analysis performed with 100 *P. acnes* isolates with the 7th set of RAPD primers showed 6 different RAPD patterns distributed among *P. acnes* positive ocular isolates is shown in figure 8h.

Pattern 1 was exhibited by 3 (3%) isolates of *P. acnes* [conjunctival swabs - 2 and eviscerated material – 1]. The products had 6 bands measuring 2500, 1500, 900, 800, 400, 100bp in size. **Pattern 2** was exhibited by 18 (18%) isolates [anterior chamber tap – 1, conjunctival scraping – 1, conjunctival swab – 14, iris tissue – 1 and corneal scraping – 1]. There were totally 7 Bands with size measuring 2000, 1200, 900, 500, 400, 300, 100 base pairs. **Pattern 3** was exhibited by 16 (16%) isolates [conjunctival swab - 12, corneal button – 1, corneal scraping – 2 and eviscerated material - 1]. The products produced 7 bands measuring 2000, 1200, 900, 600, 500, 400, 100bp in size. **Pattern 4** was shown by 41 (41%) isolates [anterior chamber tap - 1, conjunctival swab - 22, corneal button - 4, corneal scraping – 7**,** eviscerated material – 2, iris tissue – 1, lid abscess – 3, sclera nodule - 1], with 5 products of size 2000, 1200, 900, 300, 100bp respectively. **Pattern 5** was exhibited by 14 (14%) isolates. [Conjunctival swab - 11, corneal scraping – 2, lid abscess – 3, vitreous chamber tap – 1] There were totally 7 Bands measuring 1200, 1000, 800, 600, 500, 400, 100bp in size. **Pattern 6** was shown by 8 (8%) isolates [conjunctival swab - 4, corneal scraping – 1, eviscerated material – 2 and orbital implant – 1] with 7 bands corresponding to 3000, 1500, 800, 600, 500, 300, 100bp in size respectively.

The 4th pattern was found to be the most prevalent pattern among *P.acnes*. These different patterns from different specimens indicate that all are not of common origin or hospital acquired infection.

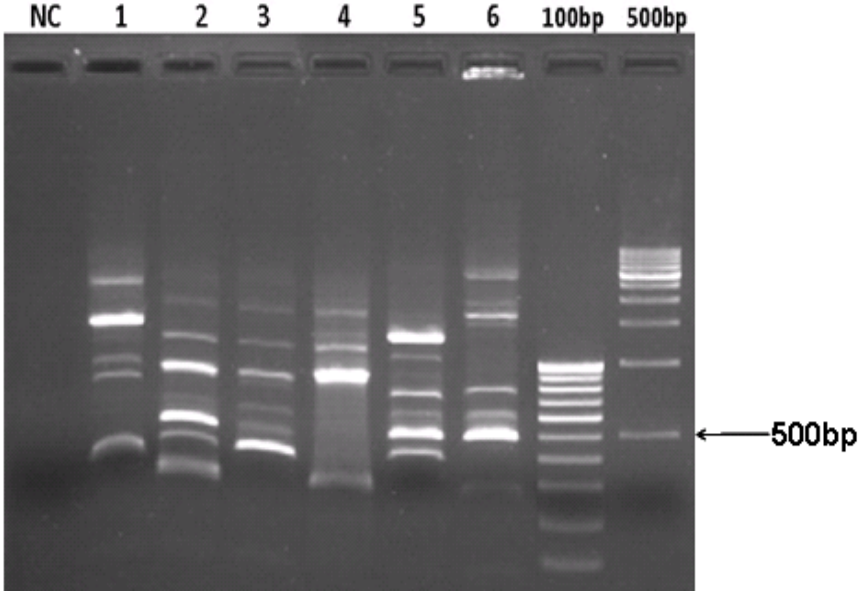


Figure 8h: Agarose Gel electrophoretogram of six different RAPD finger printing profiles of the *P. acnes* using 7th primer.

Pattern 1: 6 bands measuring 2500, 1500, 900, 800, 400, 100bp in size.

Pattern 2: 7 Bands measuring 2000, 1200, 900, 500, 400, 300, 100bp in size.

Pattern 3: 7 Bands measuring 2000, 1200, 900, 600, 500, 400, 100bp in size

Pattern 4: 5 Bands measuring 2000, 1200, 900, 300, 100bp in size

Pattern 5: 7 Bands measuring 1200, 1000, 800, 600, 500, 400, 100bp in size

Pattern 6: 7 Bands measuring 3000, 1500, 800, 600, 500, 300, 100bp in size

These different RAPD patterns obtained from different specimens indicated that they were not from a similar clone or from a same ancestral origin. Pattern 4 was the predominant type (43.0%) and majority of the isolates were from conjunctival swabs. There existed no correlation between specific banding patterns of RAPD with that of the MIC results. Higher resistance was seen among the extra ocular isolates especially the conjunctival swabs and however predominantly many conjunctival swabs exhibited pattern 4.

Reproducibility of RAPD fingerprinting:

The reproducibility of the RAPD fingerprinting technique was confirmed by comparing the reproducibility of the fingerprint patterns obtained from duplicate runs of RAPD analysis of several different *P. acnes* isolates. A single primer (7^{th} primer) was used to discriminate *P. acnes* species based on their sequence variation. The experiments were carried out twice using two different brands of thermal cyclers, and the results were resolved on the same 1% agarose gel to evaluate the reproducibility.

8.5.5. Determination of Antibiotic susceptibility by MICs:

Antibiotic susceptibility pattern of 100 *P. acnes* isolates for the 9 antibiotics tested and the MIC values are shown in Table 8e and figure 8h

Norfloxacin:

Of the 100 clinical isolates, 59 isolates (59.01%) were resistant to Norfloxacin. The most resistant isolates were associated with conjunctival swabs (80%) followed by

eviscerated material (60%) and corneal scraping (40%). The MICs for isolates ranged between <0.1 to 4mg/L.

Penicillin G:

Among 100 clinical isolates tested, 35 isolates (35.0%) were resistant to penicillin G. The degrees of resistant isolates were high with conjunctival swabs isolates (40%) followed by corneal scraping (33%) and eviscerated material (75%). The MICs for isolates ranged between <0.1 to 2mg/L.

Imipenum:

A total of 39 (39.0%) clinical isolates were resistant to imipenum out of 100 clinical isolates of *P. acnes* tested. The most resistant isolates were associated with conjunctival swabs (42%) with the MICs ranging from between <0.1 to 2mg/L.

Vancomycin:

Of the 100clinical isolates, 50 isolates (50.0%) were resistant to vancomycin. The most resistant isolates were associated with conjunctival swabs (50%) followed with eviscerated material (35%) with a MICs ranging from <0.1 to 4mg/L.

Nalidixic acid:

Of the 100 clinical isolates tested, 58 isolates (58.0%) were resistant to nalidixic acid. The most resistant isolates were associated with conjunctival swabs. The MICs ranging from between <0.1 to 4mg/L.

Ciprofloxacin: All the isolates were susceptible to ciprofloxacin.

Clindamycin:

A total of 21 (21.0%) clinical isolates were resistant to clindamycin out of 100 clinical isolates of *P. acnes* tested. Resistance to clindamycin were found to be predominant among conjunctival swabs isolates with MICs ranging from between <0.1 to 2mg/L

Cephotaxime: All the isolates were susceptible to cephotaxime.

Metronidazole: All the strains were resistant to metronidazole (100%).

