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STUDY ON BIOFILM FORMING ABILITY OF CERTAIN SPECIES OF *STAPHYLOCOCCI* AND EFFECT OF ANTIBIOTICS ON THEM

Dissertation submitted to
The Tamil Nadu Dr. M. G. R. Medical University,
Chennai

in partial fulfillment of the award of degree of

MASTER OF PHARMACY

(PHARMACEUTICAL BIOTECHNOLOGY)

Submitted by
R. SENTHILRAJ

Under the guidance of
Dr. S.KRISHNAN, M. Pharm., Ph.D.,
Professor & Head,
Department of Pharmaceutical Biotechnology

MARCH – 2010

COLLEGE OF PHARMACY
SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES
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in the Department of Pharmaceutical Biotechnology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, which is affiliated to **The TamilNadu Dr. M.G.R. Medical University, Chennai**, under my direct guidance and to my fullest satisfaction.

Dr. S. KRISHNAN, M.Pharm., Ph.D.,
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Place: Coimbatore

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Date:

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ABBREVIATIONS

ATCC	-	American Type Culture Collection
ATP	-	Adenosine Tri Phosphate
CFU	-	Colony Forming Units
CLSM	-	Confocal Laser Scanning Microscopy
CRA	-	Congo Red Agar
CTC	-	5-Cyano-2, 3-ditolyl Tetrazolium Chloride
CV	-	Crystal Violet
DMMB	-	DiMethyl Methylene Blue
EDTA	-	Ethylene Diamine Tetra Acetic acid
EPS	-	ExoPolySaccharides
FDA	-	Fluorescin Di Acetate
Ica	-	Intracellular Adhesion
MBC	-	Minimal Bactericidal Concentration
M-EDTA	-	Minocycline- Ethylene Diamine Tetra Acetic acid
MIC	-	Minimal Inhibitory Concentration
Mmol	-	Milli mole
MRD	-	Modified Robbin's Device
NCIM	-	National Collection of Industrial Microorganisms
TCP	-	Tissue Culture Plate
TM	-	Tube method
TSA	-	Tryptone Soy Agar
TSB	-	Tryptone Soy Broth
TTC	-	2,3, 5 Triphenyl Tetrazolium chloride
XPS	-	X-Ray photo electron spectroscopy
XTT	-	Sodium salts of 2,3bis (2methoxy 4nitro -5 sulpfophenyl) 2, 4 Tetrazolium 5 carboxanilide

OBJECTIVE OF THE WORK

During the past 20 years it has been reported that between 6 -14% of patients enter general hospitals develop a nosocomial infection. Overall, upto of 60% of all nosocomial infections are due to biofilms. This makes biofilm-related infections a major cause of morbidity and mortality and frequently the only solution to an infected implanted device is its surgical removal which bears additional economic and health costs. The ability of biofilm formation is one of the most important virulence factors occurring in microbes. The *Staphylococcus species* is one of the most important etiologic agents of hospital infections associated with biofilm formation on different indwelling medical devices.

A wide range of antimicrobial compounds are used to remove biofilm structure e.g. antibiotics, disinfectants or antiseptics and other non-antibiotic agents; their effectiveness depends on the properties of the biofilm forming bacteria and also the age of biofilm. Medical devices treatment with antimicrobial agents and antimicrobial locks are some methods involved in the destruction of matured biofilm or prevention of its formation.

Thus the objectives of our study were

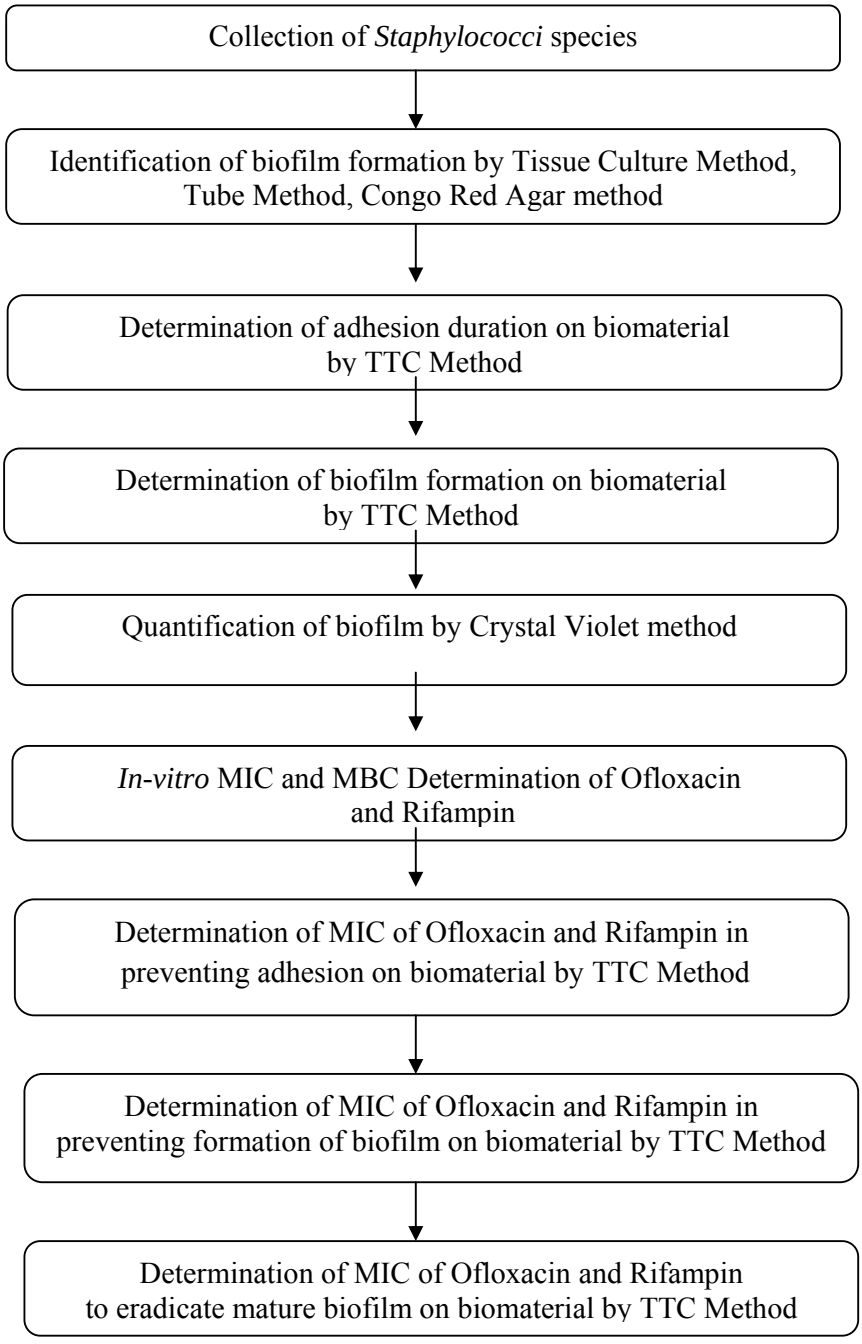
- To study biofilm forming abilities of certain *Staphylococcal sp.* by various methods like**:** Tissue Culture Plate method (TCP), Tube Method (TM) and Congo Red Agar method (CRA).

- To study the duration of adhesion and biofilm formation on biomaterial *in-vitro* by TTC method.

- To quantify the biofilm formation by using Crystal Violet method.

- Determination of Minimal Inhibitory Concentration (MIC) of Ofloxacin and Rifampin by broth dilution method and evaluate the effects of Ofloxacin and Rifampin on Adhesion, biofilm formation and eradication of mature biofilm on biomaterial *in- vitro* by TTC method.

SCHEME OF WORK



ABSTRACT

In our study, biofilm forming ability of *Staphylococcal* sp. was screened by using Tissue Culture Plate method (TCP), Tube Method (TM) and Congo Red Agar (CRA) methods. The biofilm forming abilities of three *Staphylococcal* sp. designated S-II, S-III and S-IV were compared with reference strain S-I. S-II exhibited strong biofilm producing ability when studied using all the three methods. The strain S-III exhibited strong biofilm producing ability in TCP and TM method but biofilm producing ability was poor as studied by CRA method. The strain S-IV exhibited moderate biofim producing ability in TCP method but biofilm producing ability was poor in TM and CRA methods. The adhesion process and biofilm formation on biomaterial were determined using 2, 3, 5 Triphenyl Tetrazolium Chloride (TTC) method. All strains were able to form biofilm on biomaterial between 72 to 96 hrs, irrespective of adhesion duration (S-I 12hrs, S-II 24hrs, S-III 24hrs and S-IV 48hrs). The concentration range of Ofloxacin in preventing adhesion process was found to be 3.9 to

62.5µg/ml. Biofilm formation was prevented at 15.6 to 125 µg/ml and to eradicate mature biofilm on biomaterial, 125 to 500 µg/ml was needed against all strains. The concentration range of Rifampin in preventing adhesion process was found to be 0.04 to 0.78µg/ml. Biofilm formation was prevented at 0.78 to 3.12 µg/ml and to eradicate mature biofilm on biomaterial 6.25 to 25 µg/ml concentrations was needed against all strains.

INTRODUCTION

The successes of modern medicine are closely linked to the ever-increasing use of implanted biomedical devices for the intermittent or permanent substitution of failing organs or for management of vital functions of critically ill patients in the intensive care unit. A major complication of the use of these devices is infection affecting upto millions of patients worldwide each year. Biomaterial-associated infections are caused in most instances by *Staphylococci,* mostly *Staphylococcus epidermidis* and *Staphylococcus aureus,* and do not respond properly to antimicrobial treatment, regularly requiring the removal of the infected biomaterial and leading to substantial morbidity and mortality **(Mack D., *et al* 2004).**

In the 17th century, the discovery of microbial biofilms can be attributed to Anton van Leeuwenhoek, who first observed microorganisms in the plaque on his own teeth, using his simple microscope. Later, in the 20th century, Heukelekian and Heller in 1940 and Zobell in 1943 showed that bacterial growth and activity were

considerably enhanced by the presence of a surface to which bacteria could attach and that the number of microorganisms on surfaces was significantly higher than in the surrounding medium (**Costerton, 1999).**

The biofilm can be defined as a community of bacteria, that is irreversibly attached to a biotic or abiotic surface and that is enclosed in a matrix of exopolymeric products. This matrix is composed of a mixture of extracellular polymeric substances (EPS), such as polysaccharides, proteins, nucleic acids and other substances **(Donalan R.M *et al.*, 2002).**

Bacteria and the need to form Biofilm

According to Darwin's theory of evolution, the only true driving force behind the course of action of any organism is reproductive fitness. Any action that increases proliferation will endure within a species. Therefore, when we talk about the driving force behind

biofilm formation we are asking the question ‘‘How does the biofilm mode of growth promote survival and propagation of the cell?’’ It almost seems counter-intuitive that the biofilm mode of growth could confer productive fitness advantage when one considers that biofilm bacteria have a reduced rate of growth relative to bacteria growing planktonically in broth culture. Outside of the laboratory, however, bacteria rarely, if ever, find themselves in an environment as nutrient rich as culture media and in these less-than-ideal conditions, there are a number of fitness advantages imparted by the biofilm mode of growth. The formation of bacterial biofilms must, necessarily, begin with the adhesion of a small number of bacterial cells to a surface. Many laboratory-adapted strains have virtually lost their ability to adhere to surfaces because, each time they are transferred into fresh medium, the inoculum for this operation is taken from the bulk fluid and any bacterial cells that are adherent to the walls of the vessel are left behind **(Jefferson K.K., 2004).**

Process of biofilm formation

Attachment.

