STUDIES ON INTERACTIONS BETWEEN POLYMER BIOMATERIALS AND *STAPHYLOCOCCUS EPIDERMIDIS* STRAINS

A Thesis presented
by

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In partial fulfilment of the requirement for the
degree of Doctor of Philosophy

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
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DECLARATION

I, **K. RATHINAM** hereby certify that I had personally carried out the work depicted in the thesis entitled **"STUDIES ON INTERACTIONS BETWEEN POLYMER BIOMATERIALS AND *STAPHYLOCOCCUS EPIDERMIDIS* STRAINS"** except where external help was sought and acknowledged.

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Studies on Interactions Between Polymer Biomaterials and *Staphylococcus Epidermidis* Strains

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This work is dedicated to my parents

Dr. K. Kothandaraman,

Thirumathi K. Nagalakshimi

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" Life is not so short but that there is always time enough for courtesy "

- Ralph Waldo Emerson

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" The extended abstract can supply virtually as much information as a full paper (thesis). "

- Robert A. Day

Synopsis

STUDIES ON INTERACTIONS BETWEEN POLYMER BIOMATERIALS AND STAPHYLOCOCCUS EPIDERMIDIS STRAINS

The introductory chapter deals with the statement of the problem and its significance, statement of hypothesis, assumption limitation and delimitation and definition of terms. It was Elie Metchnikoff the father of cellular immunology, who in 1893, first gave serious attention to the phenomenon - foreign bodies with infection.[1] Foreign body associated infection has been an important clinical problem not only to health care programme but also economic burden to the hospital authority and ailing patients as well. Most of the foreign body associated infection especially as a result of polymer based device implants used for diagnostic, reconstructive and other clinical conditions end with impending infection mainly caused by Staphylococcus-epidermidis[2-4] a Gram positive, coagulase-negative, sucrose fermenting staphylococcus species. It has been well established[5] that implanted polymer substrate tend to favour colonization by Staph. epidermidis. The sources of Staph. epidermidis are found to be from hospital environment, hospital personnel and other types of nosocomial infections.[6] Hence the constant surveillance of these pathogen is becoming very important in controlling the eventual foreign body associated infection due to the interaction between Staph. epidermidis and the implants. In this context the present study has great relevance in understanding the phenomenon of infection associated with polymer Biomaterials.

The microbiological survey of different possible sources of Staph. epidermidis, isolation & identification of them becomes very important so that its interaction with foreign bodies from these sources is avoided. Slime producing potential of Staph. epidermidis is also assessed and isolated from different sources so that the possible incidence of Implant associated infection is predicted & controlled.

Isolation, identification, and speciation of Coagulase- negative Staphylococci for Staph. epidermidis from different sources, their slime forming potentials, antibiotic susceptibility, adherence to implant materials and the interference by antibiotics, in vivo tissue cage experiments in Guinea pig and Rabbit models are done to hypothesize the possible mechanism of controlling/preventing the adverse bacterial biomaterial (Polymer) interaction so that infection does not occur, and if it occurs, is rendered easily curable with antibiotics/interferring solution.

Chapter II deals with the review of related Literature wherein the colonization of Staph. epidermidis on Biomaterials resulted in Implant-associated infection. Review of the literature encompasses the incidence of infection as a result of interaction of slime positive, and slime negative Staph. epidermidis with materials[7] and other clinical anecdotes that dictates the prudent control & evaluation of bacterial biomaterial interaction problems.

Chapter III deals with the Materials and Methods and the different instrumental analysis (Elec . microscopy) for adherence study and (UV spetro photometer) for Hydrophobicity/Hydrophilicity of Staph. epidermidis. Staph. epidermidis isolated and speciated from different sources such as Clinical, Aerial, Hospital Aerial. Non - hospital Aerial and carriers are subjected to Antibiotic susceptibility test[8] slime production,[9] Beta lactamase[10] production, M.I.C[11] and hydrophobicity/hydrophilicity[12] characters. Evaluation of Antibacterial effect of silver coated material was also carried out using representative strain of Staph. epidermidis. Chlorhexidine digluconate impregnated with silastic polyurethane materials were also found to have a significant antistaphylococcal effects against A182, A313, A61 and A72 (Staph. epidermidis clinical Std strains received from Australia, and Wood 46 strain (Staph. aureus) received from Switzerland).

In vivo experiments using tissue cages,[13] an attempt was made to see the phenotypic changes that happen to the injected Staph. epidermidis (Std. strain A54) in terms of their susceptibility to antibiotics, slime production and other parameters tested prior to the injection to the tissue cage both in Guinea pigs and Rabbits.

In vivo tissue cage experiments were initiated using Guinea pigs in order to study the efficacy of antibiotic effect of agents that showed significant effect in in vitro

condition. This experiment resulted in loss of 95% of implanted animals due to some unexpected factors such as severe adhesion, protrussion of edges of Tissue cages through the skin exposing them open to external entry of bacteria from aerial environment. However two experiments yielded interesting findings, ie. 1.0 mg/kg of cloxacillin given I.P to the A54 strain infected Tissue cage did not show any effect except that the susceptibility of the bacteria to Cloxacillin reduced markedly. A startling finding that 100.0 mg/kg of Cloxacillin (non toxic tolerable dose) given intramuscularly (route was changed from I.P to IM in order to get the maximum benefit out of pharmacokinetic effect of the drug) completely eradicated the intracage infected A54 Staph. epidermidis.

Chapter IV deals with the Analysis of Data with Results and Discussion.

Isolation and Identification of Coagulase negative Staphylococci from different sources showed the predominance of Staph. epidermidis.[6] The slime production was found to be maximum with Staph. epidermidis isolated from clinical isolates followed by hospital staff members. Staph. epidermidis was also found to be the leading species exhibiting maximum number of slime producing strain ie 52.5% Staph. epidermidis isolated from clinical specimens showed maximum percentage of slime production than aerial and carriers samples.[14] The conventional antibiotic

susceptibility test using Disc diffusion method indicated that isoxazolyl pencillins such as Oxacillin and Cloxacillin have maximum antistaphylococcal effects against Staph. epidermidis followed by Methicillin, Ticarcillin and Carbenicillin.[15] Silver impregnated Latex catheter material was found to have antibacterial effects against Staph. epidermidis strains such as A54 (Staph. epidermidis isolated from Blood culture) ATCC 35983 (Staph. epidermidis std strain) B 3972 (0) Std. strain and E.coli (clinical specimen). Among Quinolone antibacterial agents Ofloxacin exhibited maximum inhibiting effects against Staph. epidermidis strains.

A new era in the tissue cage implantation technique using Rabbit model has been initiated and in this procedure no protrusion of cage edges through the walls of skin was observed. Another advantage of this system is that the cage can be moved in the subcutaneous space so that injection of Bcteria & aspiration of tissue cage exudate become easy and less traumatic. Antibiotic solution such as isoxazolyl penicillins, Quinolone and other interferring solutions have been tried with this system after injecting with Standard strains of clinical Staph. epidermidis.

Chapter V deals with the summary and conclusion of the study. Predominance of Staph. epidermidis strain among the various environment and carriers would give us lot of

information that will be of high value in monitoring and preventing the occurrence of serious infection due to Staph. epidermidis strains in patients implanted with various types of medical devices.

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" Diseases is from old and nothing about it has changed . . . it is we who change as we learn to recognise what was formerly imperceptible. "

- Charcot

Introduction

CHAPTER I

INTRODUCTION

Recent advances made in Medical sciences and biomedical engineering have succeeded in the possibility of replacing a variety of defective or loss of functioning of body as a whole or part of it, with Bioimplants. Bioimplants (Fig 1.1) have emerged in last quarter of the 20th century in many fields of medicine, surgery and dentistry and have given rise to much new discipline as plastic surgery and biomaterial science. Since the early 1950's devices made with synthetic or natural biomaterial have been put into the body at ever increasing rate (Consensus Conference, JAMA, 1983). Biomaterials are defined as any substance (other than drugs) or combination of substances, synthetic or natural in origin that can be used for any period of time, as a whole or as a part of a system that treats, augments or replaces any tissue, organ or function of the body. The types of materials used for making various implants are depicted in Table 1.1. 25 million major operative procedures involving bioimplants are performed in the United States of America yearly and an equivalent number of such operations in Europe (Graves, 1989) using both temporary and permanent use of biomaterials. Millions of operative procedures such as continuous ambulatory peritoneal dialysis and the use of drug delivery catheters or fibre optic interventions also involve biomaterials. Surgical procedures like Cardiovascular, Plastic, Gynecological, Orthopaedic, Urologic and Reconstructive operations are responsible for implanting more than one million permanent biomaterial devices each year in USA alone. The major impediment to

FIGURE 1.1

BIOIMPLANTS USED AS HUMAN SPARE PARTS

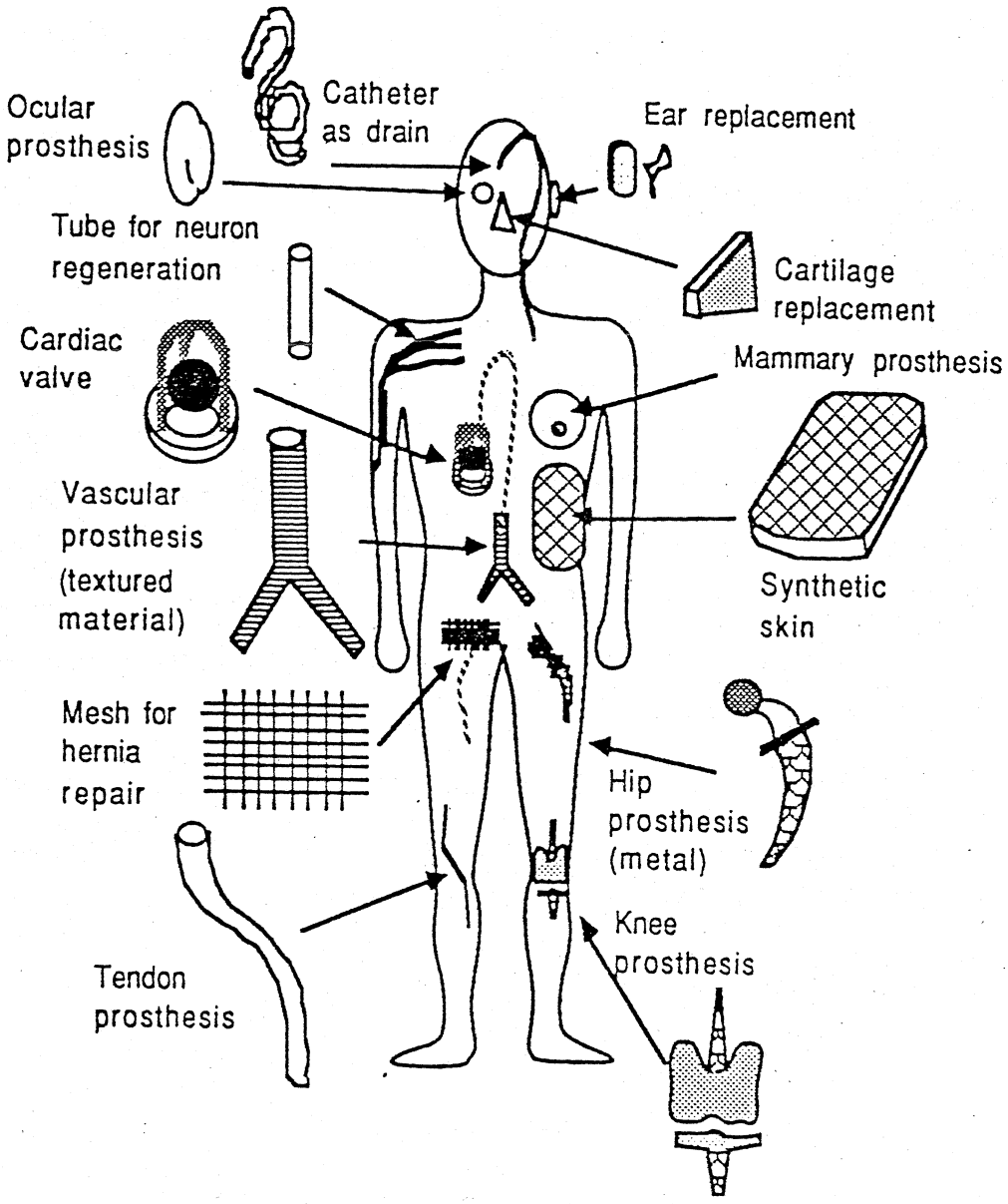


Table 1.1

POLYMER BIOMATERIALS AND THEIR APPLICATIONS

BIOMATERIALS (POLYMERS)	APPLICATION(S)
Polyamides, Polyesters, Polypropylene, Polylactic acid & Polyglycolic acid	Sutures
Polyester	Vascular grafts & surgical fabrics
Polyethylene - Ultra High molecular Weight Polyethylene	Knee, Hip and Prosthetic heart valve
Polymethylmethacrylate (PMMA)	Bone, Dental cements & Denture materials
Polytetrafluoroethylene (PTFE)	Middle Ear implants
Polyhydroxyethylmethacrylate (Poly - HEMA)	Intra-ocular lenses