Table 8e: Results of In-vitro susceptibility of 100 *P. acnes* isolates performed against nine antimicrobial agents.

Antibiotics	Susceptible MIC for range (mg/L)	MIC for isolates (mg/L)	No of isolates resistant
Ciprofloxacin	≤ 0.25 to 2 mg/L	≤ 0.1 to 4mg/L	**0 %**
Norfloxacin	≤ 0.12 to 1 mg/L	≤ 0.1 to 4mg/L	**41 (41.0%)**
Nalidixic acid	≤ 0.25 to 2 mg/L	≤ 0.1 to 4mg/L	**42 (42.0%)**
Clindamycin	≤ 0.06 to 2 mg/L	≤ 0.1 to 2mg/L	**79 (79.0%)**
Vancomycin	≤ 0.5 to 2 mg/L	≤ 0.1 to 4mg/L	**50 (50.0%)**
Cefotaxime	≤ 0.06 to 1 mg/L	≤ 0.06 to 2mg/L	**0 %**
Imipenum	≤ 0.06 to 1 mg/L	≤ 0.1 to 2mg/L	**61 (61.0%)**
Metronidazole	≤ 0.06 to 1 mg/L	≥256 mg/L	**100 (100.0%)**
Penicillin G	≤ 8 to 256 mg/L	≤ 0.1 to 2mg/L	**64 (64.0%)**

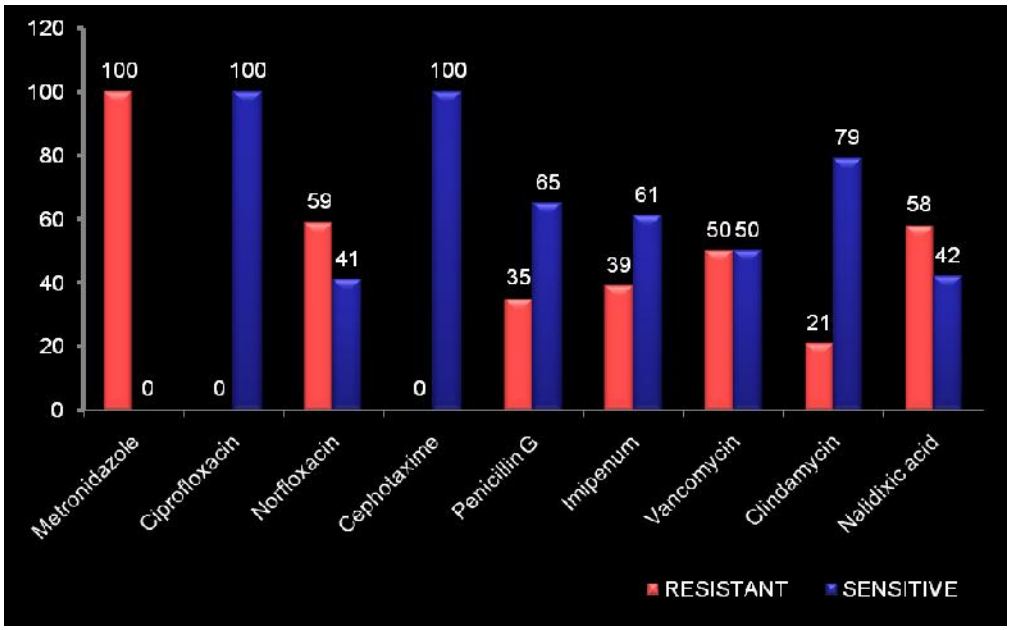


Figure 8i: Graph showing antibiotic resistance pattern (%) prevalent among ocular *P.acnes*

8.5.6. Results of *in vitro* biofilm production:

Present study indicated that many *P. acnes* strains isolated from ocular clinical specimen can form biofilms *in vitro*. Among our one hundred P. acnes isolates tested, a total of 16 isolates (16%) produced biofilm i.e. 7 conjunctival swabs, 1 corneal scraping and 3 eviscerated material (infectious endophthalmitis) isolates showed high positivity for the production of biofilm and 4 conjunctival swabs and 1 eviscerated material (infectious endophthalmitis) isolates showed moderate positivity for biofilm production. Our data also showed that the biofilm producing isolates were resistant to more than 3 antimicrobial agents compared with the *P. acnes* isolates which lacked in the production of biofilm. This could help explain the emergence of resistance due to the presence of biofilms among ocular clinical isolates of *P. acnes* antimicrobial therapy in the treatment of ocular infections. *P. acnes* isolates producing biofilm is shown in figure 8j.

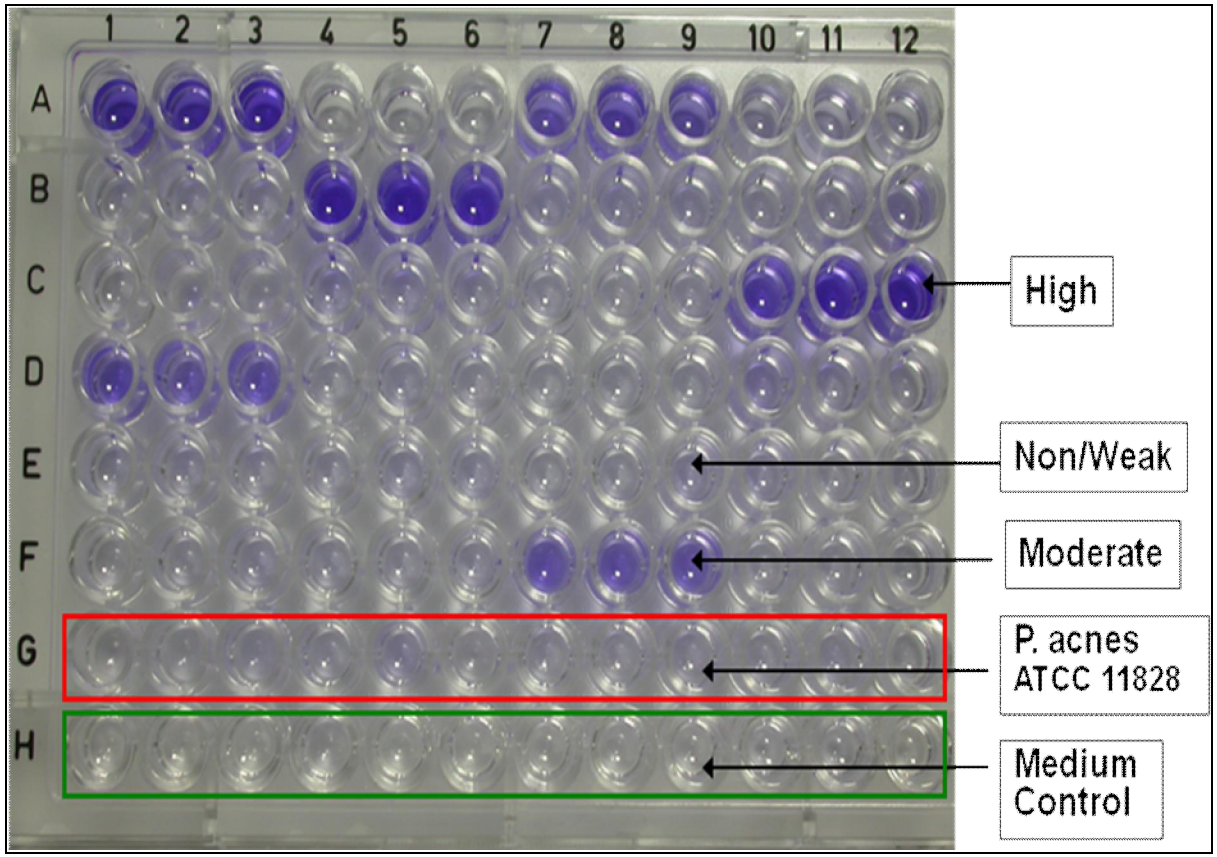


Figure 8j: 96 well plate showing Biofilm production by *P.acnes* isolates. Crystal violet staining results of *blaNDM-1* producing *A.baumannii* isolate along with the control strains and medium controls are shown.