Microcolony formation.

Formation of three-dimensional structure and maturation.

Detachment .

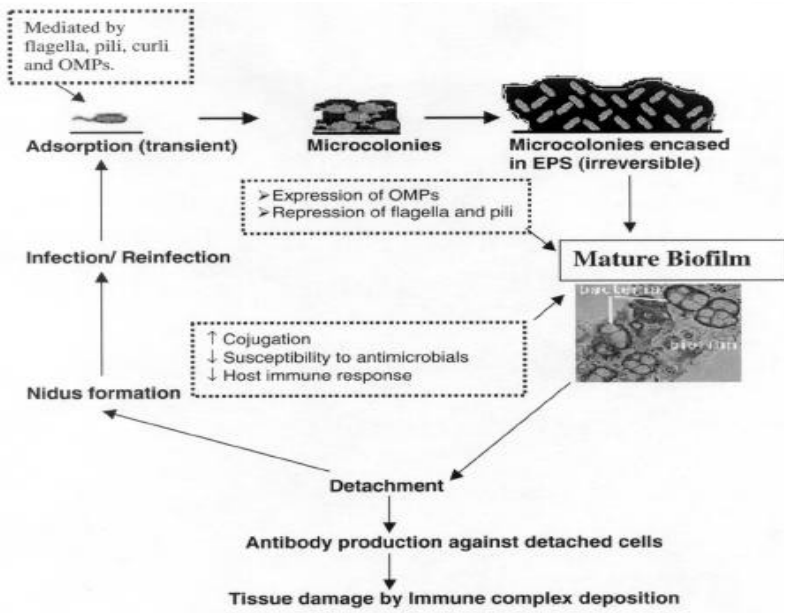


Fig. 1:s Schematic representation of steps in biofilm formation and its consequences

Attachment

The initial microbial adherence is mostly dependent on bacterial cell surface characteristics and the nature of the material surface. In its most fundamental form, bacterial adhesion can be divided into two main phases: Primary or Docking phase and the Secondary or locking phase. In some cases, an additional surface conditioning step is added to this process Conditioning occurs when the biomaterial is inserted in the human body and interacts with the surrounding environment. As a result, the native surface is modified by the adsorption of host-derived matrix proteins such as albumin, fibronectin or fibrinogen. When this process occurs, surface properties become permanently altered, in a way that the affinity of an organism for a conditioned surface can be quite different than for a naked one.

Primary adhesion is a reversible stage that occurs when the bacterial cells first meet the surface. This is determined by a number of physiochemical variables that define the interaction between the bacterial cell

surface and the conditioned surface of interest. First, the organism approaches the surface, either randomly or directed by chemotaxis and motility and when it reaches a critical proximity to the surface (usually <1 nm), the final determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two surfaces. These forces include electrostatic hydrophobic interactions, Van der Waals forces and acid-base interactions. Electrostatic interactions are likely to favor repulsion, because most bacteria and inert surfaces are negatively charged. The physical interactions are further classified as long-range and short-range interactions. The non-specific long-range forces include Van der Waals forces, electrostatic forces, acid-base interactions, and Brownian motion forces and are a function of the distance and free energy (distances >50 nm) Short-range specific interactions operate in highly localized regions of the interacting surfaces (distances <5nm) and include hydrogen bonding, ionic and dipole interactions. In accordance, as reported by Marshall in 1985, at separation distances greater than approximately 50 nm, Van der Waals forces are the only ones operative

since this distance is considered to be too large for the opposing surfaces to recognize specific surface components. However, at separation distances between approximately 10 and 20 nm, only secondary minimum interactions occur as a result of Van der Waals and electrical double layer forces and adhesion is probably reversible. At separation distances of less than about 1.5 nm, a great variety of specific or nonspecific short range forces can occur, leading to irreversible adhesion. Cell and substratum surfaces hydrophobicity can here play a major role in removing water films between the interacting surfaces enabling short-range interactions to occur. Roughness and chemical composition of the biomaterial surface itself are also important parameters involved in this initial stage of adhesion

The second stage of adhesion is the anchoring or locking phase and employs molecularly mediated binding between specific adhesions and the surface. All bacteria produce multiple adhesions, and some are regulated at the transcriptional level, permitting organisms to switch from sessile to planktonic forms under different environmental influences. At this point,

loosely bound organisms consolidate the adhesion process by producing exopolysaccharide that complex with surface materials and/or receptor-specific ligands located on pili and fimbriae or both **(An Y.H *et al.*, 1997, Dunne W.M, 2002).**

Micro colony formation

After the bacteria adhere to the inert surface/living tissue, the association becomes stable for microcolony formation. The bacteria begin to multiply while emitting chemical signals that intercommunicate among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to the formation of a microcolony.

Formation of three-dimensional structure and maturation

During the attachment phase of biofilm development, perhaps after microcolony formation,

the transcription of specific genes takes place. These are required for the synthesis of EPS. Attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded, followed by formation of water-filled channels. It has been proposed that these channels constitute primitive circulatory systems, delivering nutrients to and removing waste products from the communities of cells in the microcolonies.

Detachment

Mature biofilms can then undergo a detachment process, due to the exposure to strong mechanical and hydrodynamic forces and to quorum sensing regulation, releasing planktonic bacteria that can then colonize another region of the substratum to form new microcolonies **(Prakash B. *et al.*, 2003).**

Composition of biofilm

Concerning the biofilm composition water is considered to be the major component of the biofilm matrix up to 97% while bacteria occupy only between 10 to 50% of the total volume of the biofilm. EPS

account for 50 to 90% of the total organic carbon of biofilms. Besides polysaccharides, proteins, nucleic acids or phospholipids, non cellular materials such as mineral crystals, corrosion particles or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix.

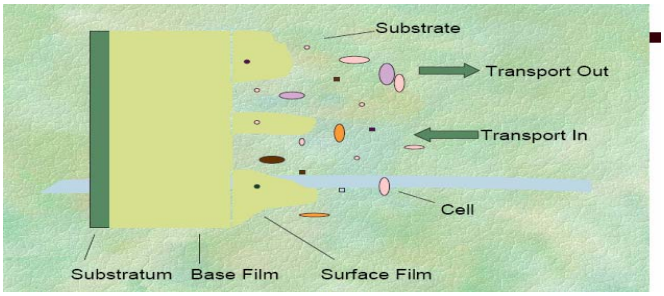


Fig.2: Composition of biofilm

In biofilms, the cells exchange information by signals, denominated "quorum sensing", which are chemical signals, used to regulate cell density-dependent gene expression. The quorum sensing signal molecules are peptides, comparable to pheromones and hormones that controls cell division and thereby the biofilm population density as well as the production of exopolymeric matrix **(Donlan R.M *et al.*, 2002).**

Regulation of biofilm formation

Substratum Effect

The extent of microbial colonization appears to increase as the surface roughness increases due to the diminished surface area and higher surface area on rougher surfaces. Maximum attachment depends upon high surface free energy or wettability of surfaces. Surfaces with high surface free energies such as stainless steel and glass are more hydrophilic. These surfaces generally show greater bacterial attachment than hydrophobic surfaces such as Teflon, Buna-n rubber and fluorinated hydrocarbon.

Conditioning Film

Solid surfaces which have been exposed in an aqueous medium become conditioned or coated with polymers from the medium. The chemical modification of surfaces affects the rate and extent of microbial attachment. The surface is converted to hydrophilic by cleaning with alkali or strong acid (4M nitric acid) of

stainless steel surfaces. Once the stainless steel is exposed to air or water, it is passivated by the formation of a chromium oxide layer. Organic soil adheres to the oxide layer, producing a conditioned substratum to which bacteria adhere. Another prime example is "acquired pellicle" which develops on tooth enamel surfaces in oral cavity. It consists of albumin, lysozyme, glycoprotein, phosphoproteins, lipids and gingival screvice fluid. Bacteria, from oral cavity, colonize pellicle-conditioned surfaces within hours of exposure to these surfaces. A number of host-produced conditioning films such as blood, saliva, tears, urine, intravascular fluid and respiratory secretions influence. The attachment of bacteria to biomaterials. The surface energy of the suspending medium may affect hydrodynamic interactions of the microbial cells with surfaces by altering the substratum effects.

Hydrodynamics

Biofilms have also been examined under various hydrodynamic conditions such as laminar and turbulent flow. It is found that biofilm response is altered in flow conditions. Biofilms grown under laminar flow

are found to be patchy and consist of rough cell aggregates separated by interstitial voids. Biofilms grown under turbulent flow cells are also patchy but are elongated "streamers" that oscillate in the bulk fluid. Association of cells with the surface also depends on cell size and cell motility.

Characteristics of Aqueous Medium

Physico-chemical characteristics of aqueous medium such as pH, nutrient levels, ionic strength and temperature, etc. may play an important role in the rate of microbial attachment to the surfaces. The bacterial attachment and biofilm formation in different aqueous systems are affected by season. This may be due to the temperature of water or other seasonally affected parameters. It is found that an increase in concentration of several cations such as sodium, calcium, lanthanum and ferric ions affects the attachment of *P. fluorescence* by reducing the repulsive forces between the cell and glass surfaces.

Horizontal Gene Transfer

Horizontal gene transfer is important for the evolution and genetic diversity of natural microbial communities. During the evolution, and adaptation of bacteria to new environment often results in the acquisition of new genetic traits via horizontal gene transfer rather than accumulation in modification of gene function by mutations. The mobile genetic element mediates horizontal gene transfer between bacteria. These elements can be conjugative plasmids, transposons or bacteriophages. Bacteria in biofilms expressing different phenotypic characters from planktonic counterparts. This is due to different genes transcribed in the planktonic and biofilm-associated phases of bacterial life cycle. Some genes may be expressed in response to a specific surface on which bacterium has chosen to settle. Many marine *Vibrio* species survive by attachment and degradation to chitin. The structural genes responsible for attachment to chitin differ from those required for attachment to abiotic, non-nutritive surfaces such as plastics and glass.

Bacillus subtilis strain harboring conjugative transposons which confers resistance to tetracycline was introduced to the system and resistance profile of biofilm bacteria was assessed. It was found that transfer of the conjugative transposons occurred within a biofilm resulting in *Streptococcus* species resistant to tetracycline. This was the first demonstration of gene transfer in an oral microbe growing in a biofilm and these findings indicate that non-oral bacteria have the potential to transfer genes to oral commensally. The transfer of TOL plasmid, which carry the genes for the degradation of toluene and the benzyl alcohol has occurred in biofilm community growing on benzyl alcohol as the sole carbon and the energy source. Virus-mediated gene transduction is another mode of gene transfer in biofilm associated microbial community.