the extended use of implanted biomaterials is infection (Table 1.2) centered around the foreign body (Gristina, 1987[a]). The rate of infection in major implant surgery is found to be 0.5 and 6.0 %, even in those receiving prophylactic antibiotics. Morbidity and cost involved in biomaterial centered infection has been estimated to exceed five to ten times than that of the original procedures. For example, unrelenting infection of the total joint replacement or vascular grafts usually require removal of the device and leads to reimplantations. Revision surgery is difficult, successful only in less than one third of cases, and may result in death and amputation. Infection rates vary from less than 0.5% for artificial hip to 6% for vascular graft implant and up to 100% for the total artificial heart (Gritina et al, 1994). The bacteria causing biomaterial centered infection are highly adaptive organisms and naturally adhere to surfaces as a survival mechanism whether surface is metallic, polymeric or biologic.

It has been well established that coagulase-negative staphylococci (CNS) are among the most commonly isolated organisms in patients with prosthetic devices (Baddour & Christensen, 1987; Baddour et al, 1986; and Gristina, 1987[b]).Among them, **Staphylococcus epidermidis (Staph.epidermidis)** is the most frequently isolated species, occurring in the majority of biomaterial associated infections (Hamory & Parisi, 1987; Archer, 1978). **Staph. epidermidis** and other CNS species, often previously discarded as culture contaminants are assuming greater importance as true pathogens (Archer, 1990). Infections caused by these organisms involved in indwelling

Table 1.2

IMPLANTS ASSOCIATED INFECTIONS

Implants	Infections
CSF Shunts	Access site infection, ventriculitis
Ocular Prosthesis	Conjuctivitis/keratitis
Dental implants	Peridontal disease,gingivitis
Cardiac pacemakers	Generator pocket infection, Endocarditis
Breast implants	Soft tissue infections
Joint prosthesis	Septic arthritis, osteomyelitis
Intra venous/arterial cathters	Septic phlebitis,bacteremia , septicemia
Vascular grafts	Graft infections
Intrauterine devices	Pelvic inflammatory disease
Hydrophilic (soft) contact lenses	Bacterial corneal ulceration (BCU)
Cardiac valves	Prosthetic valve endocarditis

foreign bodies are increasing as the number of catheters and artificial devices implanted become more numerous.

These infections are characterised by their indolence but may necessitate the removal of the catheter or device. Resistance of infecting organisms to multiple antibiotics may further complicate treatment. It has further been established that implanted polymer substrate tend to favour the colonization by **Staph. epidermidis** (Gristina et al 1987[b]). There has been a belief that **Staph.epidermidis** is one of the commensal micro-organisms present on our skin, nose and ears having no pathogenicity as such. However, when it finds an entry into our body during implantation of medical devices or later for various clinical problems converts itself from non-pathogenic **Staph. epidermidis** to pathogenic and becomes an opportunistic pathogenic bacteria.

Foreign body associated infection has become an important clinical problem not only for health care programme of a nation but also an economic burden to the hospital management as well as ailing patients. Ideally, the prevention of coagulase-negative staphylococcal infection should be based on better understanding of the epidemiology, hospital reservoirs, mechanism of transmission, virulence factors present in **Staph.epidermidis** such as slime forming potential, *in vitro* antibiotic susceptibility pattern, bacterial cell surface property such as hydrophobicity/ hydrophilicity, adherence property on foreign bodies such as catheter surfaces, the phenotypic metabolic changes induced by biomaterial surfaces and bacterial susceptibility to most sensitive antibiotic in *in vivo* condition. In this context the different sources of prevalence of coagulase-negative Staphylococci especially **Staph. epidermidis** become highly relevant in

understanding the occurrence, virulence and antibiotic sensitivity pattern so that correct regimen of treatment could be forecasted.

With these basic information efforts are made towards the isolation and identification of **Staph. epidermidis** among coagulase-negative staphylococci, isolated from different sources, antibiotic susceptibility pattern, slime producing potentials, hydrophobicity of their surface, their pathogenic characteristics such as Beta-lactamase production, minimum inhibitory concentration of Penicillin and bactericidal effects of a Beta-lactam antibiotic in *in vivo* experiments. Antibacterial agents incorporated biomaterials such as latex rubber and polyurethane materials are also investigated to demonstrate the possibility of using bacterial resistant biomaterials in clinical practice.

" Nature fits all her children with something to do, he who would write and can,t write can surely review. "

- James Russell Lowell

Review of related literature

CHAPTER II

REVIEW OF RELATED LITERATURE

The harmful effect exerted by the presence of a foreign body on the evolution of wounds has been known for a very long time. Four centuries ago Amproize Pare in his treatise dealt with wounds made by gunshot, Fierie Engines and all sorts of weapons (Johnson 1634). Galen stated that nature cannot endure a little hair in any little strange body enclosed in a wound (Worms et al.1973).Research undertaken shortly after the outbreak of World War I revealed two important items of data: First, that foreign bodies fix germs on to their surfaces (Delbet and Fiessinger,1918) and secondly, that a germ bearing foreign body can remain enclosed in the depths of a healed wound and that the germs it bears are capable of surviving in a dormant state until such time as an accidental cause triggers their return to virulence (Policard 1918).

A great many investigation have been devoted to the tissue response to foreign bodies a few of them associated with infection, most of them concerned with comparing the degree of inflammatory reactions caused by foreign bodies such as metals, silk, catgut and polymers (Elias and Epslein, 1961). Since early 1950's devices made up of synthetic or natural materials have been introduced into the human body at an ever increasing rate. Biomaterials are now used to address needs that the patient perceives in terms of rehabilitation comfort, convenience and aesthetics. It has been estimated, for example, that as many as 59,000 individuals

underwent unilateral or bilateral hip joint replacements in the United States in 1980 alone (Bisno and Waldvogel, 1989).

As with all forms of treatment, however adverse side effects are to be anticipated in implantology. In practice, one of the most frequent and serious complications of the use of these biomaterials has been the development of infection. It was Elie Metchnikoff the father of Cellular immunology who in 1893, first gave serious attention to the phenomenon- foreign bodies with infection (Metchnikoff & Lecture 1968) Coagulase-negative staphylococci (CNS) and in particular **Staph.epidermidis** are implicated as pathogens of infections associated with Indwelling catheters (Archer, 1978). Cerebrospinal shunts (Holt 1971). Prosthetic heart valve (Chemovitz et al 1985), Hip joints (Patterson and Brown 1972) and other implanted devices (Eisenberg et al 1987 and Sugarman , 1984). **Staph. saprophyticus** was the second leading pathogen after **Escherichia coli** in urinary tract infection (Maclaren and Namavar 1983).

Staph.hominis and **Staph. haemolyticus** also cause infections in man while five other species cause rare and opportunistic infections (Pfaller and Herwaldt 1988; Noble and white 1983 and Kloos and Lambe 1981). There is a growing recognition that coagulase negative staphylococci (principally **Staph.epidermidis)** is the common pathogen in patients with prosthetic devices (Sewell et al 1982 and Hagler et al 1975) occur within few weeks to months after implantation. It has been demonstrated that colonization of medical devices by **Staph.epidermidis** is the first event in the process of biomaterial associated infection (Bayston and Penny 1972).

Once interaction between **Staph.epidermidis** and implants initiated, adherence and formation of mucoid substance viz. slime formed at the interface (Ludwicka et al 1984, and Peter 1988).

Bacterial adherence on to surfaces is regarded to be an important virulence factor (Ofek & Beachay, 1980) produced by **Staph.epidermidis.** Prevalence of **Staph.epidermidis** among clinical, hospital and non hospital environments which are indirect factors for subsequent nosocomial infection including implant associated infections were demonstrated by many investigators (Krynski et al 1973, Parisi 1886, Shanmugam et al 1994, Thurn et al 1992 and Deighton et al 1988).

Slime production by **Staph.epidermidis** has been found in several investigations as adherence as well as pathogenic factors leading to foreign body associated infection.(Davenport et al 1986, Sheth et al 1985 and Christensen et al 1982). However, some investigators showed in their studies the failure of significant positive correlation between slime production and pathogenicity of implant associated infections (Kristinsson et al 1986 and Needam & Stempsey 1984).

Slime production by **Staph.epidermidis** has been associated with the ability of this organism to adhere to the surfaces of catheter and other plastic biomedical devices (Peters, 1986, Peters & Pulverer 1984 and Sheth et al 1985). Slime production potential of **Staph.epidermidis** has also been found by several investigators to be an efficient discriminator between infectious and non infectious strains (Christensen et al 1982 and Diaz Mitoma et al 1987). However other investigators have found that slime production is not an universal determinant of

those strains causing infections (Diaz Mitoma et al 1987, Kristinsson et al 1986 and Needham & Stempsey 1984). Subsequently concensus has developed that beside test for slime production in medical decision making, the additional clinical and epidemiological studies would be considered for pathogenicity of foreign body associated infection.

Antimicrobial susceptibility of coagulase - negative staphylococci particularly to antibiotic agents is extremely variable. Although commonly acquired isolates are frequently susceptible to a wide variety of antibiotics, strains isolated from hospitalised patients have been noted to be resistant to an increasing number of antibiotics (Archer 1978, Archer & Armstrong 1983 and Karchmer et al 1983) Antimicrobial resistance and *in vitro* susceptibility shown by **Staph. epidermidis** have been reviewed by investigators (Pfaller and Herwaldt 1988, Kaiser 1989).

Treatment of coagulase- negative Staphylococcal infection depends on the severity and anatomic site of infection, the presence of a foreign body prosthetic device and the results of antimicrobial susceptibility testing. Penicillin is the drug of choice for susceptible strains of **Staph.epidermidis**. If the organism is resistant to penicillin then treatment with Naficillin, Oxacillin or Cephalothin is effective. Lowry and Hammer (1983) and Karchmer et al (1983) have recommended that Vancomycin can be used empirically for the treatment of the **Staph.epidermidis** infection.

Alternative antibiotics are currently being investigated that may be useful against multiresistant isolates of coagulase -negative staphylococci. These include newer agents such as Teichoplanin and Quinolone derivatives. (Barry et al, 1987,

Archer 1990 and Galetto et al 1986). Because of the importance of adherence occurring in **Staph.epidermidis** biomaterial related infections, several investigators have suggested that production of catheters and other implants which are resistant to bacterial adherence may be an effective means of preventing these infections. (Gristina 1987, Kingston et al 1986 and Pascual et al 1986).