8.6. Discussion:

Anaerobic bacterial flora of the normal human conjunctiva has been studied by numerous authors during the past 30 years. The surface microbial flora of humans is predominantly anaerobic. The pO_2 of the central vitreous cavity is very low. Anaerobic bacteria might therefore cause endophthalmitis if introduced in sufficient numbers into the vitreous cavity from the conjunctiva during surgery or trauma (Sharma et al., 1995).

P. acnes is the predominant anaerobe isolated from ocular specimens (Perkins et al., 1975). The isolated *P. acnes* were predominantly from conjunctival swabs taken from outpatients who visited the hospital for cataract surgery. The remaining *P. acnes* isolated from other specimens belonged to the patients with a previous history of injury (due to vegetable matter, thorns and metal objects), trauma (stone) in more than 50% of cases or an insect bite in 10 % cases. Another important finding is that, in 80% of corneal scraping positive for *P. acnes,* fungus was isolated in culture and in one case of corneal scraping along with *P. acnes,* viral pathogen was reported.

Majority of *P. acnes* were from the male population with a mean age of approximately 52 years in both the population. Conventional method for detecting the *P. acnes* was in 100% concordant with the uniplex PCR specific for *P. acnes.*

Dougherty et al., 1984, have reported the co-existence of *P. acnes* in blepharitis infections. In our study we could associate *P. acnes* in 11 cases of blepharitis i.e. 1/11 cases as primary pathogen, 6/11 as secondary pathogen and 4 /11 presented as a non

pathogen. In both primary and secondary blepharitis infection *P. acnes* was isolated as polymicrobial pathogen was either bacteria or fungus. Similarly in case of dacryocystitis, the rate of *P. acnes* isolation was 10%, which is slightly higher than what was reported by Brook *et al.* (1998), where as the prevalence of *P. acnes* among dacryocystitis cases were 9.4%. As inferred by Brook *et al.* (1998), the recovered anaerobes are likely of endogenous in origin because they are members of normal oral and skin flora. Their isolation may be attributable to the specific methods for specimen collection, transportation, and cultivation for anaerobes used in our study.

In a study conducted by Essex et al., (2004), 17.6 % of *P. acnes* were proven to cause post-traumatic infections. In our study 16% of *P. acnes* were isolated from trauma cases. In all these cases, *P. acnes* was determined as primary or the secondary cause of keratitis due to corneal tear following injury with stick, insect, hammer and stone injury. In 79 cases, *P. acnes* were isolated without a previous history of infection. Yet the rate of isolation of P. *acnes* was 21% from ocular disease conditions, highlights its importance in ocular infections.

DNA based molecular techniques played a significant role in the characterization ocular *P. acnes.* Phylogenetic analysis proved all the type I A, B, C and type II *P. acnes* were prevalent among the conjunctival swabs. Type II strains of *P. acnes* were predominantly isolated from conjunctival isolates and one from corneal scraping and vitreous humour sample each. The entry of conjunctival *P. acnes* into the cornea could have led to the isolation of type II *P. acnes* from the corneal scraping.

McDowell *et al*. have demonstrated the existence of type I and type II *P. acnes* in 2005, and type III in 2008 based on phenotypic, immunological and molecular methods targeting *recA gene*. In our study, we found that type I isolates can be further typed into A, B, C based on restriction sites to *Hin6I* of *16S rRNA* gene amplified products. Theses subtypes were not specific to sites of collection of clinical specimens. Type III isolates was not encountered in our study. The identification of type-specific nucleotide differences between types I and II has also revealed that DNA sequencing can be used as an accurate method for the identification of *P. acnes* types.

McDowell *et al*. (2008) have proved that the *16S rRNA* gene is considered the 'gold-standard' for investigating the phylogenetic relationship between bacterial organisms; the inability of the same to distinguish the closely related ones posed a challenge with our isolates. Our study also suggest that *recA* gene, a protein-encoding genes with housekeeping functions, provided a better foundation for bacterial systematic and differencing closely related organisms due to a higher neutral mutation rate within such genes.

The results of the PCR-based RFLP on *16S rRNA* was 100 % in concordance with DNA sequencing results and the fermentation experiments. The characteristic banding pattern was specific for type I and type II *P. acnes.* The minimum intrasequence variation was observed among *16S rRNA* gene of *P. acnes* this region and therefore, it could be used for RFLP technique which is cost effective and rapid compared to the DNA sequencing technique for typing of *P. acnes*. The predominant

RFLP pattern was Type IB (46.0%) followed by Type IC (36.0%). Conjunctival swabs exhibited majority of Type II and IA.

PCR - RAPD fingerprinting technique is a particularly powerful taxonomical tool. It is proven that the microheterogenecity of the sequences among strains arises due to continuous point mutations and other variations in the genome, which results in various RAPD patterns. This method utilizes arbitrary oligonucleotides to prime DNA synthesis at low annealing temperatures to divulge genomic diversity. Therefore this powerful tool was applied to study the heterogenecity among *P. acnes* isolates (Lin et al., 1996).

Studies reported earlier have shown the existence of only two genotypes of *P. acnes* (McDowell et al., 2005). In our study we found a total of 6 RAPD patterns, of which pattern four was predominantly present among *P. acnes* isolates obtained from various ocular specimens. There existed no specific pattern for any particular type of specimen. Our study also showed that PCR-RAPD could be applied for large-scale genotyping of isolates of *P. acnes*.

As reported earlier by Dali *et al*., (2001) all the *P. acnes* ophthalmic isolate from our study demonstrated excellent high-level in vitro susceptibilities to ciprofloxacin and cephalosporins based on the MIC data. Nayak et *al.,* (2007) has reported that the world literature review highlights the strains of *P. acnes* showing resistance not only to ciprofloxacin, but also to fourth generation fluoroquinolones like gatifloxacin and

moxifloxacin, whereas in our study we found that isolates were resistant to norfloxacin but 100 % susceptibility was seen for ciprofloxacin (Nayak N et *al.*, 2007). Almost all the patients with *P. acnes* positivity were treated with ciprofloxacin eye drops and significant clinical response has been observed.

All *P. acnes* strains (100%) were resistant to metronidazole $\geq$256 mg/L as reported by many in literature (Dali et al., 2001, Shames et al., 2006). Ophthalmic isolates of *P. acnes* showed resistance to imepenum and vancomycin with the MIC values ranging between 0.1mg/ml to 2.0mg/ml and 0.1mg/ml to 4mg/ml respectively.

In a study conducted by Mory et al., (2005) and Oprica et al., (2005) on antibiotic susceptibility pattern of *P. acnes*, 100% susceptibility to vancomycin and penicillin was reported. All these antibiotics play a vital role in treating patients with clinically suspected *P. acnes* endophthalmitis. In our study group, 50% of isolates showed resistance to vancomycin with an MIC of $\leq$ 0.1 to 4mg/L and 64% isolates showed resistance to penicillin with an MIC of $\leq$ 0.1 to 2mg/L.