Quorum Sensing

Cell to cell signaling has recently been demonstrated to play a role in cell attachment. Intracellular communication between bacteria is generally carried out by bacterial products that are

able to diffuse away from one cell and enter into another cell. This method of intracellular signaling seems ideally suited for bacteria in a diffusion-limited environment. Production of quorum sensing molecules is known as acyl-homoserine lactone (acyl-HSL). *P. aeruginosa* mutants that do not produce acyl-HSL form biofilms in which the cells are closely packed together and easily disrupted by sodium dodecyl sulfate. The role of intracellular signaling in multispecies biofilms significantly differ from that observed in single species biofilms. These signals are broadly classified as any actively or passively transmitted bacterial products that alter the state of neighboring microbes. These might include bacterial metabolites, acryl HSLs secreted proteins, genetic material such as DNA or RNA *etc*., This signal may alter distribution of specific bacterial species in the biofilm, alter protein expression in neighboring cells, introduce new genetic trait in neighboring cells and incorporate bacteria in biofilm. In addition to above factors, properties of cell such as cell surface hydrophilicity, presence of fimbriae, and flagella and production of EPS influence the rate and extent of attachment of microbial cells **(Kokare C.R. *et al.*, 2009).**

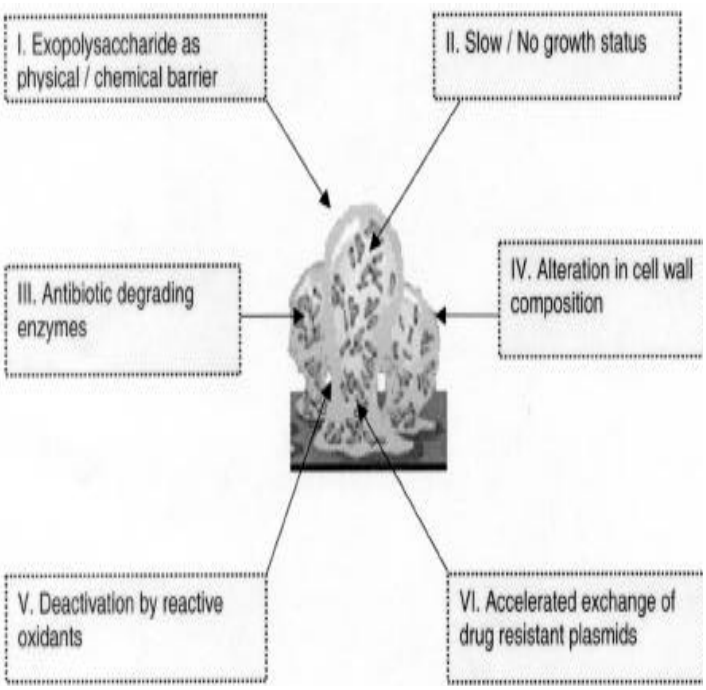


Fig. 3: Mechanism of antimicrobial resistance

The bacteria enclosed within the biofilm are extremely resistant to antibiotic treatments. Such resistance can be explained by hypotheses, not necessarily limited to the following ones.

First, the EPS secreted by biofilm bacteria, acts as a physical/chemical barrier, thus preventing penetration by antibodies or many antibiotics.

Moreover, EPS is negatively charged and functions as an ion-exchange resin which is capable of binding a large number of the antibiotic molecules that are attempting to reach the embedded biofilm cells.

Second, embedded biofilm bacteria are generally not actively engaged in cell division, are smaller in size and less permeable to antibiotics. Virtually all antimicrobials more effective in killing rapidly growing cells further transition from exponential to slow/no growth is generally accompanied by expression of antibiotic-resistant factors. Slow growth activates the RelA-dependent synthesis of ppGpp, which inhibits anabolic processes in bacterial cells56. Interestingly, ppGpp suppressed the activity of a major *E. coli* autolysin, SLT57, which would make the cells more resistant to autolysis and could explain the mechanism of tolerance to antibiotics in slowly growing cells. ppGpp inhibits peptidoglycan synthesis, which would explain the decreased levels of activity of cell-wall synthesis inhibitors under starvation conditions.

Third, antibiotic degrading enzymes such as β-lactamase may also be immobilized in the EPS matrix, so that the incoming antibiotic molecules can be inactivated effectively. It is interesting to note that biofilm cells of the *P. aeruginosa* have been shown to produce 32-fold more β-lactamase than cells of the same strain grown planktonically.

Fourth, up to 40% of the cell-wall protein composition of bacteria in biofilms is altered from Planktonic cells .The membranes of biofilm bacteria might be better equipped to pump out antibiotics before they can cause damage, or even antibiotics targets may disappear.

Fifth, the antimicrobial agent is deactivated in the outer layers of the biofilm, faster than it diffuses. This is true for reactive oxidants such as hypochlorite and Hydrogen peroxide. These antimicrobial oxidants are products of the oxidative burst of phagocytic cells and poor penetration of these may partially account for the inability of phagocytic cells to destroy biofilm microorganisms.

Sixth, Biofilms also provide an ideal for the exchange of extrachromosomal DNA responsible for antibiotic resistance, Virulence factors and environmental survival capabilities at accelerated rates, making it a prefect milieu for emergence of drug resistance pathogens **(PrakashB. *et al.*, 2003).**

Biofilm Examination and Measurement

Over the past years, several assays for biofilm quantification in microtiter plates have been described. These can be classified into biofilm biomass assays (based on the quantification of matrix and both living and dead cells), viability assays (based on the quantification of viable cells) and matrix quantification assays (based on the specific staining of matrix components).

Crystal violet (CV) staining was first described by **Christensen G.D *et al.*, (1985)** and has since then been modified to increase its accuracy and to allow biofilm biomass quantification in the entire well. CV is a basic dye, which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix. Because cells (both living and dead), as well as matrix are stained by CV, it is poorly suited to evaluate killing of biofilm cells.

The fluorogenic dye Syto9 is a nucleic acid stain, which diffuses passively through cellular membranes and binds to DNA of both viable and dead cells. As DNA is also a substantial part of the extracellular matrix,

this staining will provide information on total biofilm biomass. Syto9 has previously been used in Confocal Laser Scanning Microscopy (CLSM) studies of biofilm composition and morphology. This stain has also been used for the routine quantification of bacterial and yeast biofilm biomass.

To discriminate between living and dead cells, quantification techniques based on the metabolic activity of viable cells are available. Various viability stains involve the use of Tetrazolium salts, including 5-Cyano-2, 3-ditolyl Tetrazolium Chloride (CTC) and 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]- 2H-tetrazolium hydroxide (XTT) The XTT assay is based on the reduction of the XTT dye to a water-soluble formazan. The absorbance of the cell supernatant is proportional to the number of metabolically active microbial cells. The XTT assay has been used extensively for the quantification of viable cells in Planktonic cultures and for the quantification of bacterial and yeast biofilms. Despite its popularity, problems regarding intra- and interspecies variability have been reported. Another viability assay is based

on the reduction of Resazurin by metabolically active cells. This is a blue compound that can be reduced to pink resorufin, which is fluorescent. Although this dye, also known as Alamar Blue, is mainly used in viability assays for mammalian cells, it has also been applied extensively in susceptibility testing of fungi, Mycobacterium tuberculosis, *Staphylococcus epidermidis* and *Streptococcus* mutans. Viable microbial cells are also capable of converting non coloured non-fluorescent Fluorescein Di Acetate (FDA) into yellow, highly fluorescent fluorescein by non-specific intra and extracellular esterases. FDA has been used for measuring total microbial activity in soil and litter, as well as for the quantification of biofilm biomass.

The quantification of the biofilm matrix, an essential component of the biofilm, is also of interest. The dye 1,9-DiMethyl Methylene Blue (DMMB), originally applied for quantification of sulphated glycosaminoglycans in chondrocyt cultures has also been used to quantify *Staphylococcus aureus* biofilm matrices. DMMB forms an insoluble complexation product with sulphated polysaccharides in the biofilm matrix. The amount of dye released by adding a

decomplexation solution is spectrophotometrically measured and reflects the amount of sulphated polysaccharides present in the biofilm matrix **(Peeters E.*, et al.*, 2008** and **Pettit K.R *et al.*, 2005).**

REVIEW OF LITERATURE

Eftekhar F. *et al.*, 2009 evaluated biofilm production by *Staphylococcus epidermidis* isolates, from nosocomial infections and skin of healthy volunteers. In this work, biofilm phenotype and icaADBC gene carriage were studied in 50 *S. epidermidis* isolates. Biofilm phenotype was studied by colony morphology on Congo red agar and the Microtiter plate method. Polymerase chain reaction was employed to detect the presence of icaADBC operon. The results showed no significant difference between the two groups of isolates for the potential to form biofilms by the two phenotypic assays, produced by the two groups. On the other hand, ica gene carriage was more discriminatory and was observed in 30% of group A isolates compared to group B of 8% of the skin isolates.

Barbara V.U. *et al.*, 2009 evaluated the role of bacterial extracellular polysaccharides in biofilm. Extracellular polymeric substances (EPS) produced by microorganisms are a complex mixture of biopolymers primarily consisting of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. EPS make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix. The results showed, the key functions of EPS comprise the mediation of the initial attachment of cells to different substrata and protection against environmental stress and dehydration.

Kokare C.R *et al.,* 2009 reviewed the importance and application of biofilm. In this review authors were discussed the formation of biofilm, structure, role in microbial communities and its application in detailed manner.

Juda.M *et al.,* 2008 reported EDTA as a potential agent in preventing formation of *Staphylococcus epidermidis* biofilm on polychloride vinyl biomaterials. The 6 strains of *S. epidermidis* was found that were able to form the biofilm on PCV biomaterials, but irrespective of adhesion properties. The results showed bacteriostatic effect of EDTA against planktonic cells of the isolates in the concentration of 0.25–0.5mmol/l (MIC). The adhesion process and formation of the biofilm was inhibited by EDTA at concentrations of 1.0–2.0mmol/l. The eradication of the mature biofilm was achieved at 2.0–4.0mmol/l EDTA for two strains, while for the other four isolates, concentration of EDTA needed for eradication effect was greater 32 mmol/l.

Hardy M.T. *et al.,* 2008 examined *invitro* activity of antibiotic combinations against *Pseudomonas aeruginosa* biofilm and planktonic cultures. Micronized Tobramycin, Clarithromycin, Azithromycin, PolymyxinB, Colistin, Polylysin, Protamine and Chitosan were used. Among the antibacterial agents tested, Tobramycin and PolymyxinB had the strongest activity against planktonic cultures when tested alone. Synergistic activity was

observed for the combination chitosan/Tobramycin against planktonic cultures but not biofilms, and for the combination Tobramycin/Clarithromycin against biofilms but not planktonic cultures. Their results suggest that the combination of Clarithromycin/Tobramycin may be successful for eradicating infections involving bacterial biofilms by *P. aeruginosa.*

Mehdi H. *et al.*, 2008 reported on the investigation of alkane biodegradation using the microtiter plate method and correlated. The strain BC (*Pseudomonas*) could remove crude oil by 83%, and produces more biosurfactant, and its biofilm formation is higher compared to other strains. Bacterial adhesions to crude oil for other strains CS-2 (*Pseudomonas*), BC, PG-5 (*Rhodococcus*) and H (*Bacillus*) were 30%, 46%, 10% and 1%, respectively. PCR analysis of these four strains showed that all isolates had alk-B genes.

Naves P. *et al.*, 2008 studied correlation between virulence factors and *invitro* biofilm formation by *Escherichia coli* strains. The ability of 15 *Escherichia coli* strains to form biofilms on polystyrene plates was studied. Virulence factors-associated genes 30 were analyzed, including 15 adhesions (papC, papG and its three alleles, sfa/focDE, sfaS, focG, afa/draBC, iha, bmaE, gafD, nfaE, fimH, fimAvMT78, agn43, F9 fimbriae and type 3 fimbriae-encoding gene clusters), four toxins (hlyA, cnf1, sat and tsh), four siderophore (iron, fyuA, iutA and iucD), five proctetins/invasion-

encoding genes (kpsM II, kpsMT III, K1 kps variant- neuC, traT and ibeA), and the pathogencity island malX and cvaC. Their results showed that seven strains were classified as strong biofilm producers and the remaining eight strains were regarded as weak biofilm producers.