Bacterial adhesion with mammalian cells and artificial surfaces is promoted by the hydrophobicity of the bacterial cell surfaces (Rozgonyl et al 1990 and Hogt 1983[a]). The hydrophobic interactions between two non polar groups is of fundamental importance in both the attachment of bacteria to each other, and the bacterial adherence to tissues and biomaterials. Therefore determination of bacterial cell surface hydrophobicity may give us an information about the hydrophobic interaction between bacteria and biomaterials. However as to staphylococci, it has been shown that they seem to use different types of bonds to attach to and colonize biomaterial surfaces (Barret 1985 and Costerton et al 1985). It was reported that slime present around adhered bacteria decreased bacterial hydrophobicity (Hogt 1983[b]). Thus no direct positive or negative correlation under various conditions used, between slime production and hydrophobicity of bacteria could be made.

Over six million urinary catheters are used annually in India alone and also it has been estimated that an average of 20% of patients developed catheter associated urinary tract infections. It has been well established that catheter related infections are caused by microorganisms Viz. Gram positive bacteria especially coagulase negative staphylococci. The pathogenesis of such infection is favoured by these organisms

potential to adhere to polymer surfaces followed by colonization and the production of adherent biofilm (Slime). Slime embeds bacteria on the catheter surface rendering antibiotic treatment and host mechanisms ineffective (Sheth et al, 1985). To overcome the problem of catheter related infections, the concept of breaking the periluminal migration of bacteria into the intracutaneous segment by the application of antimicrobial substances to the catheter (Solomon & Sherertz, 1987) or the development of biocompatible and antiadhesive surfaces have been suggested (Maki 1986). As antibiotics carry the risk of bacterial resistance, the application of heavy metals such as copper and silver were investigated. Silver is well known antimicrobial agent with a strong oligodynamic activity, and different formulations have been found to be effective in topical treatment of burns (Fox,1968), severe chronic osteomyelitis (Becker & Spadaro 1978) and urinary tract infections (Liedberg & Lundeberg 1990). *In vitro* studies by incubating silver-coated polyurethane segments with 10^5 colony forming units (CFU/ml) of **Staph. epidermidis** KH^6 led to a significant decrease of cells adhering to the surface (Jans et al, 1992) silver coated nylon suture material was to exhibit antibacterial property towards well established bacterial colonies (Chu et al 1987).

Antimicrobial coated biomaterials for different applications becomes necessity due to their bacterial resistant property in medicine. One of the antibacterial agents considered has been Chlorhexidine digluconate that was proven to be effective against colonization by **Staph.epidermidis** (Hadein & Hambracus, 1993 and Shik et al 1991).

Implant associated infections are often resistant to antibiotic therapy, routine *in vitro* antibiotic sensitivity tests fail to predict therapeutic success. Therefore experimental *in vivo* test were sought that would better correlate with drug efficacy in biomaterial/device related infections.

An animal model suitable for this type of studies was developed using Polymethylmethacrylate or Polytetrafluoroethylene (Teflon) perforated cylinders namely Tissue cages (TC) implanted subcutaneously in Guinea pigs (G.pigs) Zimmerli et al 1981, 1982 and 1986). While demonstrating the drug efficacy in tissue cage experiment Zimmerli and Widmer 1990 have shown maximum i.e. 75% and minimum i.e. 0% of antibacterial effects by Rifampicin and Ciprofloxacin respectively. Cure rate against **Staph.epidermidis** correlation between *in vivo* and *in vitro* efficacy of antibacterial against biomaterial associated infection was made by Widmer et al (1990). No data is available as to the antibacterial effects of Beta-lactam antibiotics such as Cloxacillin, Oxacillin, Methicillin and Ticarcillin against **Staph.epidermidis** using TC model (Widmer et al 1990).

"It is common sense to take a method and try it : if it fails, admit it frankly and try another."

- Franklin D. Roosevelt

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

In vitro Experiments:

ISOLATION OF COAGULASE-NEGATIVE STAPHYLOCOCCI FROM HOSPITAL AND NON HOSPITAL (BIOMEDICAL TECHNOLOGY WING) AERIAL SOURCES.

One hundred and forty one hospital and one hundred and twenty non hospital Biomedical Technology Wing (BMT Wing) aerial CNS strains were collected by exposing the sheep blood agar plates (5%) for one hour at various locations. The plates were incubated at 37 ± 1^0 C for a minimum of 18 hours in an incubator. After incubation, Staphylococci like colonies white flat colonies were picked up.

ISOLATION FROM THROAT, NOSE, AND EXPOSED PART OF SKIN (FORE ARM).

Isolation of coagulase-negative staphylococci from throat, nose and skin from healthy hospital (35) and non hospital i.e. BMT Wing (51) Staff's were isolated by taking swabs (sterile) from oropharyngel area, nasal area, cleaned area of fore arm and streaked on sterile sheep blood agar plates and incubated to a minimum of 18 hours.

ISOLATIONS FROM ROUTINE CLINICAL ISOLATES AVAILABLE AT THE DIVISION OF MICROBIOLOGY, SREE CHITRA THIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, THIRUVANANTHAPURAM-695 011.

Two hundred and seven strains of coagulase-negative Staphylococci were isolated from various clinical specimens such as blood, sputum, pus, urine, CSF etc. were subcultured in sheep blood agar and subsequently in nutrient broth.

Each strain stored in semisolid Nutrient agar overlayered with sterile liquid paraffin was picked up using a sterile loop and streaked over sheep blood agar and incubated for a minimum period of 18 hours.

All these CNS strains were identified by colony morphology, Gram's staining, catalase and coagulase tests.

Colony morphology: Visual examination of the colony morphology of strains grown in blood agar was made and confirmed as Staphylococci on the basis of following criteria. Small white colony with raised flat surface were picked up and inoculated into sterile nutrient broth and incubated over night. Isolates, thus subcultured was subjected to the following tests for confirmation as Staphylococci.

a. Gram's staining:- (Hucker modification method)

A smear was prepared on a clean and grease free micro slide and fixed by mild heating by passing over the flame 2-3 times. Staining of the fixed and dried smear was done as follows sequentially.

Primary stain with crystal violet solution for one minute. Slide was rinsed with distilled water and flooded with gram's iodine solution and kept for one minute and washed with distilled water. Later the slide was flooded with acetone for a few seconds and immediately washed with distilled water. After decolourisation with acetone and wash, the slide was counterstained with safranin and kept for thirty seconds and rinsed subsequently with distilled water and dried. Microscopic observation of gram's stained smear was made under oil immersion (100X) for morphological characterisation of the bacterial strain under test. Gram positive (purple stained) cocci in clusters was identified as Staphylococci (Photographic plate no.I).

Catalase test.

One loopfull of bacterial strain grown in nutrient agar over night was introduced into a test tube containing 30% by volume hydrogen peroxide solution. If effervescence (release of oxygen) observed in the hydrogen peroxide after the insertion of bacteria, it confirms the organism as catalase positive.

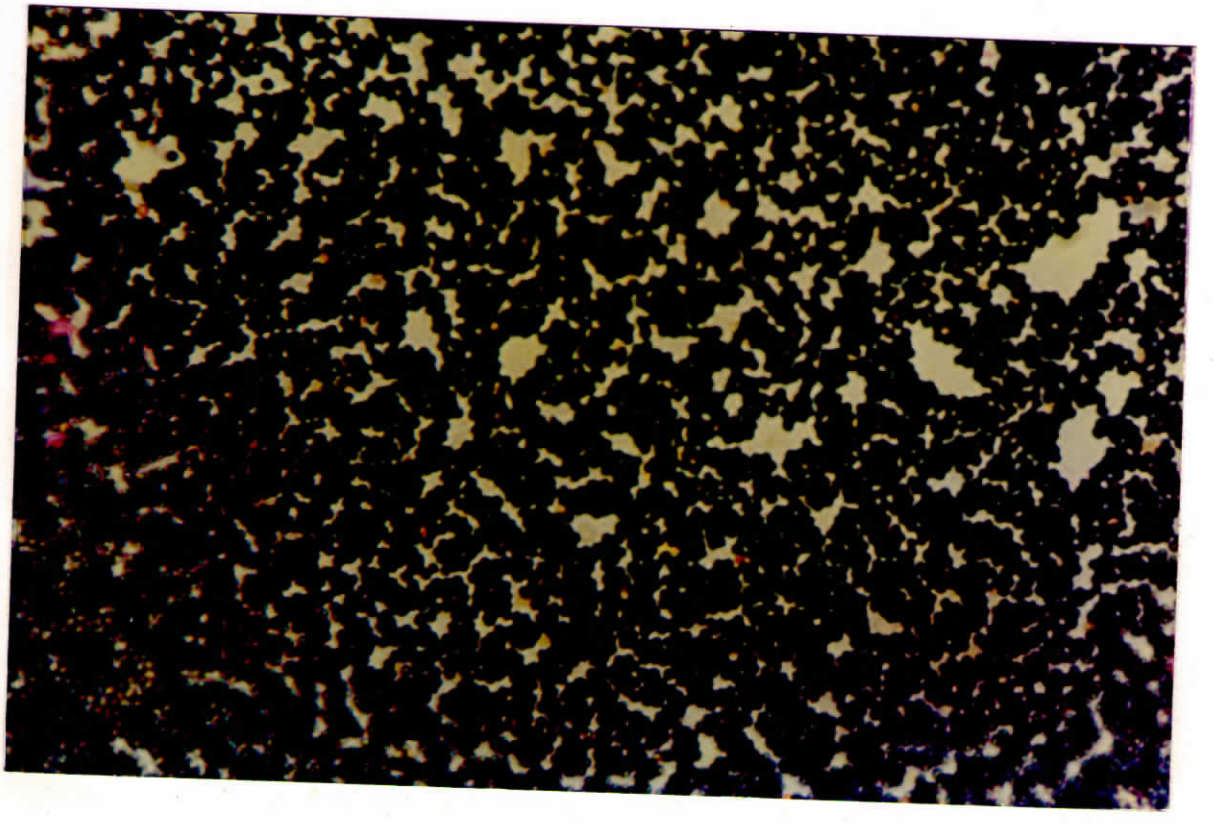
Coagulase test:

Once the strain was identified as staphylococci, it had further to be classified as coagulase- positive and coagulase- negative Styphylococci by coagulase test.

A single colony was subcultured into the nutrient broth and incubated over night (not less than 18 hours) 0.5 ml of 1:10 sterile human plasma was added and inoculated with fresh culture and incubated at 37^0C for 3-6 hours.

PHOTOGRAPHIC PLATE NO I

STAPHYLOCOCCUS EPIDERMIDIS (GRAM STAIN)



The formation of the clot (gel) confirmed the strain as coagulase positive i.e. **Staphylococcus aureus**, and strain not causing coagulation belong to the group known as coagulase negative Staphylococci.

Sugar Fermentation test (Woods et al.1987):

This is one of the tests used to speciate the coagulase-negative Staphylococci. Sugars used were Glucose, Sucrose, Mannitol, Trehalose and D(+)Xylose. Sugar fermentation medium consisted of the following ingredients and amount.

a. Preparation of nutrient broth:

Sodium chloride	-	5. 0 g
Peptone(Bacteriological)	-	10.0g
Beef extract(Lab Lamco)	-	3. 0 g
Distilled water	-	1000 ml

The above ingredients were dissolved in 1000.0 ml distilled water and mixed well. The pH of the solution was adjusted to 7.6 using Lovibond comparator. The nutrient broth thus prepared was sterilized by autoclaving at 15 lb. pressure (121 °C) for 15 minutes. To 500.0 ml of nutrient broth 2.5 g of each sugars (0.5%) indicated earlier, were added using Andrade's indicator. The solution was distributed into 10X75mm test tubes in 2 ml amounts and plugged with cotton swabs. Sterilization of

the sugar solution was carried out by autoclaving at 10 lb. pressure (110 °C) for 10 minutes.