Smith et al., (1996) and Mory et al., (2005) found that all *P. acnes* strains that were tested were highly susceptible to cefotaxime, imipenum and clindamycin. Our isolates showed 100 % susceptibility to cefotaxime. Resistance to clindamycin was about 79 % followed by 61% resistance to imepenum. This indicates the emergence of resistance to clindamycin and imepenum among ocular isolates.

Definitive treatment *P. acnes* endophthalmitis is controversial. Direct intravitreal instillation of antibiotics to treat endophthalmitis, caused by many anaerobic organisms has been shown to be safe and effective (Perkins et al., 1975). All the *P. acnes* ophthalmic isolates demonstrated excellent high-level in vitro susceptibilities to the ceftazidime which in reported in our previous study (Smith et al., 1996). Almost all the patients with *P. acnes* positivity were treated with ciprofloxacin eye drops and intravitreal injection of ceftazidime, cefazolin, amikacin and vancomycin achieved a significant response still a slight degree of vancomycin resistance was seen in our MIC results. Higher degree of resistance to antibiotics was observed among *P. acnes* isolated from conjunctival swabs followed by eviscerated material and corneal scrapping. Amphotericin B, natamycin and fluconozole were used as antifungal agents in treatment of polymicrobial infection of *P. acnes* with along with fungus. All the patients showed good clinical outcome.

Behlau et al., 2008, has reported the association of *P. acnes* with low pathogenicity and much delayed onset of pseudophakic endophthalmitis. Recently biofilm like deposits have been seen on IOLs with polymerase chain reaction confirmation. Contact between the lens and external tissues surrounding the incision has been observed to result in 26% of the lenses becoming colonized. The IOL appears to provide a niche where bacteria may attach to form a bio film. In our study the rate of biofilm formations was 16% among the *P. acnes* isolates and majority was from conjunctival swabs. All biofilm producing cells were resistant to more than 3 drugs antimicrobial agents compared with the *P. acnes* isolates which lacked in the production

of biofilm. This could help explain the emergence of resistance due to the presence of biofilms among *P. acnes* antimicrobial therapy in the treatment of ocular infections.

8.7. Conclusion:

In conclusion, this is the first study to have demonstrated the various subtypes of *P. acnes* by targeting *16S rRNA* among ocular isolates by PCR – RFLP techniques, a method, comparatively much cost effective than the DNA sequencing method, that can achieve differentiation of subtypes of *P. acnes* much earlier too. Thus within the scope of this study, we state that DNA sequencing is expensive whereas RFLP is cost effective with high sensitivity, specificity, reproducibility and is a more reliable method though there exist no correlation between *P. acnes* subtypes and pathogenesis pattern.

This study has proven that RAPD is an alternative rapid, reproducible, powerful genomic typing method for *P. acnes* and may definitely play a vital role in identifying epidemiology of *P. acnes.* In our study we could not establish any correlation between the antibiotic resistance pattern of the isolates and RAPD patterns. This is the first study to report on antimicrobial susceptibility by MIC method for 9 antibiotics along with the demonstration of PCR-RAPD on *P. acnes* with reproducibility of results and screening of correlation between both MIC results and RAPD pattern from a large number of ocular isolates of *P. acnes* in the literature. Vancomycin resistance among the *P. acnes* is being reported for the first time. Studies

at molecular level with vancomycin resistant isolates will help to understand the mechanism of resistance better.

This study also stands ahead in detection of biofilm production and comparison of the biofilm results with the results of antibiotic susceptibility profile along with the reproducibility of results. Compared to the conventional methods, the speed of molecular identification of *P. acnes* is faster. This can lead to much earlier application of the most appropriate therapy and minimize empiric use of our most powerful agents.

8.8. Future prospects:

Further investigations are required to explore the relationship with different subtypes of *P. acnes*, its inflammatory response, their interaction with the ocular cellular mechanism and their role in exhibiting both its virulence and pathogenicity. Such studies will pave way in the development of better knowledge of its pathogenicity, virulence mechanism and efficient antibiotic treatment along with clinical management of ocular *P. acnes* infections.

OVER ALL CONCLUSIONS:

Antibiotic resistance among ocular pathogens is increasing in consonance with increase of resistance among bacteria and fungi associated with systemic infections. The factors contributing to the development of drug resistance among ocular bacterial isolates includes overuse of antibiotics for systemic infections, increased drug resistance are improper dosing regimen, misuse of antibiotics and extended duration of therapy and finally the overuse of topical antibiotics in the eye. Centers for Disease Control and Prevention and World Health Organization have proposed strategies to preserve usefulness of antibiotics. In addition to global surveillance programs, adequate training and awareness programs should be implemented.

In this current study, predominant bacterial *spp.* isolated among ocular specimens in our hospital population was *Coagulase negative Staphylococci* (10.4%) followed by *Coagulase positive Staphylococci* (6.5%). Prevalence of bacterial infections was 36.2%; Prevalence of fungal infection was 11.5%; rate of PMI was 3.9%.

PCR based DNA sequencing along with dHPLC posses the ability to identify non-cultivable pathogen and detection of more than one microbial *spp.* present in ocular clinical specimens. Ours is the first study to identify the microorganisms from culture negative PCR positive intraocular specimens. PCR based dHPLC along with DNA sequencing revealed presence of 65 bacterial genomes in 35 intraocular specimens of which 33 (50.77%) were Gram positive, 27 (41.53%) were Gram negative and 5 (7.7%) were uncultured bacterium. Five novel genera were detected.

Methicillin resistant Staphylococci (MRS) has emerged as a dreaded organism for its wide range of resistance to several groups of antibiotics. Its prevalence in conjunctivitis is highly variable. Situation in case of coagulase negative staphylococci (CoNS), a common cause of keratitis and endophthalmitis, is no less precarious. The isolation rate of MRS was 63.6 per cent among our isolates and MR-CoNS constituted a larger portion of MRS. *S. haemolyticus*, followed by *S. epidermidis* was predominant CoNS isolated. PCR assay was superior in identifying intermediate and heterogeneous MR in shorter duration of time. PCR-RFLP of *gap* gene was found to be specific for staphylococcal spp.

Recently, serious concern has been initiated to analyze the factors contributing for high degree of resistance shown by multidrug resistant (MDR) Gram negative bacteria causing ocular infections. A few reports have reported the isolation of MDR enteric bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* from patients with endophthalmitis with poor visual outcome. Ours is the first extensive study conducted to both screen and to identify the predominant drug resistance encoding ESBL genes among the ocular isolates of *Enterobacteriaceae*. CTX-M-15 was demonstrated to be the common ESBL genotypes present among *Enterobacteriaceae* isolated in our hospital set up. Our study is first of its kind to carry out the PCR based molecular technique for screening *blaNDM-1* genes among *Enterobacteriaceae* isolates and our study proved the absence of *blaNDM-1* genes on the ocular isolates of *Enterobacteriaceae.* Overall rate of ESBL positivity was 31.5% in hospitalized patients and 68.5% in community acquired infections.

Reports on existence of *blaNDM-1* genes among bacteria causing systemic infections is being published in increasing frequency and whereas our study proved the absence of *blaNDM-1* genes among the ocular isolates of *Enterobacteriaceae.* Apart from screening drug resistance among *Enterobacteriaceae*, a special focus was given to swabs collected from multi organ donor (MOD) and Donor corneal rim specimens (DCR). The degree of resistance exhibited by these extra ocular specimens was studied.