Peeters E. *et al.,* 2008 compared the multiple methods for quantification of microbial biofilms grown in microtiter plates. In this study six assays for the quantification of biofilms formed in 96-well microtiter plates were optimized and evaluated, the crystal violet assay, the Syto9 assay, the fluorescein diacetate assay, the resazurin assay, the XTT assay and the DiMethyl Methylene Blue assay. Their data showed that some assays are less suitable for the quantification of biofilms of particular isolates.

Guillot P.V. *et al.,* 2007 reported *in-vitro* activities of different inhibitors of bacterial transcription against *Staphylococcus epidermidis* biofilm. Only a few antibacterial agents were relatively active against biofilm. Among the transcription inhibitors Rifampin, was the most effective molecule against biofilm-related infections.

Burton E. *et al.,* 2007 studied microplate spectrofluorometric assay for bacterial biofilms. The method involved staining of biofilms and the fluorescence of stained biofilms was measured with a fluorescent plate reader. This method was compared with a widely used microplate colorimetric

assay involving crystal violet staining. A strong linear association existed between the two methods (r 2=0.99/0.94). Being more sensitive and specific as compared to colorimetric method, the spectrofluorometric assay provides a better alternative for quantification and characterization of bacterial biofilms.

Sung B.H. *et al.*, 2006 developed a biofilm production-deficient *Escherichia coli* strain as a host for biotechnological applications. Bacteria form biofilms by adhering to biotic or abiotic surfaces. This phenomenon causes several problems. To overcome the the problems associated with biofilm, the authors created a biofilm production-deficient *Escherichia coli* strain, BD123, by deleting genes involved in curli biosynthesis and assembly, (*csgG-csgC*) colanic acid biosynthesis and assembly,(*wcaL-wza*) and type I pilus biosynthesis,(*fimB-fimH*). *E. coli* BD123 remained mostly in the form of planktonic cells and became more sensitive to the antibiotics Streptomycin and Rifampin than the wild-type *E. coli* MG1655: the growth of BD123 was inhibited by one-fourth of the concentrations needed to inhibit MG1655.

Mathur T. *et al.*, 2006 detected the biofilm formation among the clinical isolates of *Staphylococci* using three different screening methods. *Staphylococcus* spp(152) 88(57.8%) displayed a biofilm-positive phenotype under the optimized conditions in the Tissue Culture Plate(TCP) method and strains were further classified as high 22 (14.47 %) and moderate 60 (39.4 %) while in

70 (46.0 %) isolates weak or no biofilm was detected. Though Tube Method(TM) correlated well with the TCP test for 18 (11.8 %) strongly biofilm producing strains, weak producers were difficult to discriminate from biofilm negative isolates. Screening on Congo Red Agar (CRA) does not correlate well with either of the two methods for detecting biofilm formation in *Staphylococci.* The TCP method was found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation by *Staphylococci.*

Hola V. *et al.*, 2006 studied the dynamics of *Staphylococcus epidermis* biofilm formation in relation to nutrition, temperature and time. In this study the dynamics of biofilm formation was correlated with nutrition and temperature conditions during the cultivation of biofilm-positive *Staphylococcus epidermidis* isolates. The cultivation was performed on standard microtiter plates and the wells were examined for the production of biofilm every 1 up to 48 hrs. All the tested strains showed better growth of the biofilm at a temperature of 37 °C in a nutrient-richer environment. The first signs of bacterial adhesion were visible after 2–4 hrs but a very thin layer was visible after 5 hours, after 10 hrs the biofilm layer seemed to be mature – the changes in thickness were not so evident after this time. After cultivation longer than 34–42 hrs, parts of the biofilm layer started to detach, and consequently the biofilm became non-homogeneous. Their result suggests that for biofilm cultivation 12–16 hrs should be sufficient.

Merritt J.H. *et al.*, 2005 reported four basic protocols for the growth and analysis of biofilms in static systems. The microtiter plate biofilm assay was a useful method for assessing bacterial attachment by measuring the staining of the adherent biomass. The air-liquid interface assay was complementary to the microtiter plate biofilm assay in that it provides a mechanism for direct microscopic viewing of the live attached microbes. A colony-based biofilm system is also described with this system being especially useful for monitoring cell death in biofilms treated with antimicrobial agents. Kadouri Drip-Fed Biofilm Assay growth can be maintained for a much longer period, allowing formation of mature biofilms.

Cerca N. *et al.*, 2005 studied quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*. The major virulence factor of these organisms is their ability to adhere to devices and form biofilms. In this study, the initial adhesion to different materials (acrylic and glass) of 9 clinical isolates of *S. epidermidis*, along with biofilm-positive and biofilm-negative control strains, was assayed using physico-chemical interactions to analyze the basis for bacterial adherence to the substratum. X-ray photo electron spectroscopy (XPS) analysis of the cell surface elemental composition was also performed in an attempt to find a relationship between chemical composition and adhesion

capabilities. Biofilm formation on the two surfaces was evaluated by dry weight measurements. The clinical isolates exhibited different cell wall physico-chemical properties, resulting in differing abilities to adhere to surfaces. Adhesion to hydrophobic substrata for all strains occurred to a greater extent than that to hydrophilic surfaces. However, no direct relationship was found between the amount of biofilm formed and the initial adhesion extent. These results indicate that high levels of initial adherence do not necessarily lead to thick biofilm formation. These two aspects of the pathogenesis of medical device related-infection may need to be evaluated independently to ascertain the contribution of each to the virulence of *S.epidermidis* causing device-related infections.

Fux C.A. *et al.*, 2005 Studied survival strategies of infectious biofilms. Bacterial biofilms are difficult to detect in routine diagnostics and are inherently tolerant to host defenses and antibiotic therapies. The authors reviewed current concepts of biofilm tolerance with special emphasis on the role of the biofilm matrix and the physiology of biofilm embedded cells. The heterogeneity in metabolic and reproductive activity within a biofilm correlates with a non-uniform susceptibility of enclosed bacteria. Nutritional starvation and high cell density, two key characteristics of biofilm physiology, also mediate antimicrobial tolerance in stationary-phase planktonic cultures.

Stepanovic *et al.,* 2001studied the modified microtiter plate tests to investigate biofilm formation by *Staphylococci* under both static and dynamic conditions. The quantity of biofilm produced under static conditions was used as are reference. Dynamic conditions were achieved by incubating microtiter plates on a horizontal shaker. Dynamic conditions particularly affected the capacity of certain species to produce biofilm. These species included the causative agents of infections associated with a foreign body (*Staphylococcus epidermidis, Staphylococcus aureus*-ATCC 25923). Their results revealed that, dynamic conditions should be included as a parameter for evaluating biofilm formation by *Staphylococci in vitro*.

Spoering A.L. *et al.,* 2001 compared the tolerance level of biofilms versus stationary- and logarithmic-phase planktonic cells of *Pseudomonas aeruginosa* with four different antimicrobial agents. In general, stationary-phase cells were somewhat more tolerant than biofilms in all of the cases examined. They concluded that, at least for *Pseudomonas aeruginosa*, one of the model organisms for biofilm studies, the notion that biofilms have greater resistance than do planktonic cells is unwarranted. They further suggest that tolerance to antibiotics in stationary-phase or biofilm cultures is largely dependent on the presence of persister cells.

Ashby M.J. *et al.*, 1994 reported effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. Several classes of antibiotics were assessed for activity against non-growing *Escherichia coli* and cells grown as a biofilm. Antibiotics which had activity against non-growing cells also showed some activity against biofilms. The most effective antibiotics were Imipenem and Ciprofloxacin, which were also active against steady state biofilms. Their results suggest that growth rate plays a major role in mediating resistance of biofilms to antibiotics.

Drancourt M. *et al.*, 1993 reported oral Rifampin plus Ofloxacin for treatment of *Staphylococcus*-infected orthopedic implants. The authors were examined the effectiveness and safety of the combination of Rifampin plus Ofloxacin given orally for treating prosthetic orthopedic implants infected with *Staphylococci.* The combination of Rifampin administered orally plus Ofloxacin is a suitable alternative to the conventional long-term intravenous therapy for treatment of orthopedic implants infected with *Staphylococci*.

Gallimore B. *et al.*, 1991 reported natural history of chronic *Staphylococcus epidermidis* foreign body infection in a mouse model. The development and characterization of a mouse model of chronic *Staphylococcus epidermidis* foreign body infection was done with two clinical isolates that differed in degree of

extracellular slime production. Both test strains of *S. epidermidis* persisted at the implant site through the 6-month follow-up in 80% of the mice, regardless of the degree of slime production. There was no evidence of overt animal morbidity, and microbiologic assessment of other peritoneal sites did not reveal dissemination of bacteria from the infected focus. In comparison with control mice, animals harboring chronic foreign body infection presented marked neutrophilia and mild anemia.

Prosser, B.T., *et al.*, 1987 evaluated effects of antibiotics on bacterial biofilm. Antibiotics are generally not effective against biofilms. A simple method of studying the effect of antibiotics on *Escherichia coli* ATCC 25922 biofilms was established on 0.5 cm^2 catheter disks. The disks were incubated for 1 h at 37°C, washed, transferred to petri dishes containing 20 ml of broth, and incubated at 37°C for 20 to 22 h, at which time thick biofilms were established. Disks were washed, placed in broth or broth containing antibiotic, and incubated at 37°C for 4 h. The disks were removed, and viable counts were determined. Viable bacterial counts decreased from 10^3 to 10^4 CFU/cm^2 in 24 h with 400 mcg of Amdinocillin or Cefamandole per ml. A combination containing 400mcg of each antibiotic per ml decreased the viable counts to an undetectable level (<100 CFU/ cm^2) in 24 hrs.

Christensen, G.D. *et al.*, 1982 studied the adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth

surfaces. Slime production is not a generally recognized feature of *Staphylococcus epidermidis*. Through the results suggest that slime mediated adherence may be a critical factor in the pathogenesis of *S.epidermidis* infections.

MATERIALS

APPARATUS' USED

Centrifuge tubes	-	Eppendorf
Glasspipettes	-	Borosil
Micropipettes	-	Vari pippets
Petriplates	-	Borosil
Standard flask	-	Borosil
Sterile swabs	-	Hi media
Test tubes	-	Borosil
Tissue Culture Plate	-	Tarsons

CHEMICALS USED

Acetic acid Qualigens,Mumbai.	-	
Acetone	-	Qualigens,Mumbai.
Agar Agartype 1 Mumbai.	-	Hi media labs ltd,
Ammonium sulphate	-	S.d fine chemicals ltd,

		Mumbai.
Congo Red	-	Hi media labs ltd, Mumbai.
Crystal Violet	-	Qualigens, Mumbai.
Disodium hydrogen Phosphate	-	S.d fine chemicals ltd, Mumbai.
Ethanol	-	Qualigens, Mumbai.
Glucose	-	Qualigens,Mumbai.
Mannitol salt agar	-	Hi media labs ltd, Mumbai.
Mannitol salt broth	-	Hi media labs ltd, Mumbai.
Methanol	-	Qualigens, Mumbai.
Mueller Hinton Agar	-	Hi media labs ltd, Mumbai.
Mueller Hinton Broth	-	Hi media labs ltd, Mumbai.