Freshly subcultured strain of CNS was inoculated into each of the five sugar solutions and incubated for a minimum period of 18 hours at 37 °C.

Fermentation of sugars were confirmed when tubes showed change of colour from yellow to pink. Depending upon the types of sugar fermented, the CNS strains were classified into different species. **Staph.epidermidis** was speciated among other CNS species, on the basis of its fermentation positivity with Glucose and Sucrose (Table 3.1).

Once **Staph.epidermidis** was identified the strains were preserved by inoculating them in semi solid nutrient agar over layered with sterile liquid paraffin and maintained at room temperature.

Table (3.2) shows the details of **staph. epidermidis** obtained from Australia and Switzerland as standard control strains which were used in all our experiments. Standard as well as isolated CNS strains from different sources were stored in semi solid nutrient agar over layered with sterile liquid paraffin and used by subculturing them in nutrient broth or blood agar periodically.

ANTIBIOTIC SUSCEPTIBILITY TEST:

The antibiotic susceptibility tests of all CNS strains and **Staph.epidermidis** isolates from different sources was carried out as per method of Bauer et al (1966) on Mueller-Hinton (MH) agar plates. Hence antibiotic susceptibility test of a given

Table 3.1

DIFFERENTIATION OF COAGULASE-NEGATIVE STAPHYLOCOCCI ON THE BASIS OF SUGAR FERMENTATION TEST (Wood et al 1987)

Sl.No	Coagulase- negative Staphylococci	Glucose	Sucrose	Mannitol	Trehalose	Xylose
1.	**Staph.epidermidis**	+	+	-	-	-
2.	**Staph.saprophyticus**	+	+	+	+	-
3.	**Staph.cohnii**	+	-	+	+	-
4.	**Staph.hominis**	+	+	-	+	-
5.	**Staph.capitis**	+	+	+	-	-
6.	**Staph.xylosus**	+	+	+	+	+

+ Fermentation positive, - Non fermenting.

Table 3.2

DETAILS OF STAPH.EPIDERMIDIS AND STAPH.AUREUS STRAINS USED AS STANDARD CONTROLS

Sl. No	Strain No	Staphylococcus species	Sources	Slime Production	Hydrophobicity/Hydrophilicity
1.	A 54	**Staph.epidermidis**	Melbourne, Australia	Strongly Positive	Hydrophobic
2.	A 61	-do-	-do-	Positive	-do-
3.	A 72	-do-	-do-	Strongly Positive	-do-
4.	A 74	-do-	-do-	Positive	-do-
5.	A 77	-do-	-do-	Strongly Positive	-do-
6.	A 182	-do-	-do-	Negative	-do-
7.	A 283	-do-	-do-	-do-	-do-
8.	A 310	-do-	-do-	Positive	-do-
9.	A 313	-do-	-do-	Weakly positive	-do-
10.	A 315	-do-	-do-	Negative	-do-
11.	A 332	-do-	-do-	Positive	-do-
12.	Wood 46	**Staph.aureus**	Basel, Switzerland	-do-	-do-
13.	ATCC 35983	**Staph.epidermidis**	-do-	Strongly Positive	-do-
14.	B 3972 (O)	**Staph.epidermidis**	-do-	-do-	-do-
15.	B 3972 (V)	**Staph.epidermidis**	-do-	-do-	-do-

organism is used to determine the sensitivity and resistant pattern of a given bacteria to antibiotics/antimicrobial agents. Results of these tests are used for the selection of correct antibiotic for treatment against the infecting organism. It also provides information as to the resistant pattern occurred by the organism to a particular antibiotic indicating the use of alternative agent. Hence it has become an important technique in most of medical microbiology laboratories. The antibiotic discs used were obtained from M/s Pasteur diagnostic , Surat, India.

Mueller-Hinton (MH) agar was made from ready made media obtained from M/s Hi Media Pvt. Ltd. Bombay, India after the reagent was dissolved and autoclaved at 121 °C for 15 minutes, cooled to 55 °C. MH agar subsequently poured into 100 mm diameter sterile petridishes for uses as media for antibiotic susceptibility tests.

The composition of Mueller-Hinton agar is as follow:

Beef heart infusion	-	30.00 g
Casein acid hydrolysate	-	01.75 g
Starch (soluble)	-	00.15 g
Agar	-	01.70 g
Distilled water	-	100.0 ml
Final pH at 25 ^{0}C	-	7.4 $\pm$ 0.2

20 clinical strains of **Staph.epidermidis** and 20 aerial **Staph.epidermidis** were tested against 10 types of Beta- lactam antibiotics (Table 3.3). 25 strains of **Staph.epidermidis** of clinical sources and 39 aerial **Staph.epidermidis** were exposed to Fluoroquinolones (Table 3.3) .These strains were grown in nutrient broth and incubated at 37°C for 1 to 2 hours (A single colony of a small fraction of culture of nutrient agar plate was inoculated in to the above broth).

Lawn culture:-

MH agar plates to be used were kept in the incubator for drying and labeled with strain number, date of inoculation and the names of the antibiotics (6 to 10 numbers).

Sterile swabs (cotton dipped in the test strains grown in broth) were squeezed on the sides of the test tubes and spreaded over MH agar plates. Overflowing was avoided, since it would interfere with zone of inhibition by the neighboring antibiotic disc.

Inoculated MH plates (lawn culture) were kept inside the incubator at 37°C for about 15 to 20 minutes for drying. Consequently antibiotic discs (10 types of Penicillins and 3 types of Fluroquinolones) were placed in their respective places (marked earlier) inside the MH agar plate and incubated overnight at 37 °C.

Table 3.3

ANTIBIOTICS USED FOR SUSCEPTIBILITY TEST AND THEIR INTERPRETATION

Sl. No	Antibiotics used	Disc Potency	Sensitive ≥mm	Intermediate mm	Resistant ≤mm
1	Ampicillin	10mcg	29	21-28	20
2	Azlocillin	75mcg	18	15-17	14
3	Carbenicillin	100mcg	23	18-22	17
4	Cloxacillin	1mcg	15	10-14	9
5	Methicillin	5mcg	14	10-13	9
6	Mezlocillin	75mcg	29	-	28
7	Oxacillin	1mcg	13	11-12	10
8	Penicillin	10unit	29	21-28	20
9	Piperacillin	100mcg	29	-	28
10	Ticarcillin	75mcg	15	12-14	11
11	Norfloxacin	10mcg	17	13-16	12
12	Ciprofloxacin	5mcg	16	14-15	13
13	Ofloxacin	2mcg	16	14-15	13

Reading of sensitivity test:

The reading of the inhibitory zones around each antibiotic disc was done under bright light with dark background using a clear transparent plastic scale. The diameter of the inhibitory zones were noted and recorded. The results of antibiotic susceptibility tests were interpreted as a. Sensitive b. Moderately sensitive and c. Resistant i.e. S, M.S (Intermediate) and R respectively with the help of interpretation Table 3.3.

MINIMUM-INHIBITORY CONCENTRATION (MIC) OF PENICILLIN G AGAINST STAPH. EPIDERMIDIS STRAINS.

Minimum inhibitory concentration (MIC) i.e. the lowest concentration of an agent to completely prevent the growth of a given bacteria was carried out as per the method of Aldridge et al, (1983). Briefly, to carry out MIC, agar diffusion method was used by preparing two fold dilution (Table 3.4) of the antibiotics (Penicillin G) in sterile, melted and cooled MH agar (MHA). Each dilution was poured in to labeled petridishes, mixed gently well and allowed to solidify. Two to three hour old nutrient broth cultures were inoculated by means of a 2 mm diameter inoculation loop on each plates and incubated over night. MIC was ascertained for a given strain when no growth in an agar plate having a particular concentration of penicillin.

Staph.epidermidis isolated from aerial samples (37 nos.) and clinical isolates (44 nos.) were subjected to this study.

Table 3.4

ANTIBIOTIC DILUTION SCHEDULE FOR M. I. C. DETERMINATION

Penicillin Vial: 10,00,000 units: Dilute this in 5.0 ml of Sterile Dist. water to make 2,00,000 units / ml (Stock)

Sl No.	Tube Code No.	Stock Dil	Stock Vol.	Diluent Vol.	Dilution obtained	Dilution in agar plate (1:10)
1	1 a (vial)	200,000	2.0 ml	2.0 ml	100,000	10,000
2	2a	100,000	1.0ml	9.0 ml	10,000	1000
3	3a	10,000	2.0ml	2.0 ml	5,000	500
4	4a	5,000	2.0 ml	2.0 ml	2,500	250
5	5a	5,000	1.0 ml	4.0 ml	1,000	100
6	6a	5,000	1.0 ml	9.0 ml	500	50
7	7a	2,500	1.0ml	9.0 ml	250	25
8	8a	1,000	1.0 ml	4.0 ml	200	20
9	9a	1,000	1.0 ml	9.0 ml	100	10
10	10a	100	2.0 ml	2.0 ml	50	5
11	11a	50	2.0 ml	2.0 ml	25	2.5
12	12a	100	1.0ml	4.0 ml	20	2.0
13	13a	100	1.0 ml	9.0 ml	10	1.0
14	14a	10	2.0 ml	2.0 ml	5	0.5
15	15a	5	2.0 ml	2.0 ml	2.5	0.25
16	16a	2.5	2.0 ml	2.0 ml	1.25	0.125

A correlative study was made between MIC values of these bacterial strains Beta-lactamase production using Iodimetric method (Sykes, 1978). Determination of MIC values together with detection of Beta-lactamase production will yield useful information for initiating effective antibiotic therapy for an underlying infection as well as understanding / controlling hospital acquired infection due to CNS species, especially **Staph.epidermidis**, a recently emerging leading pathogen among patients with implants.

Correlative study between Penicillin susceptibility and Beta-lactamase production was carried out using 49 strains **Staph.epidermidis** from clinical sources and 37 strains of from aerial samples. Study on Penicillin susceptibility pattern and Beta-lactamase production of **Staph.epidermidis** becomes highly relevant in treating patients with implant associated infections.

SLIME DETECTION AMONG STAPH.EPIDERMIDIS STRAINS ISOLATED FROM DIFFERENT SOURCES.

The virulence of coagulase- negative staphylococci (CNS) is not well understood but probably multifactorial. The predominant CNS strain especially **Staph.epidermidis** associated with diseases produce a wide range of extracellular polysaccharides- Slime, which is believed to facilitate the establishment of infection near/on the surface of implanted foreign bodies (Bayston & Penny, 1972). The composition of slime is (Endl et al, 1983):

1. Glycerol phosphate,

2. Alanine,

3. Glucose and

4. Glucosamine.

This study describes slime producing potentials of **Staph. epidermidis** isolated from clinical, aerial, and carrier sources and different methods of Slime detection by test tubes, Congo red agar and microplate.