Our study showed that the prevalence of antibiotic resistance was significantly in higher rates among Gram positive group of organisms. Gram negative isolates from DCR and MOD showed 29.1% positivity for ESBL by conventional and molecular method. A *blaNDM -1* carrying *Acinetobacter baumannii* was isolated from a multi organ donor. Hence antibiotic susceptibility testing of DCR and conjunctival swabs from multi organ donor prior to transplantation will aid in understanding antibiotic pattern and the molecular methods for resistance screening will help in reducing the turnaround time for understanding the resistant pattern and initiation of appropriate antibiotic therapy following penetrating keratoplasty.

In our study, the correlation between *blaNDM–1* positivity, and biofilm production with molecular interactions was inferred through *in silico* docking studies which proved its attribution to high level resistance. Further our study also highlighted the potential of 162-166 region in *blaNDM-1* as a vulnerable site for drug target since *blaNDM-1*, has become a significant threat to human health due to the extensive drug

resistance, leaving few or no therapeutic options thus becoming a major public health problem throughout the world, particularly in India.

P. acnes is the most predominant anaerobe isolated from ocular specimens in our hospital set up. Phylogenetic analysis of *P. acnes* proved that type I A, B, C were prevalent among the isolates studied. Type II strains of *P. acnes* were predominantly isolated from only conjunctival isolates. Our study also proved PCR-RFLP, to be a less expensive, sensitivity and specific technique for subtyping *P. acnes*. Biofilm producing cells possessed increased resistance. This could explain the emergence of resistance due to presence of biofilms in treatment of ocular infections. RAPD analysis was proved to be an alternative rapid, reproducible tool for genomic typing method for *anaerobes.* Compared to conventional methods, molecular method is faster and leads to much earlier application of appropriate therapy and minimizes empiric use of most powerful agents.

Overall our study has proved that, application of nucleic acid - based molecular methods are more sensitive to identify the bacterial agent causing ocular infections and to detect drug resistance among ocular bacteria compared to phenotypic method, and polymerase chain reaction (PCR) method is superior to the commonly used conventional techniques in the rapid and accurate diagnosis

SPECIFIC CONTRIBUTIONS:

- Rate of prevalence of bacterial infections along with the prevalence of polymicrobial infections has been reported for the first time ever.
- Our is the first study to show the application of PCR/dHPLC approach for direct detection and identification of bacterium from the Eubacterial PCR amplified products of aqueous and vitreous aspirates from patients with endophthalmitis and to differentially identify the culture negative cases and initiate appropriate therapy.
- PCR assay was superior in identifying intermediate and heterogeneous Methicillin Resistance *Staphylococcus* spp. in shorter duration of time. PCR-RFLP of *gap* gene was found to be specific for typing Staphylococcal spp..
- This is the first extensive study to have screened ocular isolates for the detection of predominant drug resistance encoding ESBL genes. *CTX-M-15* was the common ESBL genotypes and *blaNDM-1* genes was absent on the ocular isolates of *Enterobacteriaceae.*
- Correlation between *blaNDM–1* positivity, and biofilm production with molecular interactions was inferred through *in silico* docking was proved for the first time in Indian literature.
- Type I A, B, C genotypes were prevalent among the all the *P. acnes* isolates included in the current study and type II strains of *P. acnes* were predominantly isolated from conjunctival swabs only. This is being reported for the first time in our study.
- No association was observed between genotypes of *P. acnes* and the site of infection / their degree of resistance to drugs.
- PCR based molecular method has been found more sensitive in the detection of bacterial pathogens causing infections as well monitoring drug resistance exhibited by them in Indian scenario for the first time in our study.

FUTURE SCOPE OF WORK:

- ✓ Study period would further extended to have a correct knowledge about prevalence of pathogens among ocular clinical specimens and the rate of occurrence of PMI.

- ✓ PCR technique for simultaneous detection *femB* and *mecA* gene should be carried out along with routine conventional technique will aid in rapid detection of resistance from clinical isolates of Staphylococci.

- ✓ Detection of ESBL genes should be further extended among *Enterobacteriaceae* isolated from non-ocular specimens and the prevalent genotype pattern and their drug resistance pattern should be monitored.

- ✓ Povidone-iodine usage prior removing the donor eye and better storage media with antibiotic coverage will reduce the microbial contamination of the donor eye.

- ✓ *In silico* studies should be further explored which will aid in understanding its role in drug resistance in *blaNDM–1* producing strains.

- ✓ Further investigations are required to understand the relationship with different subtypes of *P. acnes*, its inflammatory response, their interaction with the ocular cellular mechanism and their role in exhibiting both virulence and pathogenicity.

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APPENDIX 1

Collection and processing of ocular clinical specimens.

1.1 COLLECTION OF CONJUNCTIVAL SWAB:

- Patient is requested to look up, the lower eye lid is pulled down using thumb with an absorbing tissue paper and moistened swab is rubbed over the lower conjunctival sac from medial to lateral side and back again.
- Sterile cotton tipped swab can be moistened in HBSS/Brain heart infusion broth (BHIB).
- One swab is used for smear preparation and second swab is transferred to the transport medium containing HBSS.
- Hundred µl of specimens from HBSS is inoculated onto each Blood agar (BA) Chocolate agar (CA) medium, Brucella blood agar (BBA).

1.2. COLLECTION OF CORNEAL SCRAPINGS:

- A drop of local anaesthetic without preservative is instilled , and with the help of the slit lamp or operating microscope , the edge of the ulcer is firmly scraped using Bard Parker blade No.15' after removal of debris or discharge in the vicinity.
- Several scrapings are collected and used in a sequence to inoculate culture media in the form of 'C' curves and prepare smears

1.3. COLLECTION OF CORNEAL BUTTON:

- The corneal button removed by surgery is sent to Microbiology Laboratory in a sterile container immediately.
- The corneal button and the corneal biopsy tissues are cut into small bits in a small sterile petridish following all aseptic precautions and inoculated onto various culture media and crushed smears are also made to be used for various staining methods.

1.4. COLLECTION OF INTRAOCULAR SPECIMENS:

- Uncontaminated VF is aspirated by a syringe connected to the suction port of the vitreous cutter at the beginning of vitrectomy.
- A sterile disposable needle is fixed to the syringe, the air in it expelled carefully without causing aerosols and the needle is capped with a sterile rubber bung and sent to the laboratory immediately.
- AH samples (150-200ml) are collected aseptically in a tuberculin syringe with a 30G needle.
- Vitreous aspirate is transported in a tube with a rubber cork inside.
- After the air in the syringe is expelled to prevent inactivation of anaerobic bacteria, the needle is fixed onto a sterile rubber bung and placed in a sterile large test tube container, which is immediately transported to the laboratory.
- Specimens should be ideally processed within 15 – 30 minutes after collection. Transport media of the conventional kind have no place in the transport of these intraocular specimens.

1.5. Processing of ocular specimens

Corneal scrapings were collected by the ophthalmologist after application of local anesthetic. The culture plates were inoculated in the form of 'C' curves by the ophthalmologist and the smears were processed for detection of fungal etiology in the microbiology laboratory. About 50 -150µl of aqueous humour and 100 - 200 µl of vitreous humour was received in the laboratory for microbiological investigations. On receipt, the specimen was divided into two equal portions. One half was used for DNA extraction and the other half was subjected to conventional methods of direct smear and culture. The specimens (AH and VF, biopsy, corneal scrapings) were processed within 30 minutes after collection for direct microscopy and culture of bacteria and fungi. Direct microbiological investigations were carried out on cytospinned smears (Cytospin 2 Shandon, Southern Products Limited, Cheshire, England) of intraocular fluids and stained by KOH-Calcofluor white for detection of fungus and Gram's stain for detection of bacteria.