Non absorbent cotton cotton Ltd	-	Ramraju surgical
		Rajapalayam.
Sodium chloride Qualigens,Mumbai	-	
Sodium Dihydrogen Phosphate	-	S.d fine chemicals Ltd
		Mumbai
Sucrose	-	Qualigens,Mumbai.
Tetrazolium salt Mumbai.	-	Hi media labs ltd,
Trypticase soy broth Mumbai.	-	Hi media labs ltd,

INSTRUMENTS USED

Alpha digitoc	-	Technico
Autoclave	-	Universal Autoclave
Centrifuge	-	Eppendorf
Digital balance	-	Shimadzu
Heating mantle	-	Guna enterpirses

Hot air oven - Technico

Incubator - Technico

Microscope - Motic

UV-Spectrophotometry - Shimadzu

Vertical laminar flow hood- Technico

DRUGS USED

Ofloxocin - Pure sample gift from SKN Organics Laboratories Pvt.

Ltd Chennai.

Rifampin - Pure sample gift from SKN Organics Laboratories Pvt.

Ltd Chennai.

METHODOLOGY

Microorganisms Used

The reference strain used in the study was *Staphylococcus aureus* ATCC25923 **(Stepanovic *et al.*, 2001)** which was obtained from NCIM, Pune. One Clinical isolate *Staphylococcus aureus* and two *Staphylococcus epidermidis* were obtained from the Dept. of Microbiology, Sri Ramakrishna Hospital, Coimbatore. The strains were represented as S-I, S-II, S-III and S-IV.

Maintenance of culture

The selected strains were confirmed for their purity and identity by Grams staining method and by their characteristic biochemical reactions like catalase and coagulase test **(Mackie and McCartney 14th edition 1996)**. The selected strains were preserved by subculturing them periodically on Nutrient agar slants and storing them under frozen condition. For the study fresh 24 hrs broth cultures were used.

Standardization of inoculum

All organisms were grown overnight (24 hrs) at 37° C on Nutrient Agar (NA) and harvested during the stationary growth phase. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth 24 hrs at 37°C. Inoculum was standardized by matching the turbidity of the culture to 0.5 McFarland standard. This was produced by mixing 0.5ml of 0.048 M $Bacl_2$ (1.175% w/v Barium Chloride dehydrates) with 99.5 ml of 0.36 N H_2SO_4.) This produced an inoculating suspension of approximately 2.0×10^6 (CFU/ml) for bacteria.

A. IDENTIFICATION OF BIOFILMS

The ability of all the four strains to form biofilm was assessed by the following,

(i) Tissue Culture Plate (TCP) Method

Procedure

The *Staphylococcus* isolates were inoculated into 3-5ml of Trypticase soy broth supplemented

with1%glucose (TSB) and grown to stationary phase. The culture was diluted 1:100 in the same media. Individual wells of sterile polystyrene 96 well tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures with broth alone serve as control. After inoculation the tissue culture plates were covered and incubated for 48-72 hrs at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed three-four times with 0.2 ml of phosphate buffer saline (PBS) pH 7.4 to remove free-floating 'planktonic' bacteria.

The biofilms formed by adherent 'sessile' organisms were stained with Crystal Violet (0.7 % w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent cells formed biofilm on all sides of the well and were uniformly stained by Crystal Violet.

(ii) Tube Method

In Tube method TSB (2ml) was inoculated with loopful of microorganism from overnight culture plates and incubated for 48-72 hrs at 37°C. The tubes were decanted and washed with PBS and dried. Dried tubes were stained with crystal violet (0.7%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate and 3-was considered as strong. The experiment was performed in triplicate and repeated three times.

(iii) Congo Red Agar (CRA) Method

The composition of Congo red agar media is given below:

Brain Heart Infusion broth - (37 gms/L)

Sucrose - (50 gms/L)

Agar No.1 - (10 gms/L)

Congo Red Stain-(0.8 gms/L).

Congo Red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated with *Staphylococcus* isolates and incubated aerobically for 24 -48 hrs at 37°C.

Positive result is indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result. The experiment was performed in triplicate and repeated three times **(Mathur T. *et al.*, 2006 and Freeman D.J., *et al.*, 1989).**

B. DETERMINATION OF ADHESION TO THE BIOMATERIAL *in-vitro* BY TTC (2, 3, 5 –TRIPHENYL TETRAZOLIUM CHLORIDE) METHOD

The ability of adhesion of the *Staphylococcus* isolates on biomaterial (catheter) was studied *in-vitro* by TTC method. It's based on the ability of living cells to reduce tetrazolium salt to red formazan Precipitate **(Gallimore *et al.*, 1991).** The standardized bacterial suspensions (according to 0.5 McFarland standard) in sterile PBS were incubated with appropriate biomaterial for 1, 2, 4, 8, 12, 24, 48 and 72 hrs at 37°C. Non adherent cells were removed by careful rinsing of catheter fragment with sterile PBS and then resuspended in TSB medium with one drop of 1% TTC, followed by overnight incubation at 37°C. The minimal time needed for adhesion process was determined visually by an appearance of red formazan precipitate, on the biomaterial surface (catheter) and in the medium.

C. DETERMINATION OF BIOFILM FORMATION ON THE BIOMATERIAL *in-vitro* BY TTC METHOD

The ability of formation of biofilm by the *Staphylococcus* isolates on biomaterial (catheter) was

studied *in-vitro* also by TTC method **(Gallimore *et al.*, 1991)**. The standardized bacterial suspensions (according to 0.5McFarland standard) in TSB were incubated with appropriate biomaterial for 24 hrs at 37°C. Nonadherent cells were removed by careful rinsing of catheter fragment with sterile PBS and then resuspended in fresh TSB. Medium changing and catheter washing procedures after overnight incubation at 37°C were repeated three-five times. Finally, one drop of 1% TTC solution was added, followed by overnight incubation at 37°C. Biofilm formation was determined visually on the basis of an appearance of red formazan precipitate both on the catheter surface and in the medium **(Juda M. *et al.*, 2008).**

D. QUANTIFICATION OF BIOFILM BY CRYSTAL VIOLET METHOD

Stock solution of 0.1%Crystal Violet was prepared by using 33%w/v Acetic acid. From the stock solution, different concentrations in the range of 0.5 to 10µg/ml were prepared. Absorbance was measured at 570nm; using 33% w/v acetic acid was used as blank.

After biofilm formation on biomaterial in different hrs, it was thoroughly washed with PBS then placed in room temperature for one hour. The biomaterial was stained with Crystal Violet for 30 minutes. 10ml of 33% w/v Acetic acid was used to extract the stained Crystal Violet and absorbance was measured at 570nm using 33% Acetic acid as blank **(Merritt J.H.*et al* 2005).**

E. DETERMINATION OF MINIMAL INHIBITORY CONCENTRATION (MIC) OF OFLOXACIN AND RIFAMPIN

The MIC was performed by the broth dilution method. The 2-fold serial dilutions of the antimicrobial agents were made from the concentrations of Ofloxacin ranged from (500-0.24 µg/ml) and Rifampin (50 - 0.02 µg/ml). All tubes were inoculated with 50 µl volume of bacterial culture, which contains 10^5 CFU/ml. They were incubated overnight (18 to 24 hrs) at 37°C and examined for growth indicated by turbidity MICs were recorded as the lowest drug concentrations that completely inhibited visible growth of bacteria. Positive and negative controls were also kept for comparisons. The determinations of MICs were performed in

triplicates for each organism and the experiment was repeated where necessary.

F. DETERMINATION OF MINIMAL BACTERICIDAL CONCENTRATION (MBC) OF OFLOXACIN AND RIFAMPIN

The MBC was performed by the streak plate method. After MIC determination, a loopful of broth was collected from those tubes which did not show any growth and inoculated on sterile Trypticase Soy Agar by streaking. Test organisms to serve as control.

Plates were then incubated at 37°C for 24 hrs. After incubation the concentration at which no visible growth was seen was noted as the minimal bactericidal concentration **(Victor Lorian *3rd edition* 96-100).**

G. THE EFFECT OF OFLOXACIN AND RIFAMPIN ON ADHESION TO BIOMATERIAL *in- vitro* BY TTC METHOD

In order to assay the effect of Ofloxacin and Rifampin on adhesion, the standardized bacterial suspensions (according to 0.5 McFarland standard) in sterile PBS containing Ofloxacin (500-0.24µg/ml) and

Rifampin (50-0.02µg/ml) were incubated with appropriate biomaterial for 12–48 hrs (according to the minimal time needed for adhesion of each strain to biomaterial) at 37°C. Then, a drop of 1% TTC solution was added, followed by overnight incubation at 37°C. The minimal concentration of Ofloxacin and Rifampin inhibited adhesion process was determined visually as the concentration where the red formazan precipitate was notfound, neither on the catheter surface nor in the medium.

H. THE EFFECT OF OFLOXACIN AND RIFAMPIN ON BIOFILM FORMATION ON BIOMATERIAL *in-vitro* BY TTC METHOD

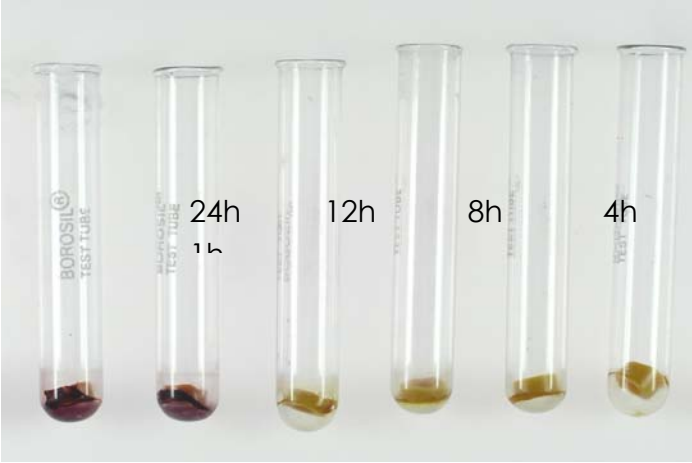
In order to assay the effect of Ofloxacin and Rifampin on biofilm formation, the bacterial suspensions in TSB containing Ofloxacin (500-0.24µg/ml) and Rifampin (50-0.02µg/ml)were incubated with appropriate biomaterial for 72 and 96 hrs at 37°C, with medium changing and catheter washing process as described above. Then, a drop of 1% TTC solution was added, by overnight incubation at 37°C. The minimal concentration of Ofloxacin and Rifampin that inhibited

biofilm formation was determined visually as the concentration where the red formazan precipitate was not found neither on the catheter surface nor in the medium.

I. THE EFFECT OF OFLOXACIN AND RIFAMPIN ON MATURE BIOFILM ON BIOMATERIAL *in- vitro* BY TTC METHOD

In order to assay the effect of Ofloxacin (500-0.24µg/ml) and Rifampin (50-0.02µg/ml) on biofilm eradication, the mature biofilm was incubated in the presence of Ofloxacin and Rifampin for 24 hrs and then a drop of 1% TTC solution was added, followed by overnight incubation at 37°C. The minimal concentration of Ofloxacin and Rifampin eradicated the mature biofilm, determined visually as the concentration, at which the red formazan precipitate was not found, neither on the catheter surface nor in the medium (**Juda M *et al.*, 2008).**

Fig.7: *In-vitro* determination of adhesion on biomaterial by TTC method



Red Formazan precipitate of TTC indicates adhesion of S-I on biomaterial.