80 clinical, 17 hospital staff, 141 hospital environment, 51 BMT staff (non hospital staff) and 121 BMT environmental (non-hospital environment) isolates of **Staph.epidermidis** were selected for slime detection by Congo red agar plate, Test tube and Microplate method.

a. Congo red agar (CRA) method:

Congo red agar (CRA) was prepared and used as described by Freeman et al (1989). Briefly CRA consisted of brain-heart infusion broth 37%, sucrose 5%, agar 1%, and Congo red 0.8%. CRA plates were prepared immediately before use, inoculated with test strains and then incubated aerobically at 37 oC for 24 to 48 hours. Dry, black, crystalline colonies were regarded as slime producing strains whereas mucoid, pink/brown or "Bull's eye" colonies were regarded as non slime producing strains (Photographic plate no.II)

PHOTOGRAPHIC PLATE NO II

SLIME DETECTION BY DIFFERENT METHODS

A. Congo red agar plate method.

(a) Strong positive +++

(b) Positive ++ or +

(c) Negative -

B. Test tube method.

(a) Strong positive +++

(b) Positive ++ or +

(c) Negative -

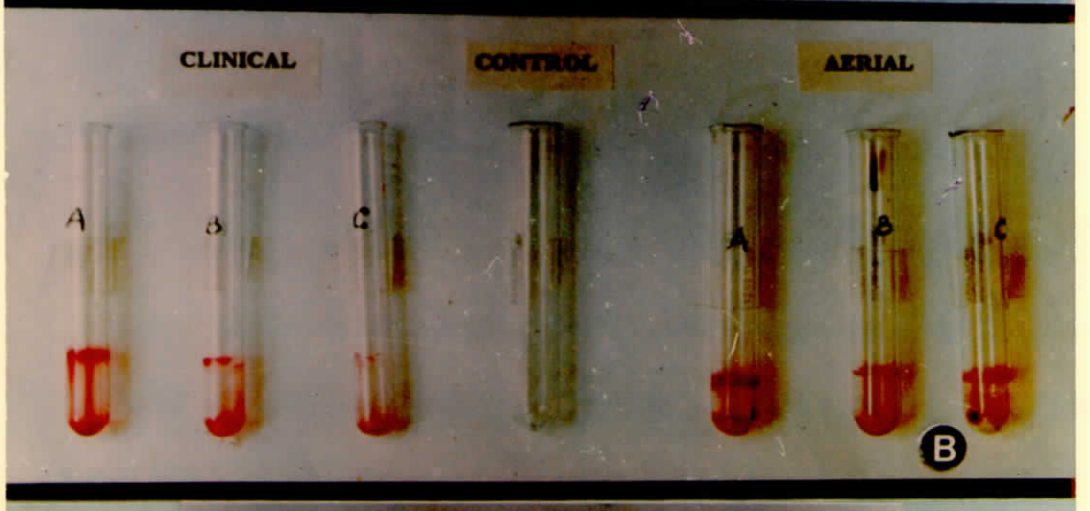
C. Microplate method.

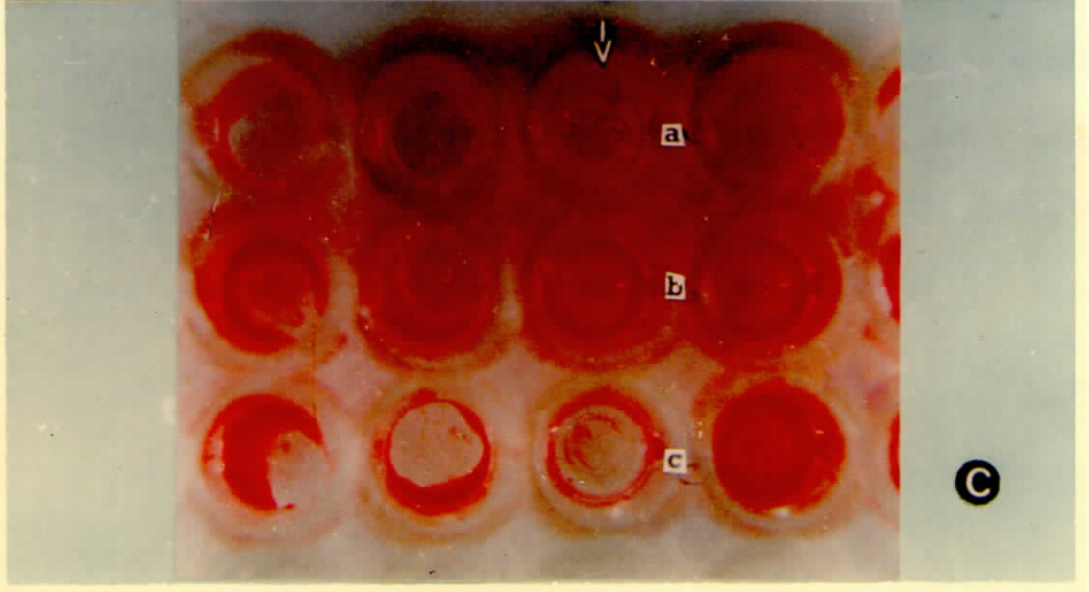
(a) Strong positive +++

(b) Positive ++or +

(c) Negative -

PLATE II





b. Slime detection by test tube method:

Slime production by this method was determined by examining over night Soyabean casein digest medium . Culture for a visible film of adherent growth which was stained with 0.1% Safranin for 10 seconds (Christensen, 1982). Test tubes were incubated in a tilted position to facilitate reading and Slime production was recorded as weak (+), moderate (++) or strong (+++), according to the density of the adherent film assessed visually (Photographic plate no II). Strain A54 of **Staph.epidermidis** which is strong slime positive supplied by Dr. MA Deighton, Melbourne, Australia was included as positive control in our study.

C. Microplate method:

Sterile microplates were used by filling 100 to 200 micro liter of sterile soyabean casein digest media in to each well. **Staph. epidermidis** isolated from different sources, were inoculated using tip of a sterile plastic tips and incubated over night. After incubation the content of the microplates were removed without agitation using Pasteur pipette or plating over dry filter paper. Subsequently sufficient amount of 0.1% safranin was poured slowly in to each well and immediately as aspirated gently and dried for 10 to 30 minutes in an incubator. The slime scoring was done as described in the test tube method(Photographic plate noII).

HYDROPHOBICITY/HYDROPHILICITY STUDIES OF STAPH.EPIDERMIDIS STRAINS:

Infections associated with prosthetic implants or medical devices are most commonly caused by coagulase-negative Staphylococci (Garvey, 1980). Bacterial adhesion on the surfaces is regarded to be one of the important virulence factor (Ofek and Beachey 1980). Bacterial interactions with mammalian cells and artificial surfaces is promoted by the hydrophobicity of the cell surface (Wardstrom et al 1981). Among various methods, to study the adherence of **Staph.epidermidis** to hydrocarbons such as salt aggregation test, hydrophobic interaction chromatography and water contact angle, the adherence to bacteria to hydrocarbon i.e. P-xylene was preferred. The aim of this study was to determine the hydrophobicity of **Staph.epidermidis** isolated from different sources and correlated with other virulence factor such as slime production.

72 clinical isolates of **Staph.epidermidis** 125 **Staph.epidermidis** isolated from aerial (both hospital and non hospital environments) were subjected for the determination of hydrophobicity by their adherence to P-xylene as per the method of Rosenberg (1984).

Analytical grade P-xylene (P-Xylene LR, SDS Fine Chemicals, Pvt. Ltd., India). was added to 3 ml of each bacterial suspensions that had been adjusted to an Optical density (OD) 540 of 0.5. The suspension was vortexed for 60 seconds and left for phase separation for 30 minutes. The aqueous phase of each strain was removed

and its OD at 540 nm was measured using Beckman Model 35, Spectrophotometer, USA. The percentage of hydrophobicity was expressed as

$$1-\left(\frac{\text{OD 540 after phase separation}}{\text{OD540 of original suspension}}\right)\times 100$$

The strains of **Staph.epidermidis** were recorded hydrophilic when percentages found between 0 and 6.5 and hydrophobic if more than 6.5.

IN VITRO *EVALUATION OF THE ANTIMICROBIAL EFFECTS OF A SILVER INCORPORATED LATEX CATHETER MATERIAL.*

Over six million urinary catheters are being used in India alone. It has been estimated that an average of 20% patients developed catheter associated urinary tract infections (UTI). Hence there is an urgent need for elimination or reducing the incidence of such infections using antibacterial agents incorporated catheter materials (Solomon & Sherertz 1987).

Catheter related infections are caused by microorganisms that invade the intracutaneous tract during catheter insertion or from the exit site while catheter is in place. Gram-positive bacteria, especially coagulase-negative **Staph.saprophyticus**, are the predominant pathogens (Janson et al 1994). To overcome the problem of catheter- related UTI infections, the concept of breaking the periluminal migration of bacteria into the intracutaneous segment by the application of antimicrobial substances to the catheter (Solomon & Sherertz 1987) has been putforth.

As bacteria possess the risk of antibiotic resistance, the application of heavy metal such as copper and silver on the catheter surfaces was investigated. Silver compounds are well known for antimicrobial property having a strong "Oligodynamic" activity and have been effective in treatment of UTI (Liedberg & Lundeberg 1990).

An investigation was made with silver incorporated latex rubber material for its antibacterial effects against **Staph.aureus**, **Staph.epidermidis** and **E.coli.**

Silver oxide(0.6 to 1.0%) was incorporated into latex rubber material by a simple and rapid chemical method (Process for the development of silver oxide incorporated antimicrobial polymers. Indian patent pending). Test materials with silver and corresponding controls having no silver incorporation were tested for their antimicrobial effects against the following bacterial strains.

Staph.aureus	(Wood 46)
Staph.epidermidis	(A54)
Staph.epidermidis	(ATCC 35983)
Staph.epidermidis	(B3972 (O)
E.coli	(Clinical isolates from UTI patients)

The method used for studying the antibacterial effect was that of Bauer et al (1966). Briefly, lawn cultures of these bacteria were made in MH agar plates and allowed to dry for 10 to 15 minutes. After drying, the latex materials with silver, control latex and standard antibiotic i.e. Cloxacillin disc were placed carefully on the MH plates and incubated at 37 oC for over night.

At the end of incubation period the zone of inhibition around the latex materials and standard antibiotic discs were observed and recorded.

STUDY ON ANTIBACTERIAL EFFECT OF CHLORHEXIDINE DIGLUCONATE COATED SILASTIC POLYURETHANE (ANGIOFLEX) MATERIALS.

Bioimplants incorporated with antimicrobial agents are a need of the hour in controlling gruesome foreign body associated infection (FBAI) in clinical settings. Here an attempt was made to develop five different types of polyurethane (Angioflex) and tested for their in vitro antibacterial effects against different standard clinical strains of viz. **Staph. aureus** (Wood 46, obtained from Dr. Zimmerli, Basal, Switzerland) and four strains of **Staph.epidermidis** viz. A182, A313, A61 and A72 (obtained from Dr. MA Deighton, Melbourne, Australia).

Angioflex (Polyuretane material obtained from Applied Biomedical Corporation, Danver MA, USA) were made into strips of 1X10 mm and subjected to different types of surface treatment (Coating) as shown in Table 3.5. Chlorhexidine digluconate (Received from M/s Sigma Chemicals Co., USA) was used here to coat the material as antibacterial agent. Glow discharge treatment was done as per the method of Sharma et al (1987). Before coating with the antibacterial agent, the polymer strips were sterilized by autoclaving at 20 psi for 7 minutes. Antibacterial effects of these materials against different types of bacteria (Table 3.2) was carried out using disc diffusion methods (Bauer et al 1966).

Table 3.5

CHARACTERIZATION OF PU MATERIALS USED

Material No.	Surface Characters
1.	Bare Angioflex (Silastic Polyurethane)
2.	Bare Angioflex with glow discharged
3.	Bare Angioflex, coated with chlorhexidine digluconate
4.	Chlorhexidine digluconate coated Angioflex glow discharged
5.	Chlorhexidine coated Angioflex glow discharged with second coating of chlorhexidine digluconate.

SCANNING ELECTRON MICROSCOPIC (SEM) STUDY OF SILVER INCORPORATED SILICONE MATERIAL SURFACE FOR THE ADHERENCE OF STAPH.EPIDERMIDIS.

The aim of this experiment was to demonstrate the interference of adherence of **Staph.epidermidis** on the biomaterial surfaces by silver incorporated silicone material.

Control and silver incorporated (Test) silicone tubes were used in this study after they were sterilised by Gamma radiation sterilization . The sterility of the material was confirmed by inoculating the sterilised material in sterile thioglycollate broth and Soyabean casein digest media (as per USP method). The sterile control and test materials were inoculated into test tube containing sterile nutrient broth. 10^{4} CFU of A54 (**Staph.epidemidis** having strong slime producing property) was inoculated into each of these tubes and were incubated at 37 ^{0}C and 22 ^{0}C respectively. After 24 hours incubation, the content of the tubes were gently decanted off and the control and test materials were washed gently using sterile 0.1M phosphate buffered saline (PBS) pH 7.4 and dried at 37 ^{0}C for 10 to 15 minutes. The materials were consequently subjected to the following steps before SEM study was initiated.