Inoculation of media for culture of bacteria and fungi is to be carried out first, because the number of organisms is likely to be low. The AH and VF being normally sterile fluids, do not require selective media for culture. For culture, a variety of media are included for the favorable growth of aerobic and anaerobic bacteria and fungi. However, the number of media included depends on the volume of the sample available. For inoculation onto solid media, 1-2 drops of the specimen is expressed through the needle onto the agar plate. The material is not spread with a loop to reduce the possibility of contamination. Liquid media are also inoculated with 2 - 3 drops. The culture media

used for isolation of bacteria and fungi were blood agar and MacConkey agar (incubated aerobically at 37^0C),Chocolate agar (10% Carbon -di-oxide), Brucella blood agar (BBA, 37^0C at anaerobic work station), Sabouraud's dextrose agar (SDA) incubated at 25^0C in a cooling incubator and liquid media brain heart infusion broth (BHIB), Thioglycollate broth incubated at 37° C. Culture plates and specially the liquid medium BHIB were incubated for a period of one month to isolate fungus. The fungal isolates were identified using standard mycological methods.

APPENDIX 2

DNA extraction methods, DNA quantification, Primer reconstitution and Agarose gel electrophoresis

2.1. DNA EXTRACTION METHODS:

2.1.1. Modified Guanidine iso thiocyanate method of DNA extraction:

Reagents:

a) Lysostaphin (1µg/µl)
b) 10.0 M Guanidine iso thiocyanate (11.816g/10ml)
c) 0.1 M EDTA (0.372g/10ml)
d) 7.5 mM Ammonium acetate (5.8 mg/10ml)
e) Proteinase k (1µg/10µl)
f) Chloroform: Isoamyl alcohol (24:1)
g) Isopropanol
h) 70% Ethanol
i) Milli Q Water.

Procedure:

- 20 mg of proteinase K was weighed and dissolved in 1 ml of Milli Q Water.
- Five µl of lysostaphin (1µg/µl, obtained from Sigma-Aldrich Company- L0761- *Staphylococcus simulans*, USA) and incubated at 37°C for 1 hour.
- To it 50µl of proteinase K (1µg/10µl) was added to the bacterial culture suspension
- Incubated at 37°C for 1 hour.
- To it 150µl of each Guanidine iso thiocyanate, EDTA, 7.5 mM Ammonium acetate was added.
- Four hundred and fifty µl of Chloroform: Isoamyl alcohol mixture was added to the specimen.

- ❖ Vortexed, mixed well and centrifuged at 15,000 rpm for 15 minutes. (Cooling centrifuge)
- ❖ The supernatant was taken and the aqueous layer and transferred to another vial.
- ❖ To it added equal volumes of isopropanol. (String of DNA can be seen)
- ❖ Incubated at -20°C for 1-2 hours.
- ❖ The specimen was centrifuged at 15,000 rpm for 20 minutes. (Cooling centrifuge)
- ❖ Discarded the supernatant.
- ❖ The deposit was washed with 250µl of 70% ethanol.
- ❖ Washing was done by centrifugation at 10,000 rpm for 10 minutes thrice.
- ❖ The pellet was air dried in oven, so that no vapour of ethanol is left behind.
- ❖ After pellet was completely dried, it was reconstituted with 50µl of milli Q water.
- ❖ It was immediately stored at -20°C until it was further used for PCR

2.1.2. Bioneer kit method of DNA extraction:

Reagents:

a. Proteinase K
b. Binding buffer
c. Isopropanol
d. Washing buffer-1
e. Washing buffer-2
f. Elution buffer

Procedure:

- ✓ From stock (20mg/1ml) 20µl of proteinase K was added to 1.5 ml tube.
- ✓ To the tube, 200µl of uniform bacterial culture suspension /clinical specimen was added.
- ✓ To it 200µl of the binding buffer (GC) was added to the sample and immediately mixed by vortexing.
- ✓ The entire mixture was incubated at 60°C for 10 minutes.

- ✓ After the incubation period, 100µl of isopropanol was added to the tube and mixed well by pipetting.
- ✓ Carefully transferred the lysate into the upper reservoir of binding column tube without wetting the rim.
- ✓ Closed the tube and centrifuged at 8,000 rpm for 2minutes.
- ✓ The tube was opened and transfers the binding column tube to a new 2 ml tube for filtration.
- ✓ Then 500µl of washing buffer-1 was added without wetting the rim.
- ✓ The tube was closed and centrifuged at 8,000 rpm for 3minutes.
- ✓ The tube was then and the content from 2 ml tube collected tube was poured into the discard jar.
- ✓ Carefully added 500µl of washing buffer-2 without wetting the rim.
- ✓ The tube was closed and centrifuged at 8,000 rpm for 3min.
- ✓ Centrifugation was carried once more at 12,000 rpm for 5mins to remove the traces of ethanol.
- ✓ The binding column was transfered into new 1.5 ml tube.
- ✓ To it 100µl of elution buffer was added on to the column and wait for 3-5 minutes.
- ✓ The column was then centrifuged at 10,000 rpm for 3minutes for elution.
- ✓ Discarded the binding column and stored the DNA at -20°C for future use.
- ✓ Calculated the amount of DNA by finding the OD at 260nm.

2.1.3. Qiagen Mini kit method of DNA extraction:

Reagents:

a. Proteinase K
b. Auto Lysis buffer (AL)
c. Ethanol
d. Washing buffer-1 (AW -1)
e. Washing buffer-2 (AW -1)
f. Elution buffer (AE)

Reconstitution of Buffer:

AW1 Buffer: To be reconstituted with 25 ml Ethanol (96-100%).

AW2 Buffer: To be reconstituted with 30 ml Ethanol (96-100%).

Procedure:

- Initially, 20µl of proteinase K was added to 1.5 ml tube.
- To it 200µl of the specimen is added along with 200 µl of the Lysis buffer (AL)
- The solution was vortexed and incubate at 56ºC in a water bath for 10 minutes
- To the solution 200 µl of 95-100% Ethanol is added.
- The content was mixed gently and the solution is transferred to the column.
- The column was centrifuged at 8,000 rpm for 1 minute.
- The column was replaced with a fresh collection tube and was washed with 500µl of wash buffer 1 (AW1) by centrifuging at 8,000 rpm for 1 minute.
- The column was replaced with a fresh collection tube and was washed with wash buffer 2 (AW2) by centrifuging at 14,000 rpm for 3 minutes.
- The eluted solution is discarded and the column is centrifuged at 14,000 rpm for 1 minute.
- The column was replaced with a sterile vial and to the column 200µl of the elution buffer (AE) is added and incubated at room temperature for 1 minute.
- The column is centrifuged at 8,000 rpm for 1 minute. The eluted DNA was stored at -80ºC.

2.2. QUANTIFICATION OF DNA:

- One µl of the extracted DNA was dissolved in 999µl of water.
- The entire volume is taken for DNA quantification.
- The DNA was quantified spectrophotometrically at 260nm and 280nm.
- The reading at 260nm gives the nucleic acid concentration.
- The ratio of readings at OD 260nm/ 280nm gives the purity of the nucleic acid.