Table No 4: Adhesion duration of S-I on biomaterial

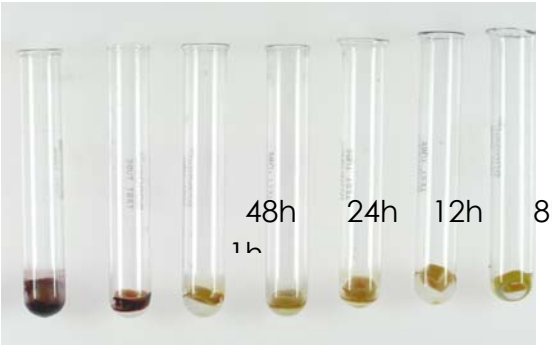
S.No	Duration in hrs	Appearance of Red Formazan precipitate
1	1	-
2	2	-
3	4	-
4	8	-
5	12	Present
6	24	Present

The red formazan

precipitate appeared on the biomaterial after 12 hrs incubation at 37°C indicating the minimal time needed for adhesion in S-I strain to be 12 hrs.

Fig.8: *In-vitro* determination of adhesion

on biomaterial by TTC method



Red Formazan precipitate of TTC indicates adhesion of

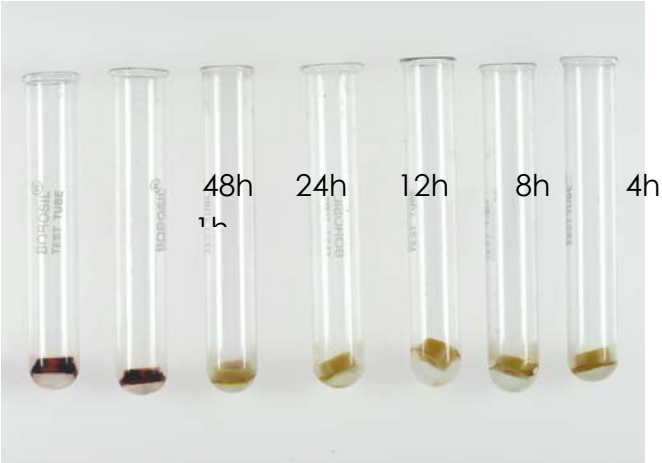
S-II on biomaterial

Table No 5: Adhesion duration of S-II on biomaterial

S.No	Duration in hrs	Appearance of Red Formazan precipitate
1	1	-
2	2	-
3	4	-
4	8	-
5	12	-
6	24	Present
7	48	Present

The red formazan precipitate appeared on the biomaterial after 24hrs incubation at 37°C indicating the minimal time needed for adhesion in S –II strain to be 24hrs.

Fig.9: *In- vitro* determination of adhesion on biomaterial by TTC method



Red Formazan precipitate of TTC indicates adhesion of S-III on biomaterial

Table No 6: Adhesion duration of S-III on biomaterial

S.No	Duration in hrs	Appearance of Red Formazan precipitate
1	1	-
2	2	-
3	4	-
4	8	-
5	12	-
6	24	Present

7	48	Present

The red formazan precipitate appeared on the biomaterial after 24hrs incubation at 37°C indicating the minimal time needed for adhesion in S –III strain to be 24hrs.

Fig.10: *In- vitro* determination of adhesion on biomaterial by TTC method

Red Formazan precipitate of TTC indicates adhesion of S-IV on biomaterial

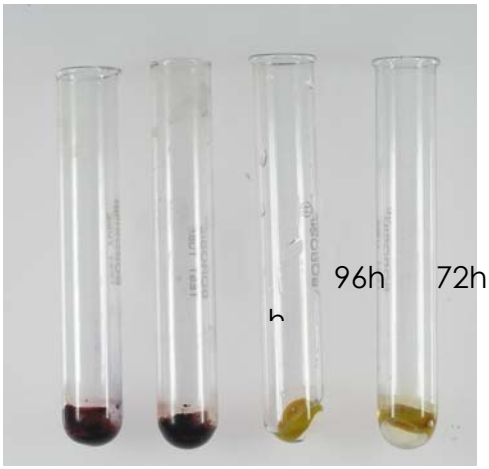
TableNo7: Adhesion duration of S-IV on biomaterial

S.No	Duration in hrs	Appearance of Red Formazan precipitate

1	1	-
2	2	-
3	4	-
4	8	-
5	12	-
6	24	-
7	48	Present
8	72	Present

The red formazan precipitate appeared on the biomaterial after 48hrs incubation at 37°C indicating the minimal time needed for adhesion in S –IV strain to be 48hrs.

Fig.11: *In- vitro* determination of biofilm formation on biomaterial by TTC method



Red Formazan precipitate of TTC indicates biofilm formation of S-I on biomaterial

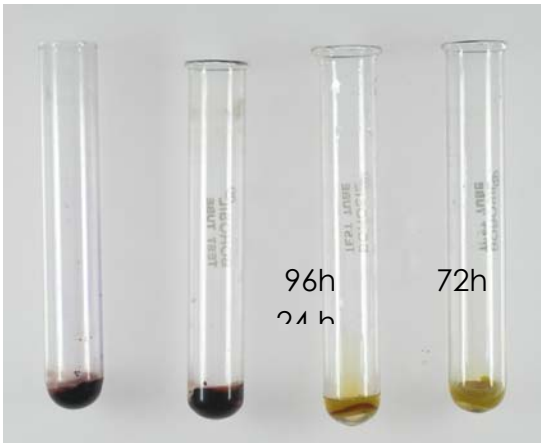
Table No 8: Biofilm formation duration of S-I on biomaterial

S.No	Duration in hrs	Appearance of Red Formazan precipitate
1	24	-
2	48	-
3	72	Present
4	96	Present

The red formazan precipitate appeared on the biomaterial after 72hrs incubation at 37°C indicating the minimal time needed for biofilm formation by S-I strain to be 72hrs.

Fig.12: *In- vitro* determination of biofilm formation

on biomaterial by TTC method



Red Formazan precipitates of TTC indicates biofilm formation of

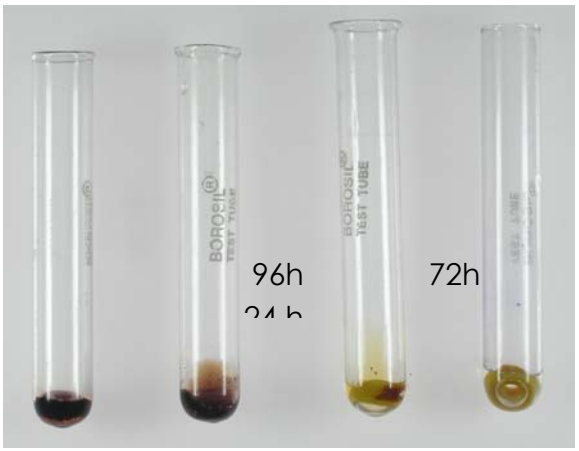
S-II on biomaterial

Table No 9: Biofilm formation duration of S-II on biomaterial

S. No	Duration in hrs	Appearance of Red Formazan precipitate
1	24	-
2	48	-
3	72	Present
4	96	Present

The red formazan precipitate appeared on the biomaterial after 72hrs incubation at 37°C indicating the minimal time needed for biofilm formation by S-II strain to be 72hrs.

Fig.13: *In- vitro* determination of biofilm formation on biomaterial by TTC method



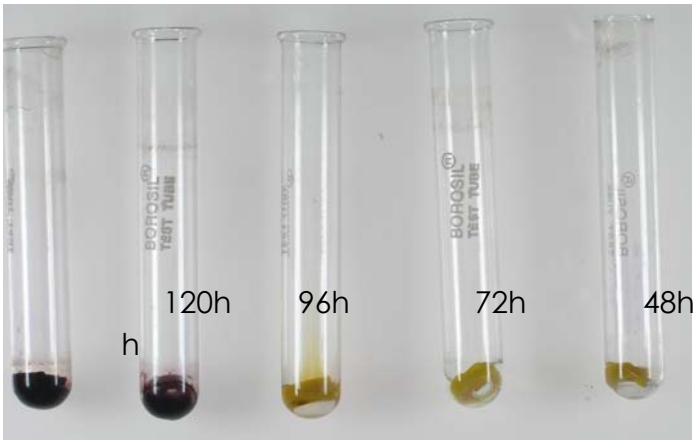
Red Formazan precipitate of TTC indicates biofilm formation of S-III on biomaterial

Table No10: Biofilm formation duration of S-III on biomaterial

S. No	Duration in hrs	Appearance of Red Formazan precipitate
1	24	-
2	48	-
3	72	Present
4	96	Present

The red formazan precipitate appeared on the biomaterial after 72hrs incubation at 37°C indicating the minimal time needed for biofilm formation by S-III strain to be 72hrs.

Fig.14: *In- vitro* determination of biofilm formation on biomaterial by TTC method



Red Formazan precipitate of TTC by biofilm formation of S-IV on biomaterial

Table No11: Biofilm formation duration of S-IV on biomaterial

S. No	Duration in hrs	Appearance of Red Formazan precipitate
1	24	-
2	48	-
3	72	-
4	96	Present
5	120	Present

The red formazan precipitate appeared on the biomaterial after 96hrs incubation at 37°C indicating the minimal time needed for biofilm formation by S-IV strain to be 96hrs.

QUANTIFICATION OF BIOFILM BY

CRYSTAL VIOLET METHOD

Table No12: Standard Graph for assay of crystal

Violet by Spectrophotometric method

S. No	Concentration µg/ml.	Absorbance at 570nm
1	1	0.1467
2	2	0.2872
3	4	0.5989
4	6	0.8866
5	8	1.2057
6	10	1.5531

Graph No1: Standard Graph for Assay of Crystal Violet by Spectrophotometric Method

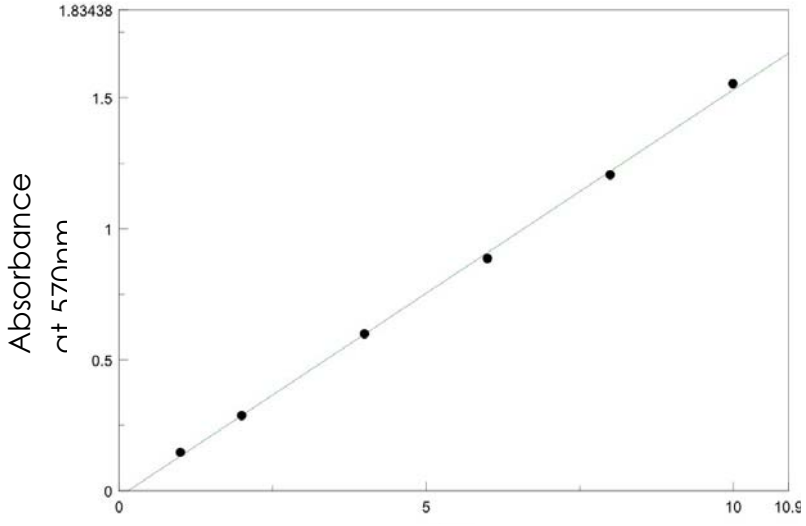


Table No13: Quantification of biofilm in S-I by

Crystal Violet method

S. No	Time in hrs	Absorbance at 570nm	Concentration of Crystal Violet µg/ml
1	72	0.4697	3.17
2	96	0.5205	3.50
3	120	0.5783	3.87

The concentration of Crystal Violet at different time interval was interpolated from the calibration graph (page no.53). The results were correlated with amount of biofilm formed.

Table No14: Quantification of biofilm in S-II by

Crystal Violet method

S. No	Time in hrs	Absorbance at 570nm	Concentration of Crystal Violet µg/ml
1	72	0.4731	3.19
2	96	0.5333	3.58
3	120	0.5964	3.99

The concentration of Crystal Violet at different time interval was interpolated from the calibration graph (page no.53). The results were correlated with amount of biofilm formed.