1. The adhered bacteria were fixed in 2.0 ml of 3% gluteraldehyde for 24 hours.

2. After gluteraldehyde fixation, the materials were washed 2 to 3 times with sterile PBS.

3. Osmium tetraoxide treatment : Materials were treated with 2.0 ml of 1.0% Osmium tetraoxide and kept at 4.0 °C for 3 to 4 hours. Care was taken to avoid spillage of osmium tetraoxide since it is a toxic chemical.

4. Osmium tetraoxide treated materials were washed with PBS (pH 7.4) 2 to 3 times and treated with 50%, 60%, 70%, 80%, 90%, and 100% acetone solution and kept for few hours at 4°C.

Steps 1 to 4 were the procedure done for fixing the adhered bacteria on the surfaces of control as well as test materials.

Treatment of bacteria fixed materials were further subjected to the following processes.

1. Treatment of isoamyl acetate

2. Critical point drying using liquid carbon dioxide at 73 psi at 31 °C for 30 minutes.

3. Gold ion sputtering (coating).

Critical point drying.

The specimen dehydrated with acetone were subjected to a pretreatment fluid viz. isoamyl acetate having a low volatility and then dried with liquid carbon dioxide using a critical point drier (Model HCP-2, Hitachi, Japan) to avoid deformation of specimens. The specimens thus prepared by this method is in a much better condition than the one which has undergone natural drying.

Ion sputtering (coating).

The specimens fixed in an aluminum stub were ion-sputtered in an Ion sputter unit (Model E- 101, Hitachi, Japan) for three minutes and were viewed under SEM for adherence of A54 (**Staph. epidermidis**) on heir surfaces using SEM Model S-2400, Hitachi, Japan. The magnification selected was 2K.

In vivo *Experiments:*

TISSUE CAGE EXPERIMENT- SHORT TERM ADMINISTRATION OF CLOXACILLIN IN THE PREVENTION OF INFECTION DUE TO STAPH.EPIDERMIDIS.

The risk of bacterial infection in the vicinity of a foreign body such as sutures and metallic or polymeric implants has been repeatedly documented in Cardiovascular (Kloster, 1979), Orthopedic (Charnley , 1972) and Plastic reconstructive surgery (Courtiss et al 1979). **Staph. epidermidis** is most frequently implicated as etiologic agent (Hunter and Dandy,1977). Little is known about the microbiologic determination of prosthetic infections, however, the efficacy of preventive short term antibiotic therapy suggests a change in bacterial behavior during the establishment of foreign body infection control programme. *In vitro* antibiotic susceptibility tests using antibiotic discs has its own limitation due to lack of pharmacokinetic phase, protein binding characteristics and reticuloendothelial system. Hence in order to understand the efficacy of well proven antibiotic agent namely Cloxacillin against **Staph. epidermidis** an *in vivo* animal model has become an imperative preposition.

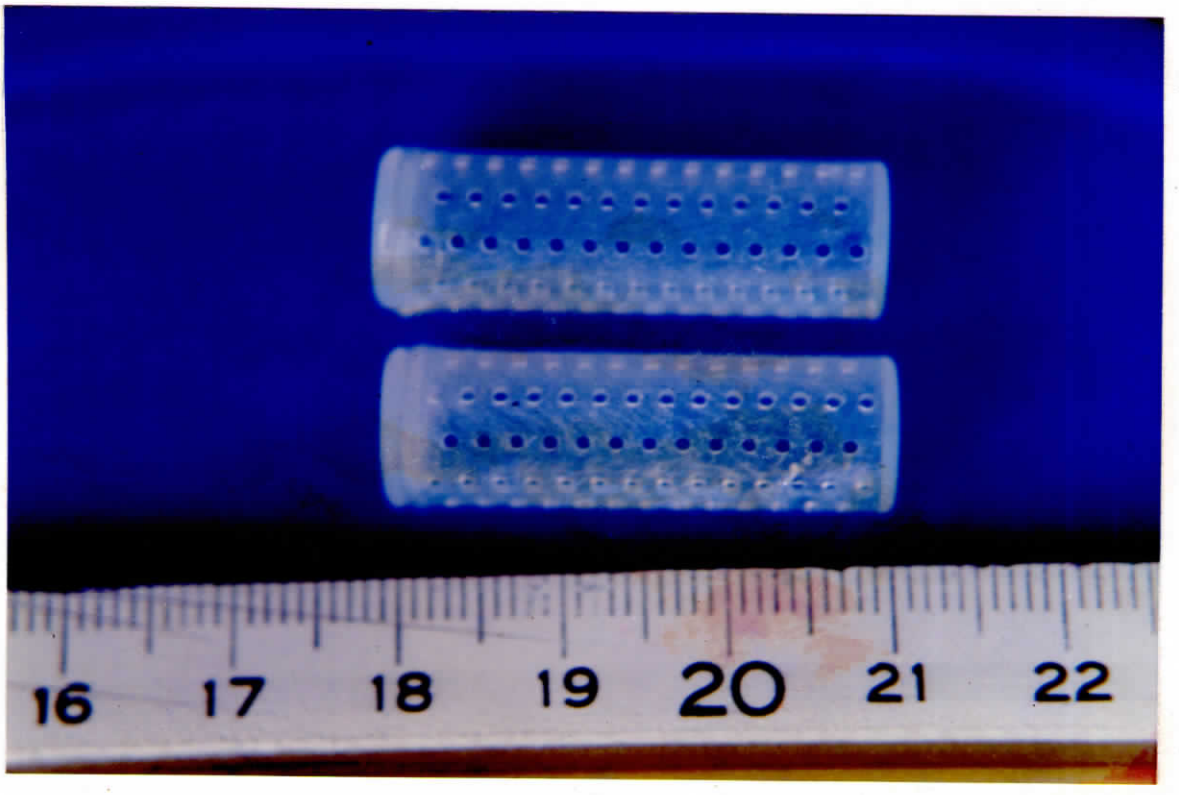
In the present study, initially we used an experimental model involving G.pigs (Zimmerli et al, 1982) and later on employing rabbit model to address these questions and doubts associated with *in vitro* testing. In particular, the anti staphylococcal activity of one of the Beta-lactam antibiotics Cloxacillin was evaluated in preventing the infection caused by **Staph. epidermidis** which was proven to exhibit 100% sensitivity to **Staph.epidermidis** by *in vitro* disc diffusion test.

G.pigs, weighing between 400 and 600 grams were used in this study. These animals were caged singly and allowed free access to food and water throughout the experiments. Before surgery the flanks were shaved by surgical clippers and the skin were disinfected with 70% ethyl alcohol. After induction of intraperitonial anaesthesia by injecting 40.0mg of pentabarbitone / kg body weight and using a strictly aseptic technique, a 4cm median dorsal incision was made and subcutaneous pocket was made by blunt dissection. Sterilised (by autoclave method) tissue cages (Photographic plate no V) were implanted into these sites and the skin was closed with interrupted sutures, which were removed after 5 days of surgery (photographic plate no VI). To a group of three G.pigs **Staph.epidermidis** (A54) 10^4 CFU/ml was injected into subcutaneously implanted Tissue cages. The antibiotic susceptibility pattern of **Staph.epidermidis** was studied before and 24 hrs after aspiration of Tissue cage fluid towards Cloxacillin, Ofloxacin, Ciprofloxacin and Norfloxacin antimicrobial agents. The aim of this experiment was to observe whether any phenotypic variation takes place in the bacteria as a result of metabolic changes in *in vivo* condition.

PHOTOGRAPHIC PLATE NO V

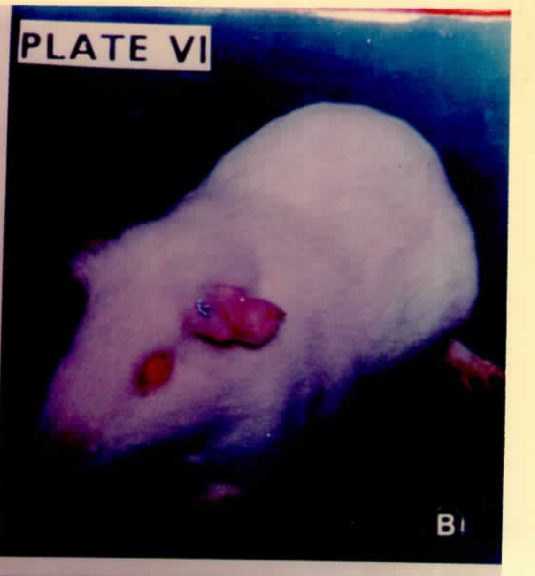
TISSUE CAGE

Polytetrafluoroethylene cylinders of 30 x 10mm having 250 regularly spaced holes of 0.8mm diameter.

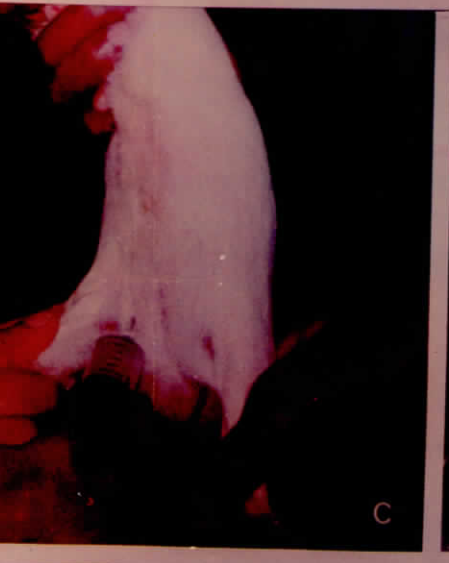


A

PLATE VI

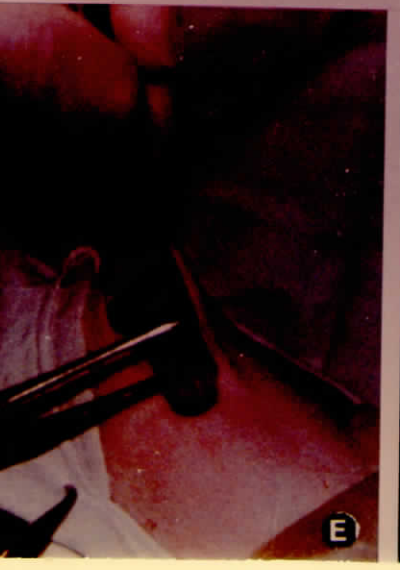


B



C

D



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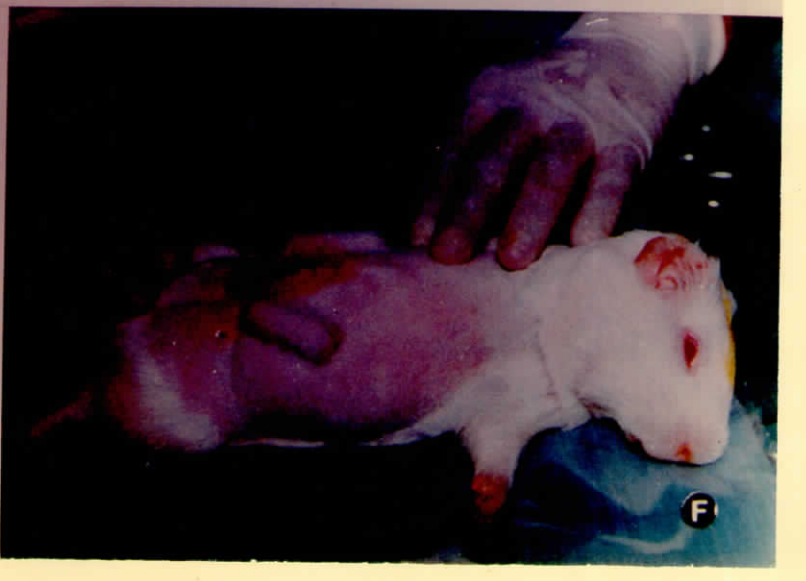