2.3. PRIMER DILUTION:

The lyophilized primer was reconstituted in 100µl of sterile Milli Q water. One microlitre of this primer was diluted to one millilitre in Milli Q water and was spectrophotometrically read at 260nm. One optical density (OD) corresponds to 33µg/ml of primer. The concentration of primer was found from the corresponding OD value. Based on the molecular weight and concentration of the primer it was diluted to the required concentration as mentioned in the detailed procedure for each of the PCRs in the methodology section.

2.4. AGAROSE GEL ELETROPHORESIS –

Preparation of reagents:

2.4.1. Preparation of 10 x TBE buffer:

Tris : 54.1g

EDTA : 3.65g

Boric acid : 27.8g

MQW : 500ml

pH : 8.00

Add Tris to 200ml of milli Q water and dissolve it completely. Then add EDTA and Boric acid. The addition increases the level of water. Remaining volume should be made up with milli Q water so that the final volume is 500ml.

2.4.2. Preparation of Tracking Dye for agarose gel electrophoresis*:*

Solution A - 0.1% Bromophenol blue in 1x TBE buffer

Solution B - 40% sucrose in 1x TBE buffer

Prepare solution A and B separately and equal volume was mixed.

2.4.3. Preparation of 2% Agarose Gel:

- 2 % Agarose gel was prepared in Tris Boric acid EDTA buffer by melting in the Agarose the oven.
- 20 mg of the Ethidium Bromide was added to the melted Agarose in a final concentration of 0.5mg/ ml and mixed well.
- This was cast onto a gel trough already sealed on both the sides with cellophane tape and fixed with gel combs of the desired well size
- The gel was allowed to solidify in dark to reduce the photo activation of Ethidium Bromide
- The entire 5µl of the sample mixed along with 5 µl of the Bromophenol blue and loaded onto the wells.
- The amplified products were loaded in order and results were read comparing the molecular weight marker (*Hinf I* digest or 100bp marker)
- The molecular weight marker loaded contains 1µl of molecular weight marker, 7µl of 1 X TBE and 2 µl of bromophenol blue.
- The electrophoresis was run at 150 volts for half an hour.
- Then gel was seen under the transilluminator under UV illuminescence.

APPENDIX 3

Protocol for Cycle sequencing of and purification of amplified products

3.1. SEQUENCING IN ABI PRISM 310/3100 AVANT GENETIC ANALYZER:

Sequence of PCR amplified DNA is deduced with the help of ABI Prism 3100 AVANT genetic analyzer that works based on the principle of Sanger dideoxy sequencing.

3.1.1. Requirements:

1. Forward and reverse primers.
2. ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit:
    a) Deoynucleotide triphosphate
    b) Ampli *Taq* DNA polymerase
    c) FS, Thprophosphatase
    d) Magnesium chloride
    e) Dye terminators and buffer

3.1.2. Principle:

The enzymatic PCR reaction involves amplification of the target gene sequence in multiple copies with the help of *Taq* polymerase enzyme that helps in the addtiion of the nucleotides to the 3'end thus resulting in chain elongation through the formation of phosphodiester bond between the 3' hydroxyl end at the growing end of the primer and the 5' phosphate group of the incoming deoxynucleotide. Thus a block in this phospho di ester bond results in abrupt chain termination that is aided by the addition of dideoxy nucleotide tri phosphate. This method was devoloped by Sanger et al in 1981. Thus at the end of the reaction, a pool of products with the base pair length ranging from 1 to the total product size were separated by electrophoretic methods.

Fluroscent based detection by automated sequencer adopts Sangers method and incorporates the fluorescent dyes into DNA extension products using 5'-dye labeled primers or 3'-dye labeled ddNTPs (dye terminators called commercially as RRMIX). The dye terminators contain a fluorescein donor dye, 6 -carboxyfluorescein (6, FAM), linked to one of the four dichlororhodamine acceptor dyes. The excitation maximum of each dye label is that of the fluorescent donor and the emission spectrum is that of the Rhodamine acceptor. Each dye emits light at a different wavelength when extited by an argon ion laser. The donor dye is optimized to absorb the excitation energy of the argon on laser in the PE amplified Biosystem DNA sequencing instrument. All four colours and therefore all four bases can be detected and distinguished in a single gel lane or capillary injection.

The amplified products with the dye at the terminated 3'end were subjected to capillary electrophoresis by an automated sample injection. The emitted flurorescence from the dye labels on crossing the laser area were collected in the rate of once per second by cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing that are analysed by software called as the sequence analysis softwares (Sequence Navigator in ABI 310 and seqscape manager in ABI 31000 AVANT machine).

Table 1: Four nucleotide bases with the respective acceptor dyes and colour emission.

Terminator	Acceptor dye	Color of raw data on ABI prism 310 electrophoretogram	Analyzed data
A	Dr6G	Green	Green
C	Dr0X	Red	Blue
G	Dr11O	Blue	Black
T	dTAMRA	Black	Red

Table 2. Protocol for cycle sequencing reaction:

Components	Volume (µl)
Amplfiied products	2.0
Sequence buffer	2.0
Primer (2pmoles/µl)	2.0
RRMIX	2.0
Water	2.0

3.1.3. Cycle sequencing profile:

Initial denaturation	- 96°C for 1min	
Denaturation	-96°C for 10 seconds	25 cycles
Annealing	-50°C for 5 seconds	
Extension	-60°C for 4 minutes	
Final extension	- 4°C	

3.1.4. Purification of cycle sequencing products:

The extension products were purified to remove the unincorporated dye terminators before sujecting the samples to capillary electrophoresis. Excess dye terminators in sequencing reations obscure data in the early part of the sequence and can interfere with base calling.

1) To the 10 µl of cycle sequencing product, 10 µl of MQW, 2 µl of 3 M Sodium acetate (pH 4.6) , 2 µl of 125 mM EDTA and 50 µl of chilled ehanol was added in a vial
2) The vials were cyclomixed, spun down and incubated at room temperature for 15 minutes.
3) After 15 minutes of incubation the vial was spin down at 12,000 rpm for 20 minutes to precipitate the amplified product and remove the unutilized ddNTPs, primer (short length molecules) etc..

4) The pellet was washed twice with 250µl of 70 % of chilled ethanol and centrifuged at 10,000 rpm for 10 minutes.
5) The vial containing the pellet was then dried at 37°C for 1 hour until no ethanol vapor is left behind.

Before loading the sample into the sequencer machine 20µl of Hidi formamide was added to the dried pellet vial cyclomixed and spun down. The samples were heated at 90°C for 3 minutes and then loaded in the sample tray provided in the ABI 3100 AVANT Genetic Analyzer (Applied Biosystem, USA.) with polymer POP7 and sequenced. The sequences were then analyzed in Sequence Navigator software (version 1.0.1; ABI Prism 310). The sequences were then analyzed with the help of BIO EDIT sequence alignment software and CHROMAS and finally blasted with NCBI to identify the species and to find the homology.

3.1.5. PREPARATION OF PURIFICATION REAGENTS

- **500mM EDTA:** 18.6 g of EDTA in 100 ml Milli Q water
- **125mM EDTA:**1µl of 500mM EDTA + 3 µl Milli Q water
- **3 M sodium acetate: (pH 4.6) :** 2.46g of sodium acetate was first dissolved in 5 ml of water and then pH was adjusted to 4.6 with acetic acid. Then the volume was made upto 10ml with water.