Table No15: Quantification of biofilm in S-III by Crystal Violet method

S. No	Time in hrs	Absorbance at 570nm	Concentration of Crystal Violet µg/ml
1	72	0.3886	2.63
2	96	0.4386	2.97
3	120	0.4856	3.27

The concentration of Crystal Violet at different time interval was interpolated from the calibration graph (page no.53). The results were correlated with amount of biofilm formed.

Table No16: Quantification of biofilm in S-IV by Crystal Violet method

S. No	Time in hrs	Absorbance at 570nm	Concentration of Crystal Violet µg/ml
1	96	0.4359	2.95
2	120	0.4913	3.31

The concentration of Crystal Violet at different time interval was interpolated from the calibration graph (page no.53). The results were correlated with amount of biofilm formed.

Determination of Minimal Inhibitory Concentration

Table No17: Minimal Inhibitory Concentration (MIC) of Ofloxacin

S. No	Microorganisms		MIC µg/ml
1	*Staphylococcus aureus*	S-I	0.4
2	*Staphylococcus aureus*	S-II	15.6
3	*Staphylococcus epidermidis*	S-III	3.9
4	*Staphylococcus epidermidis*	S -IV	15.6

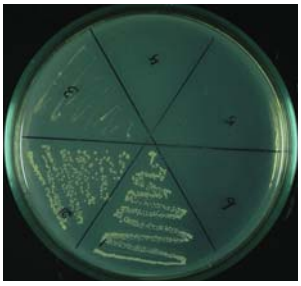
Table No18: Minimal Inhibitory Concentration (MIC)

of Rifampin

S. No	Microorganisms		MIC µg/ml
1	*Staphylococcus aureus*	S-I	0.39
2	*Staphylococcus aureus*	S-II	0.09
3	*Staphylococcus epidermidis*	S-III	0.04
4	*Staphylococcus epidermidis*	S -IV	0.09

The MIC of Ofloxacin against planktonic cells of S-I, SII, S-III, and S-IV isolates was 0.4, 15.6, 3.9, 15.6µg/ml respectively and MIC of Rifampin against S-I, SII, S-III and S-IV was 0.39, 0.09, 0.04 and 0.09µg/ml respectively.

Determination of Minimal Bactericidal Concentration
Fig.15: Minimal Bactericidal Concentration of Ofloxacin

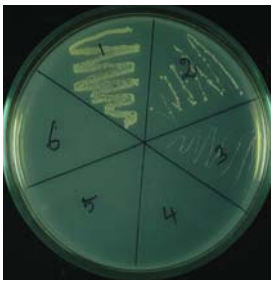


S- I

1- Control , 2 -0.48 µg/ml, 3 -

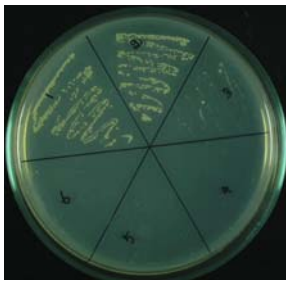
S- II

1- Control , 2 - 15.6 µg/ml, 3 -31.2 µg/ml



S- III

1- Control, 2 –3.9µg/ml, 3 -7.8 µg/ml,



S- IV

1- Control , 2 - 15.6 µg/ml, 3 -31.2

Table No19: Minimal Bactericidal Concentration

(MBC) of Ofloxacin

S.No	Microorganisms		MBC µg/ml
1	*Staphylococcus aureus*	S-I	1.9
2	*Staphylococcus aureus*	S-II	125
3	*Staphylococcus epidermidis*	S-III	15.6

4	*Staphylococcus epidermidis*	S -IV	62.5

The MBC of Ofloxacin against *Staphylococcus* S-I, S-II, S-III and S-IV were found 1.9,125, 15.6 and 62.5µg/ml respectively.

Determination Minimal Bactericidal Concentration

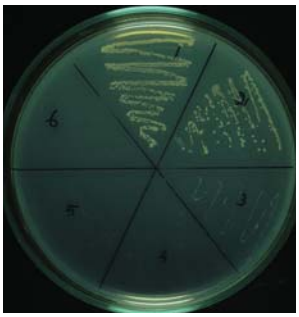
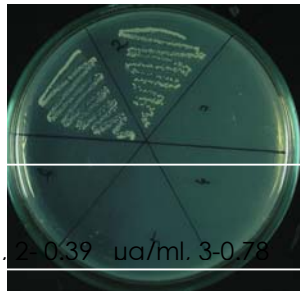
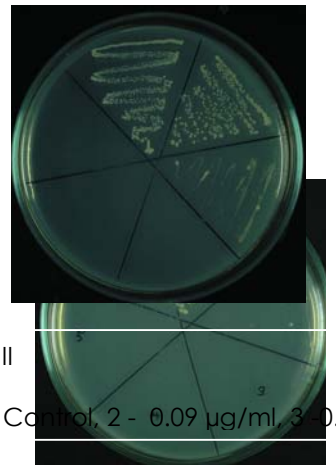


Fig.16: Minimal Bactericidal Concentration of Rifampin



S- I

1- Control, 2- 0.39 µg/ml, 3-0.78



S- II

1- Control, 2 - 0.09 µg/ml, 3 -0.19 µg/ml,

S- III

1- Control, 2 - 0.04 µg/ml, 3 –

S- IV

1- Control, 2 - 0.09 µg/ml, 3 –

Table No.20: Minimal Bactericidal

Concentration (MBC) of Rifampin

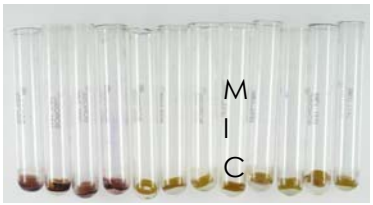
S.No	Microorganisms		MBC µg/ml
1	*Staphylococcus aureus*	S-I	1.56

2	*Staphylococcus aureus*	S-II	0.39
3	*Staphylococcus epidermidis*	S-III	0.09
4	*Staphylococcus epidermidis*	S -IV	0.19

The MBC of Rifampin against *Staphylococcus* S-I, S-II, S-III and S-IV showed in the following 1.56, 0.39, 0.09 and 0.19µg/ml.

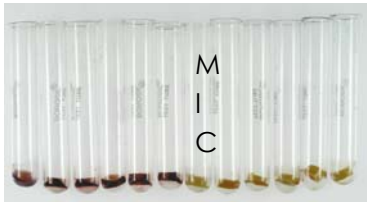
Fig.17: Effect of Ofloxacin in preventing *in-vitro*

adhesion of *Staph*.on biomaterial by TTC Method

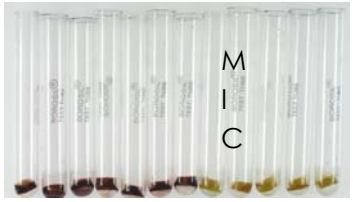


S-I

S-II



S-III



S-IV

Red Formazan precipitate of TTC not found in tubes containing Ofloxacin at MIC and above it indicating prevention of adhesion of S-I,S-II, S-III and S-IV on biomaterial.

Table No21: MIC of Ofloxacin in preventing

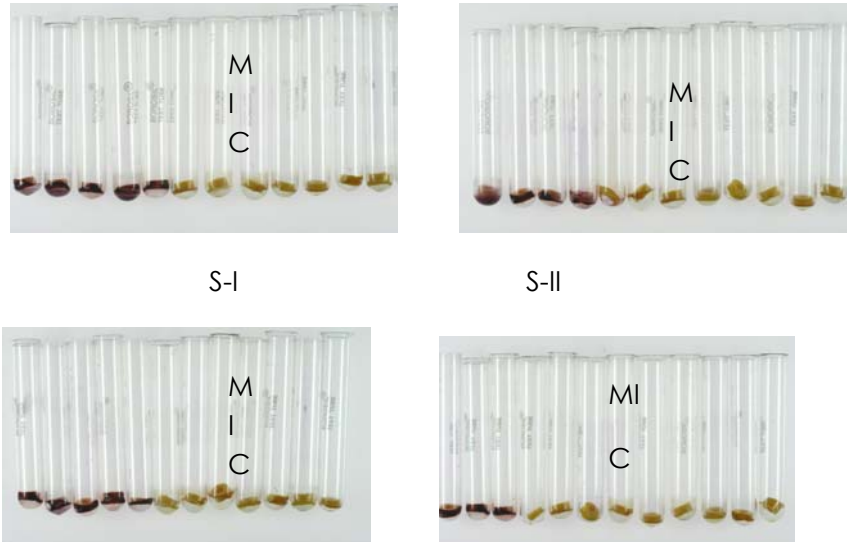
adhesion on biomaterial

S. No	Microorganisms		MIC in µg/ml
1	*Staphylococcus aureus*	S-I	3.9
2	*Staphylococcus aureus*	S-II	62.5
3	*Staphylococcus epidermidis*	S-III	15.6
4	*Staphylococcus epidermidis*	S -IV	31.2

From the Fig no:17, The tubes containing S-I at 3.9 µg/ml, S-II at 62.5 µg/ml, S-III at 15.6 µg/ml and S-IV at 31.2 µg/ml indicates the MIC value, at and above which the tubes did not show formation of red formazan precipitate either on the biomaterial surface or in the medium. It confirms the above mentioned concentration of Ofloxacin was found to be sufficient in preventing the adhesion on biomaterial.

Fig.18: Effect of Rifampin in preventing *in-vitro*

adhesion of *Staph*. on biomaterial by TTC Method



S-III S-IV

Red Formazan precipitate of TTC not found in tubes containing Rifampin at MIC and above it indicating prevention of adhesion of S-I, S-II, S-III and S-IV on biomaterial.

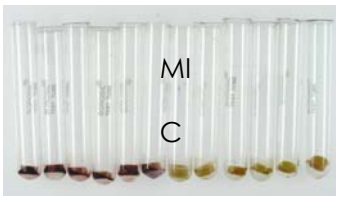
Table No22: MIC of Rifampin in preventing

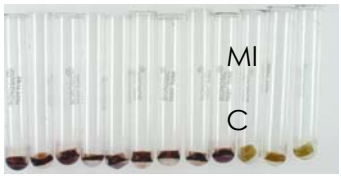
adhesion on biomaterial

S. No	Microorganisms		MIC µg/ml
1	*Staphylococcus aureus*	S-I	0.78
2	*Staphylococcus aureus*	S-II	0.39
3	*Staphylococcus epidermidis*	S-III	0.78
4	*Staphylococcus epidermidis*	S -IV	0.19

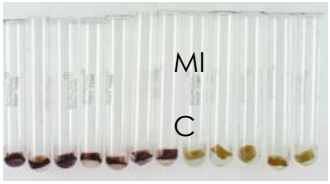
From the Fig no:18 The tube containing S-I at 0.78µg/ml, S-II at 0.39µg/ml, S-III at 0.78µg/ml and S-IV at 0.19µg/ml indicates the MIC value, at and above which the tubes did not show formation of red formazan precipitate either on the biomaterial surface or in the medium. It confirms the above mentioned concentration of Rifampin was found to be sufficient in preventing the adhesion on biomaterial.

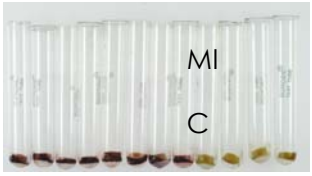
Fig.19: Effect of Ofloxacin in preventing *in-vitro* biofilm formation of *Staph*. on biomaterial by TTC Method





S-I S-II





S-III S- IV

Red Formazan Precipitate of TTC not found in tubes containing Ofloxacin at MIC and above it indicating prevention of biofilm formation of S-I, S-II, S-III and S-IV on biomaterial.