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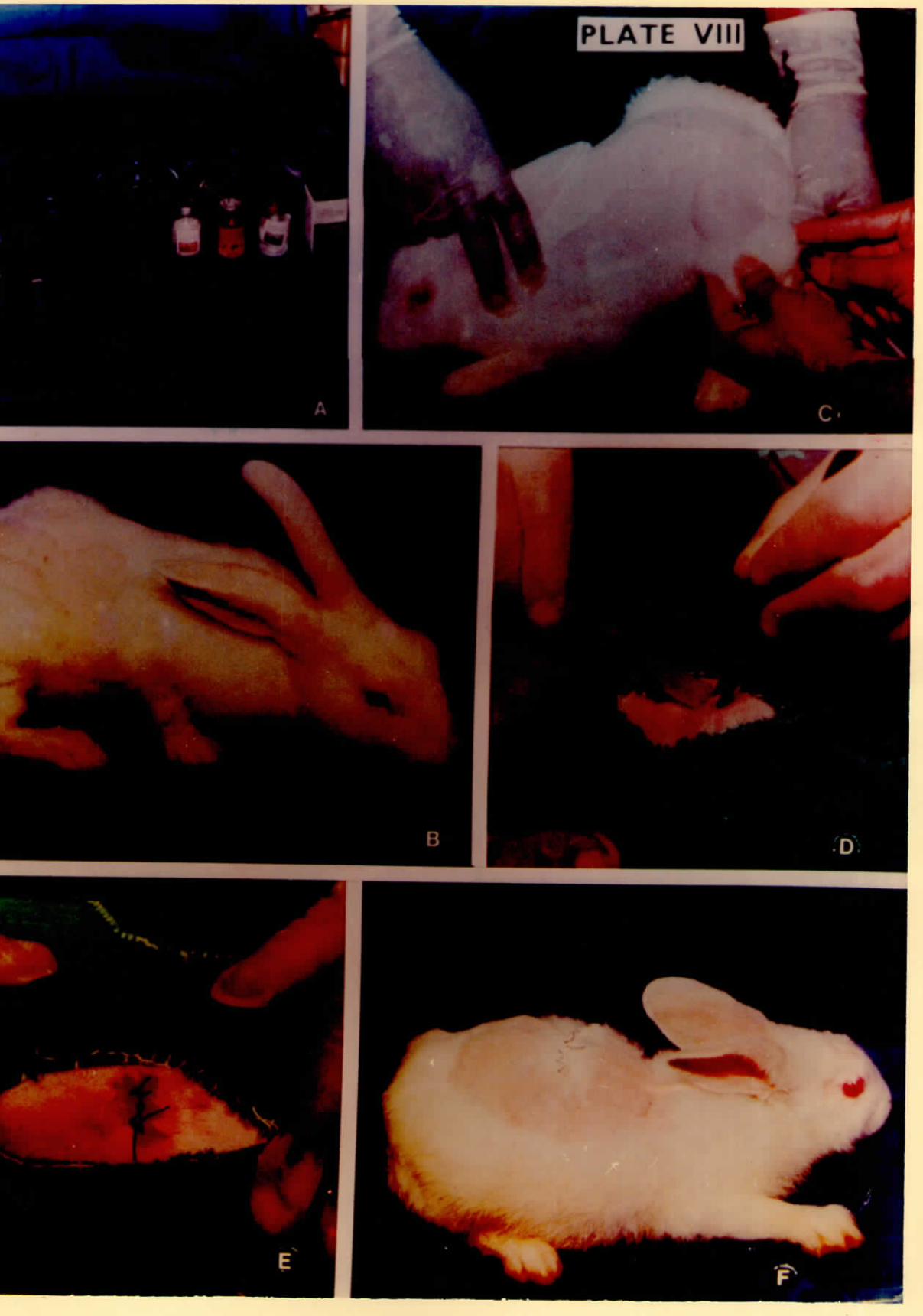
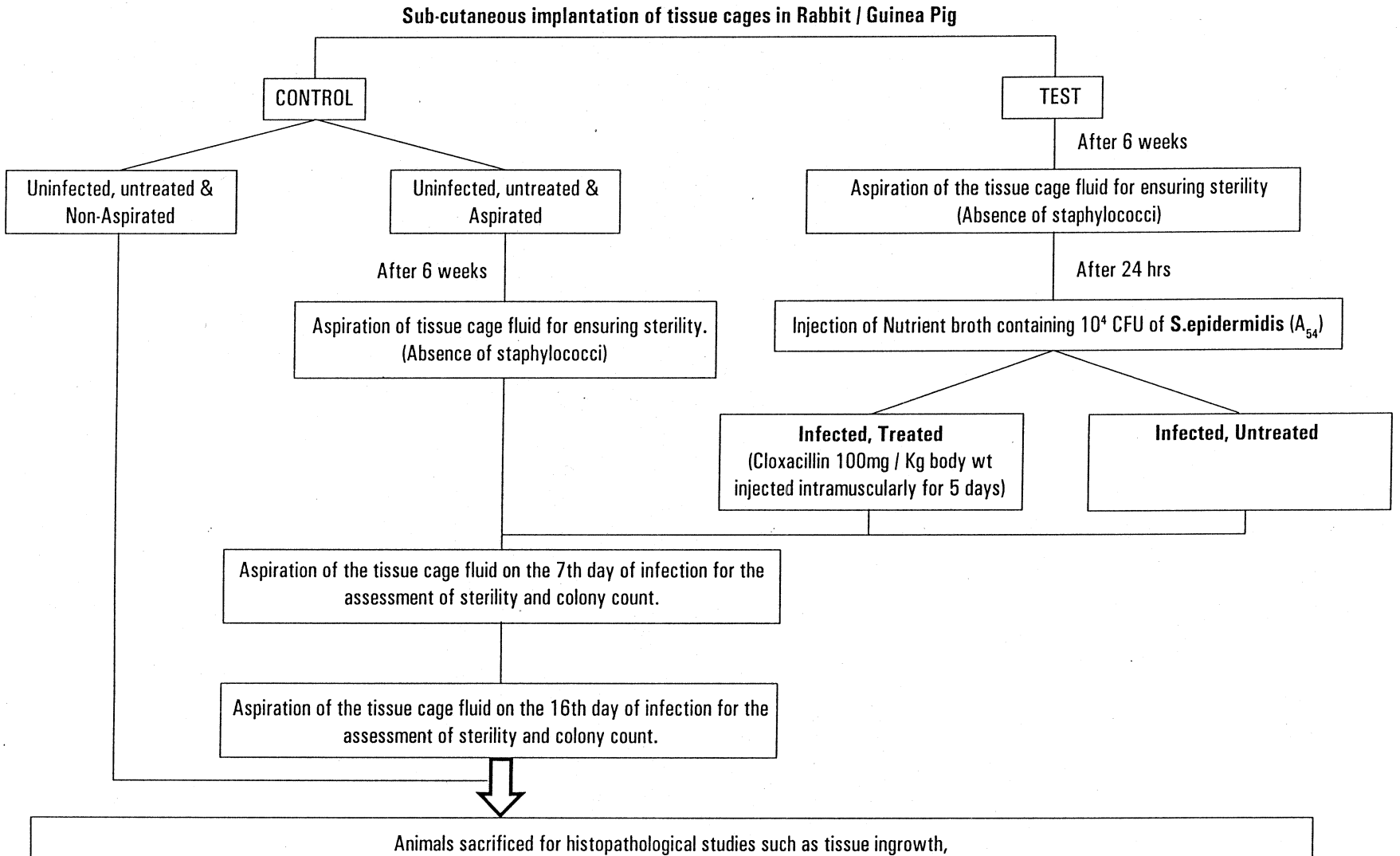


PLATE VIII
A
C
B
D
E
F

DESIGN OF TISSUE CAGE EXPERIMENTS



" I pass with relief from the tossing sea of cause and theory to the firm ground of results and fact. "

-Sir Winston S. Churchill

" Men are never so likely to settle a question rightly as when they discuss it freely."

-Thomas Bobington, Lord Macaulay

Results and Discussions

CHAPTER IV

ANALYSIS OF DATA

A. Results

***In vitro* experiments:**

A. Isolation of CNS species from patients, hospital staff, hospital environment, BMT wing staff and BMT wing environment.

Table 4.1 shows the results of CNS isolated from hospitalised patients (207), hospitalised staff (35), hospital environment (141), BMT wing Staff (51) and BMT wing environment (121). Speciation of these CNS species resulted in the identification of **Staph.epidermidis** 190 (34.2%), **Staph.saprophyticus** 141 (25.4%), **Staph.hominis** 174 (31.3%) and **Staph.cohnii** 50 (9.0%).

B. Antibiotic susceptibility test:

Antibiotic susceptibility pattern tests of **Staph.epidermidis** isolated from aerial and clinical isolates depicted in Graph No.4.1 and Table 4.2 respectively against ten different types of Beta-lactam antibiotics and Fluoroquinolone antimicrobial agents.

C. Correlative study between minimum inhibitory concentration values against Penicillin and Beta-lactamase production with **Staph.epidermidis** isolated from aerial and clinical isolates.

Table 4.3 illustrates that 84.6% of aerial and 20.6% of clinical strains of **Staph.epidermidis** correlated with low MIC values and Beta-lactamase positivity.

Table 4.1

PREVALENCE OF CNS SPECIES ISOLATED FROM PATIENTS,HEALTHY STAFF AND ENVIRONMENTS

Sources	Total strains	Staph.epidermidis	Staph.saprophyticus	Staph.hominis	Staph.cohnii
Hospitalised patients	207	80 (38.6%)	55 (26.5%)	61 (29.4%)	11 (5.3%)
Hospital Staff	35	17 (48.5%)	4 (11.4%)	11 (31.4%)	3 (8.6%)
Hospital Environment	141	41 (29.0%)	40 (28.3%)	40 (28.3)*	20 (14.1%)
BMT Staff (non-hospital Staff)	51	18 (35.3)	10 (19.6%)	17 (33.3%)	6 (11.8)
BMT environment (non hospital environment)	121	34 (28.0)	32 (26.0%)	45 (37.3%)	10 (8.2%)
Total	555	190 (34.2%)	141 (25.4%)	174 (31.3%)	50 (9.0%)

* One strain each of **Staph.haemolyticus** and **Staph.xylosus** have also been isolated.