LIST OF PUBLICATIONS

1. J. Malathi, **M. Sowmiya**, K. Lily Therese, HN. Madhavan. Application of PCR based – RFLP technique for species Identification of ocular isolates of methicillin resistant *Staphylococci* (MRS). *Indian J Med Res.* 2009;130(1):78-84

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3. **Sowmiya M**, Malathi J, Madhavan HN, Padma Priya P, Therese KL. Ocular *Propionibacterium acnes*: A study on Antibiotic Susceptibility Profiling and their epidemiological pattern. *The Internet journal of Microbiology*, 2011;9(2). DOI: 10.5580/fd5.

4. Malathi J, **Sowmiya M**, Khetan V, Therese KL and Madhavan HN. Complication of *Salmonella* Bacteremia in a Case of Treated Fungal Endophthalmitis. *Case Reports in Ophthalmological Medicine.* 2012. Vol. 2012, Article ID 198637, 3 pages, doi:10.1155/2012/198637

5. **Sowmiya M**, Malathi J, Vaidehi T, Therese KL, Rajagopal R, Padmanaban P, *et al.* A Study on Isolation Rate and Prevalence of Drug Resistance among Microorganisms Isolated from Multiorgan Donor and Donor Corneal Rim along with a Report on Existence of *blaNDM-1* among Indian Population. *Curr Eye Res.* 2012;37(3):195-203.

6. **Sowmiya M,** Umashankar V, Muthukumaran S, Madhavan HN, Malathi J. Studies on New Delhi Metallo-Beta-Lactamse-1 producing *Acinetobacter baumannii* isolated from Donor Swab in a tertiary eye care centre, India and structural analysis of its antibiotic binding interactions. *Bioinformation.* 2012;8(10):445-452.

7. **Sowmiya M**, Malathi J, Madhavan HN. Screening of Ocular Enterobacteriaceae Isolates for Presence of Chromosomal Blandm-1 and ESBL Genes: A 2-Year Study at a Tertiary Eye Care Center. *Invest Ophthalmol Vis Sci.* 2012;53(9):5251-7.

LIST OF PRESENTATIONS

1. **Oral presentation** at ASIA-ARVO International meeting on Research in Vision and ophthalmology, 2009, at Hyderabad, India on January 2009 on paper entitled "Analysis of mutations in QRDR region of *gyrase A* gene of bacteria belonging to Enterobacteriaceae isolated from ocular specimens by PCR based DNA sequencing"

2. **Poster presentation** at National conference on "Emerging trends in Life Science Reaserch" held at BITS, Pilani, 2009.

3. **Poster presentation** at IERG conference on "Application of Polymerase Chain Reaction (PCR) based DNA Sequencing for the Detection of Extended Spectrum of Beta Lactamases (ESBL'S) Genes among Ocular Specimens" held at LVPEI, Hyderabad, 2009.

4. **Oral presentation** at MICROCON 2010 at Swabhoomi, Koltata by IAMM National conference on "Genotypic Analysis of *Propionibacterium acnes* Ophthalmic Isolates and Their Antibiotic Susceptibility Profiling.

5. **Oral presentation** at Indian Association of Medical Microbiologists TN & PY Chapter Meet held on July 2011 at Aravind Eye hospital, Madurai on the paper entitled on "Prevalence of Extended Spectrum Beta-Lactamase (ESBL) producing and integrons carrying Gram negative bacilli belonging to *Enterobacteriaceae* among Ocular isolates - A hospital based study" in the CME on Ocular Infections 2011"

6. **Oral presentation** at Indian Association of Medical Microbiologists held on November 2011 at Institute of Medical Sciences, BHU, Varanasi on the paper entitled on **"**A hospital based study on microbiological and DNA based molecular analysis of *Propionibacterium acnes isolated from* ocular specimens**".**

BRIEF BIOGRAPHY OF THE CANDIDATE

Ms. M. Sowmiya, completed her under graduation B.Sc Microbiology from Valliammal college for women in the year 2004. She joined the off campus MS Medical Laboratory Technology conducted by BITS, Pilani and graduated in the year 2007. She worked as a Junior Scientist at L&T Microbiology Research Centre, Chennai from July 2007 to August 2007. She joined as a Senior Research Fellow at L&T Microbiology Research Centre in September 2007 till date. Meanwhile she registered for PhD in BITS, Pilani in November 2008 under the guidance Dr. J. Malathi. She has made 4 oral and 2 poster presentations in National and International conferences. She has 7 publications. She has been responsible for conducting practical and theory classes in microbiology and immunology for the under graduate (BS Optometry) and post graduate (MSMLT) students registered under off campus courses of BITS Pilani. She was also involved in training the post graduate students from other institutes in various molecular biological techniques. She has totally 8 awards. She was awarded the Dr H. N. Madhavan Endowment Award for the Best Outgoing Student -2007 in MSMLT, Ranbaxy Laboratories Ltd. Immunodiagnostic Division Endowment Award for the Best performance in Clinical immunology, Hi Media Laboratories Pvt. Ltd Endowment Award for the Best performance in Clinical Microbiology, Bangalore Genei Pvt. Ltd. Endowment award for the Best performance in Clinical Genetics, Biochemistry Research Dept Silver Jubilee year – Endowment award for the Best performance in Biochemistry internship, Sankara Nethralaya Silver Jubilee award - Best performance in Diagnostic Microbiology, Best Oral presentation award in ASIA-ARVO 2009, International meeting on Research in Vision and ophthalmology, 2009, at Hyderabad, India on January 2009 on paper entitled “Analysis of mutations in QRDR region of *gyrase A* gene of bacteria belonging to Enterobacteriaceae isolated from ocular specimens by PCR based DNA sequencing and finally VRF – IAMM Young Best Paper Award TN & PY 2011 for the paper entitled on “Prevalence of Extended Spectrum Beta-Lactamase (ESBL) producing and integrons carrying Gram negative bacilli belonging to *Enterobacteriaceae* among Ocular isolates - A hospital based study” in the CME on Ocular Infections 2011” of Indian Association of Medical Microbiologists TN & PY Chapter Meet held on 23rd July 2011.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. J. Malathi completed her PhD from Birla Institute of Technology and Science in 2003. At present she is the Reader in the Department of Microbiology, L & T Microbiology Research Centre, Vision Research Foundation, Chennai. She has 13 years of rich experience in Medical Microbiology with 14 year postgraduate teaching in Medical microbiology with 34 publications in national and international journals. She is a recognized Ph. D guide in BITS-Pilani, and SASTRA. She had been involved in the initiation of Reverse Transcriptase PCR and Real Time PCR development of nucleic acid–based molecular biological methods in L & T Microbiology Research Centre, Vision Research Foundation, Chennai. She is Principal Investigator of 2 projects and Co- Principal Investigator of 4 Research projects sanctioned by ICMR and DBT. Her areas of special interest are development of rapid diagnostic tests based on molecular techniques for rapid detection of infectious agents and diagnostic DNA chip for infectious diseases and to study the Molecular epidemiology of specific infectious diseases particularly related to ophthalmic infections, studying bacterial pathogenic mechanism and drug resistance. She has 40 publications in peer reviewed National and International journals. Two patents have been applied. She had done 25 presentations in National & International conferences, Invited academic lectures, National & International Symposia; CME programmes. She is a Life Member of Indian Association of Medical Microbiologists.