TableNo23: MIC of Ofloxacin in preventing

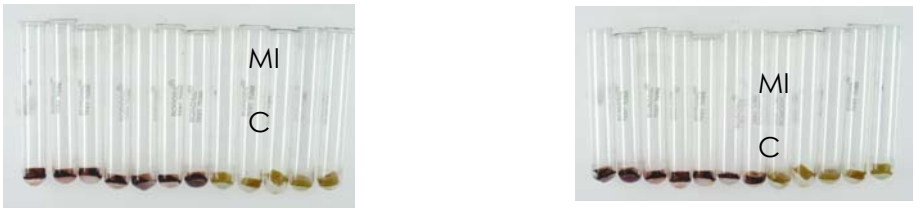
biofilm formation on biomaterial

S.No	Microorganism		MIC µg/ml
1	*Staphylococcus aureus*	S-I	15.6
2	*Staphylococcus aureus*	S-II	125
3	*Staphylococcus epidermidis*	S-III	31.2
4	*Staphylococcus epidermidis*	S -IV	62.5

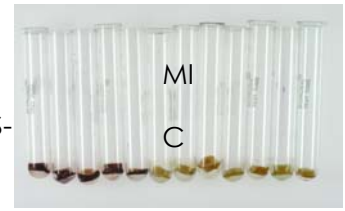
From the fig no:19 The tubes containing S-I at 15.6 µg/ml, S-II at 125 µg/ml, S-III at 31.2 µg/ml and S-IV at 62.5µg/ml indicates the MIC value, at and above which the tubes did not show formation of red formazan precipitate either on the biomaterial surface or in the medium.

It confirms the above mentioned concentration of Ofloxacin was found to be sufficient in preventing the biofilm formation on biomaterial.

Fig.20: Effect of Rifampin in preventing *in-vitro* biofilm formation of *Staph*.on biomaterial by TTC Method



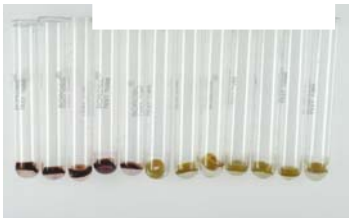
S-I



S-

S-II

S-III



S-IV

MI

C

Red Formazan precipitate of TTC not found in tubes containing Rifampin at MIC and above it indicating prevention of biofilm formation of S-I, S-II, S-III and S-IV on biomaterial.

Table No24: MIC of Rifampin in preventing

biofilm formation on biomaterial

S.No	Microorganisms		MIC µg/ml
1	*Staphylococcus aureus*	S-I	3.12
2	*Staphylococcus aureus*	S-II	3.12
3	*Staphylococcus epidermidis*	S-III	0.78
4	*Staphylococcus epidermidis*	S-IV	0.78

From the Fig no:20, The tubes containing S-I at 3.12 µg/ml, S-II at 3.12 µg/ml, S-III at 0.78 µg/ml and S-IV at 0.78µg/ml indicates the MIC value, at and above which the tubes did not show formation of red formazan precipitate either on the biomaterial surface or in the medium. It confirms the above mentioned concentration of Rifampin was found to be sufficient in preventing the biofilm formation on biomaterial.

Fig.21: Effect of Ofloxacin in preventing *in-vitro* mature biofilm of *Staph*.on biomaterial by TTC Method

S-I

S-II

S-III

S-IV

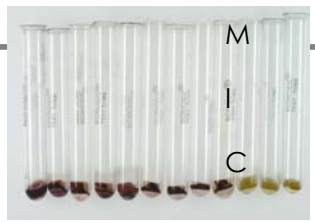
Red Formazan precipitate of TTC not found in tubes containing Ofloxacin at MIC and above it indicating prevention of mature biofilm of S-I,S-II, S-III and S-IV on biomaterial.

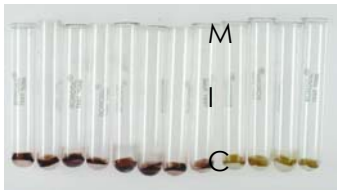
Table No25: MIC of Ofloxacin to eradicate mature biofilm on the biomaterial

S.No	Microorganisms		MIC µg/ml
1	*Staphylococcus aureus*	S-I	250
2	*Staphylococcus aureus*	S-II	500
3	*Staphylococcus epidermidis*	S-III	250
4	*Staphylococcus epidermidis*	S-IV	125

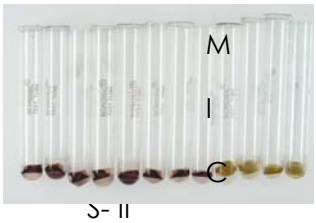
From the Fig no:21 The tubes containing of S-I at 250µg/ml, S-II at 500µg/ml, S-III at250µg/ml and S-IV at 125µg/ml indicates the MIC value, at and above which the tubes did not show formation of red formazan precipitate either on the biomaterial surface or in the medium. It confirms the above mentioned concentration of Ofloxacin was found to be sufficient to eradicate mature biofilm on biomaterial .

Fig.22: Effect of Rifampin in preventing *in-vitro* mature biofilm of *Staph*.on biomaterial by TTC Method





S- I



S- II

S- III

S- IV

Red Formazan precipitate of TTC not found in tubes containing Rifampin at MIC and above it indicating prevention of mature biofilm of S-I,S-II, S-III and S-IV on biomaterial.

Table No26: MIC of Rifampin to eradicate mature biofilm on the biomaterial

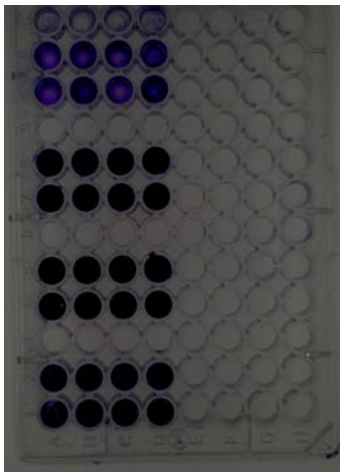
S.No	Microorganisms		MIC µg/ml
1	*Staphylococcus aureus*	S-I	12.5
2	*Staphylococcus aureus*	S-II	25
3	*Staphylococcus epidermidis*	S-III	6.25
4	*Staphylococcus epidermidis*	S-IV	6.25

From the Fig no: 22 The tubes containing S-I at 12.5µg/ml, S-II at 25µg/ml, S-III at 6.25µg/ml and S-IV at 6.255µg/ml indicates the MIC value, at and above which the tubes did not show formation red formazan precipitate either on the biomaterial surface or in the medium. It confirms the above mentioned concentration of Rifampin was found to be sufficient to eradicate mature biofilm on biomaterial.

RESULTS AND DISCUSSION

Fig.4: Screening of biofilm producers

by Tissue Culture Plate method



High and moderate slime producers differentiated with Crystal Violet staining in 96 well tissue culture plate.

Table No1: Tissue Culture Plate method

S.No	Microorganisms		Report
1	*Staphylococcus aureus*	S-I	Strong
2	*Staphylococcus aureus*	S-II	Strong
3	*Staphylococcus epidermidis*	S-III	Strong
4	*Staphylococcus epidermidis*	S -IV	Moderate

From the Tissue Culture Plate method (Fig.4) it is clear that strains S-I, S-II and S-III were uniformly stained by crystal violet on all sides of the tissue culture plate, which indicates strong biofilm producing ability. Strain S-IV showed moderate biofilm producing ability because the intensity of stained crystal violet was less in comparison.

Fig.5: **Screening of biofilm producers by Tube Method**

S-I, S-II, S-III: High, S-IV- Non biofilm producer & C-control.

Table No2: Tube Method

S. No	Microorganisms		Report
1	*Staphylococcus aureus*	S-I	Strong
2	*Staphylococcus aureus*	S-II	Strong
3	*Staphylococcus epidermidis*	S-III	Strong
4	*Staphylococcus epidermidis*	S -IV	Weak

Values are mean ± S.E.M (n=3)

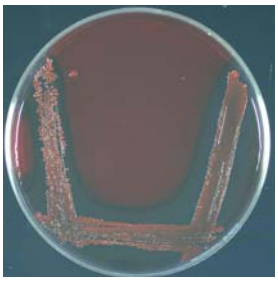
In tube method the strains S-I S-II, and S-III formed thin lined film on sides of the wall and bottom of the test tubes which indicates strong biofilm producing ability. Strain S-IV showed lesser intensity of stained crystal violet in comparison.

Fig.6: Screening of biofilm producers

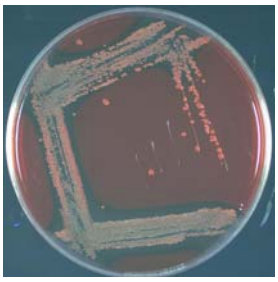
by Congo Red Agar method

S-IV Pink colonies

S-I Black colonies



S-III Pink colonies



S-II Black colonies

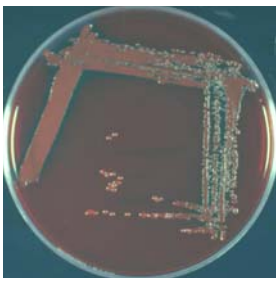


Table No3: Congo Red Agar method

S.No	Microorganisms		Report
1.	*Staphylococcus aureus*	S-I	Strong
2.	*Staphylococcus aureus*	S-II	Strong
3.	*Staphylococcus epidermidis*	S-III	Weak
4.	*Staphylococcus epidermidis*	S -IV	Weak

Values are mean ± S.E.M (n=3)

In CRA method S-I and S –II strains displayed black colonies with dry crystalline consistency confirming the organisms to be strong slime producers. S-III and S-IV displayed pink colonies which confirm they are weak slime producers.

SUMMARY AND CONCLUSION

Biofilm forming ability has been recognized as an important virulence factor in *Staphylococcal* spp. facilitating their persistence in the host, evading its defences and allowing bacterial survival at high antimicrobial concentrations (**Olivera M. *et al*., 2006).**

Biofilm producing ability does not appear equally in all strains (S-I, S-II, S-III and S-IV). The differences observed could be a result of the growth conditions used, lack of ability of some biofilm to adhere to polystyrene plates or due to different adherence mechanisms**(Vasudevan .P *et al.*, 2003).**

The strains S-I, SII, S-III, and S-IV adhered to the biomaterial at 12, 48, 48 and 72, hrs respectively. The duration for biofilm formation on the biomaterial was almost 72 hrs in all the 3 strains except S-IV which took 96 hrs for biofilm formation. There was no direct relationship between the amount of biofilm formed and the initial adhesion extent. High levels of initial adherence do not necessarily lead to thick biofilm formation. (**Nuno Cerca *et al*., 2005).**

Rifampin and Ofloxacin that were used in this study, exerted inhibitory action on all the tested strains with respect to preventing adhesion, biofilm formation and in eradication of the matured biofilm formed. Both these drugs could be used together with possible synergistic result. The methods used in this work could be

used to study the effect of more effective newer antibacterial agents against biofilm forming organisms. Genotyping of biofilm forming organisms could also be used in their identification. This could be used along with phenotype based identification methods that have been used in this study. Our study could have better clinical implication if supported with genotypic characterization of biofilm forming organisms, especially clinical pathogens. Antimicrobial combinations could be tested for possible synergism against biofilm forming organisms whose eradication in clinical setting, continues to remain a challenge for the physician.

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