GRAPH 4.1

ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF STAPH.EPIDERMIDIS ISOLATED FROM AERIAL AND CLINICAL SOURCES

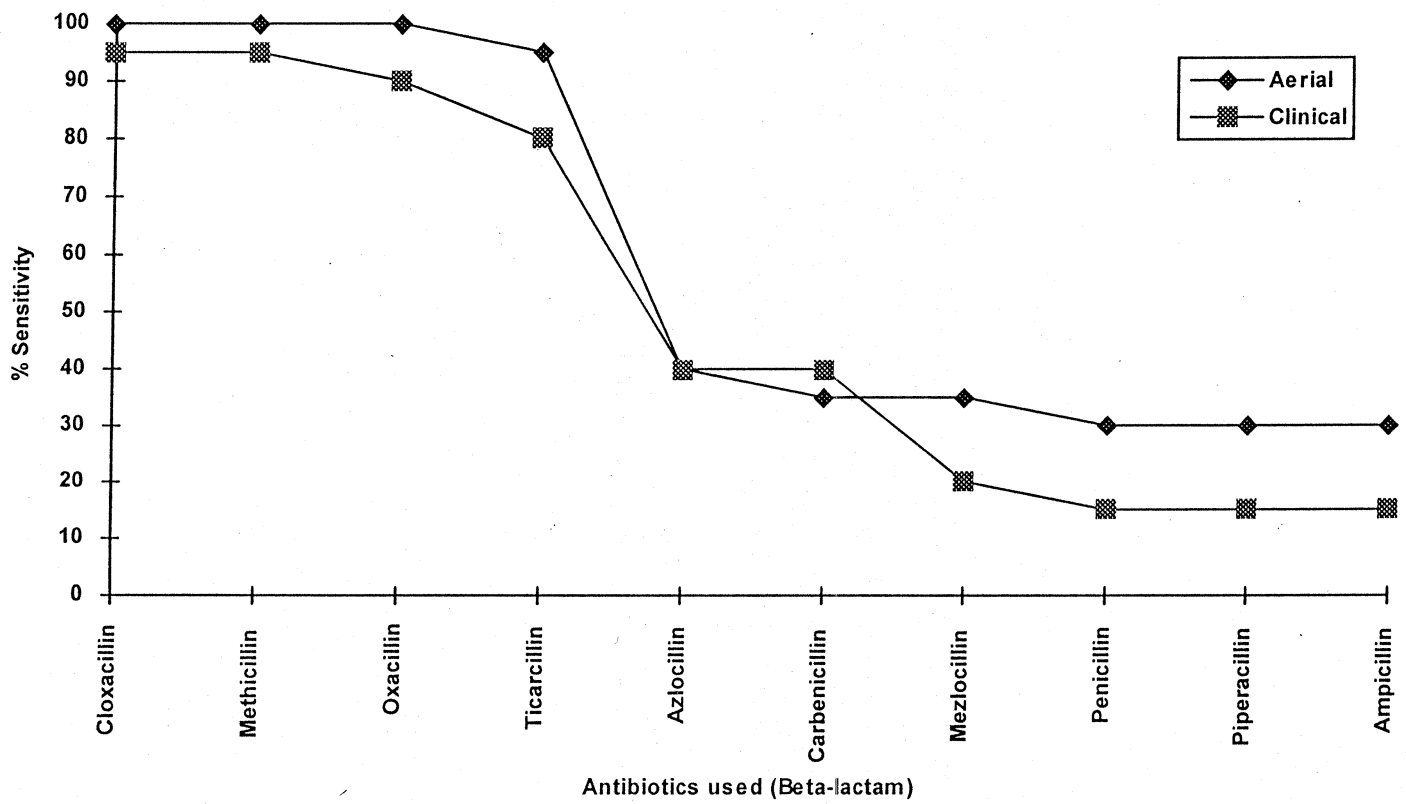


Table 4.2

SENSITIVITY RATE OF STAPH.EPIDERMIDIS STRAINS AGAINST FLUOROQUINOLONES

Sources	No.of strains used	**Ofloxacin**	**Ciprofloxacin**	**Norfloxacin**
1.Clinical	32	32 (100%)	30 (93.7%)	27 (84.3%)
2. Aerial	39	39 (100%)	38 (97.4%)	30 (76.9%)

Table 4.3

CORRELATION BETWEEN BETA-LACTAMASE PRODUCTION & M I C VALUES AGAINST PENICILLIN WITH STAPH.EPIDERMIDIS

Sources	No.of Strains tested	Total No.of Beta-lactamase producing strains	Low MIC (0.125-50 units) & Beta- lactamase positive	High MIC value(100-2000 units) & Beta-lactamase positive
Aerial samples	37	13(35.1%)	11(84.61%)	2(15.38%)
Clinical samples	44	29(65.9%)	6(20.6%)	23(79.3%)

However, 15.3% of aerial and 79.3% of clinical **Staph.epidermidis** strains produced correlation with high MIC and Beta-lactamase positivity.

Correlation study between Penicillin sensitivity and Beta-lactamase production of clinical (49 nos.) and aerial (37 nos.) isolates of **Staph.epidermidis** is given in Table 4.4. 48.9% of clinical **Staph.epidermidis** and 35.1% of aerial **Staph.epidermidis** showed positive correlation between Penicillin resistant and Beta-lactamase positivity.

D. Slime detection among **Staph.epidermidis** strains isolated from different sources .

Table 4.5 vividly depicts the supremacy of Congo red agar method (56.8%) in detecting slime among **Staph.epidermidis** isolated from different sources, followed by test tube method (38.9%) and microplate method (32.6%).

E. Hydrophobicity /Hydrophilicity studies on **Staph.epidermidis** strains isolated from different sources.

56.9% (41) of clinical and 10.4% (13) of aerial **Staph.epidermidis** showed hydrophobicity whereas 43% (31 nos.) and 89.6% (112) of clinical and aerial **Staph.epidermidis** produced hydrophilicity respectively (Table 4.6).

F. *In vitro* evaluation of the antibacterial effects of a silver incorporated latex catheter material.

Silver incorporated latex rubber showed antibacterial effects against **Staph. aureus(Wood 46), Staph.epidermidis**(A54), **Staph.epidermidis**(ATCC 35983), **Staph.epidermidis** (B3972(o)) and **E.coli** (a clinical isolate) as shown in Table

Table 4.4

CORRELATION BETWEEN PENICILLIN SENSITIVITY AND BETA-LACTAMASE PRODUCTION OF CLINICAL AND AERIAL ISOLATES OF STAPH.EPIDERMIDIS

Sources of isolation	No.of strains used	Penicillin sensitive & Beta-lactamase negative	Penicillin resistant &Beta-lactamase positive	Penicillin sensitive &Beta-lactamase positive	Penicillin resistant &Beta lactamase negative
Clinical	49	5 (10.2%)	24 (48.9%)	0	20(40.8%)
Aerial	37	10(27.0%)	13(35.1%)	3(8.1%)	11(29.7%)

Table 4.5

COMPARATIVE EVALUATION OF SLIME PRODUCTION BY STAPH.EPIDERMIDIS SPECIES ISOLATED FROM DIFFERENT SOURCES

Sources	No.of strains	**Staph. epidermidis**	Slime by Congo Red Agar plate	Slime by test tube method	Slime by microplate method
Hospital Patients	207	80 (38.6%)	48 (60.0%)	32 (40.0%)	28 (53.0%)
Hospital staff	35	17 (48.5%)	10 (58.8%)	7 (41.0%)	6 (53.2%)
Hospital environment	141	41 (29.0%)	25 (61.0%)	15 (36.5%)	13 (31.7%)
BMT staff (non hospital staff)	51	18 (35.2%)	9 (50.0%)	7 (38.8%)	5 (27.7%)
BMT environment (Non-hospital environment)	121	34 (28.0%)	16 (47.0%)	13 (38.9%)	10 (29.4%)
Total	555	190 (34.2%)	108 (56.8%)	74 (38.9%)	62 (32.6%)

Table 4.6

STUDIES ON HYDROPHOBIC AND HYDROPHILIC PROPERTIES OF STAPH.EPIDERMIDIS ISOLATED FROM DIFFERENT SOURCES.

Sl. No.	Sources of isolation	No.of **Staph. epidermidis** studied	No.of strains showing Hydrophobicity(%)	No.of strains showing Hydrophilicity(%)
1	Clinical (Blood, CSF, Pus,Urine)	72	41 (56.9)	31(43.0)
2	Aerial (Hospital and non-hospital-BMT wing)	125	13(10.4)	112(89.6)

4.7. Photographic plate no III clearly demonstrate the antibacterial effects of latex against **Staph.epidermidis** (clinical isolates).

G. Study on antimicrobial effects on Chlorhexidine digluconate coated silastic polyurethane (Angioflex) material.

Table 4.8 shows the antibacterial effects of silastic polyurethane materials tested against five different types of Staphylococci. It is observed that Bare Angioflex coated with Chlorhexidine digluconate (single coating) exhibited maximum antibacterial efficacy against all bacteria tested.

H. Scanning Electron Microscopic studies of silver incorporated Silicone material surface adherence property of **Staph.epidermidis**.

Photographic plate no.IV clearly showed the maximum adherence of **Staph.epidermidis** (A54) on control Silicone catheter materials (Silver not incorporated) and very minimum (resistant) on silver incorporated Silicone.

***In vivo* experiments:**

Tissue cage experiment- Short term administration of Cloxacillin in the prevention of infection due to **Staph.epidermidis**.

Results of microbiological monitoring of Tissue Cage fluids (Interstitial fluid) is shown in Table 4.9. Control groups (I & II) did not reveal the presence of **Staph.epidermidis.** Test (Group III infected, untreated and aspirated) showed presence of **Staph.epidermidis** (A54).However Test (Group IV infected, treated and

Table 4.7

ANTIBACTERIAL EFFECT OF SILVER INCORPORATED LATEX CATHETER MATERIAL.

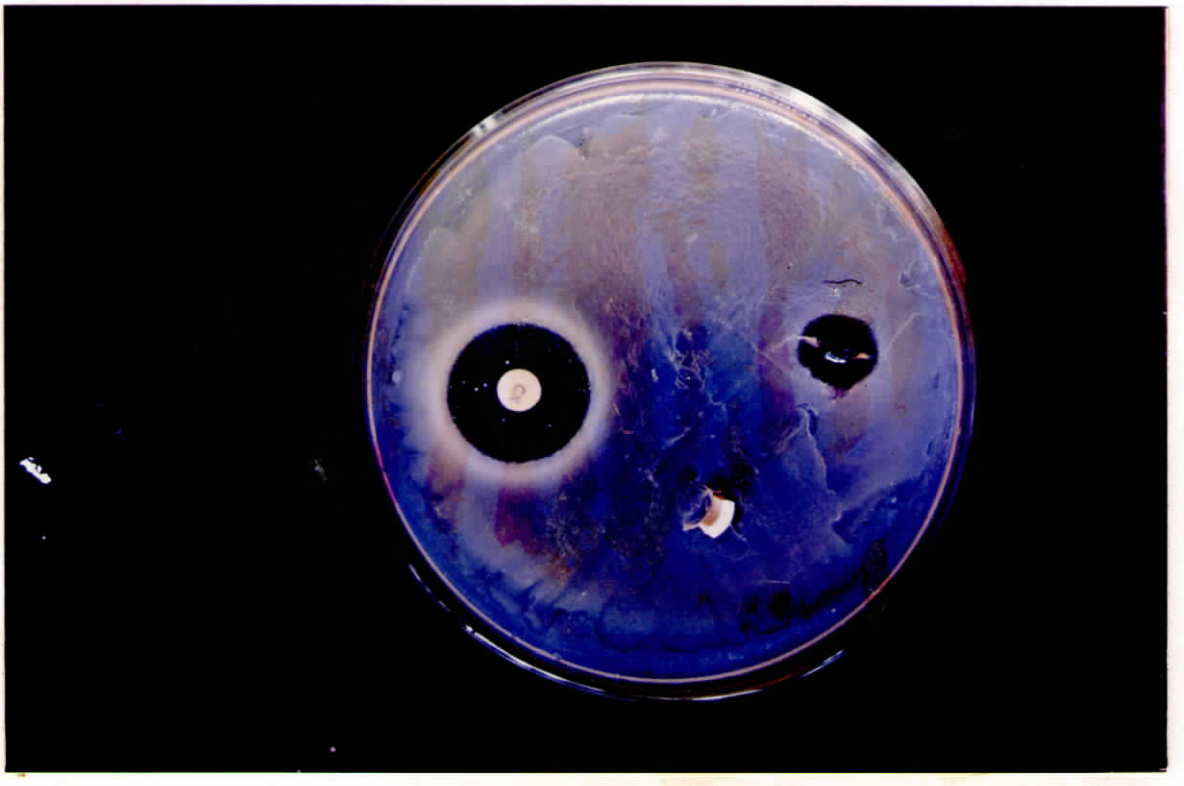
Bacteria used	Silver incorporated latex	Control latex
Staph.aureus (Wood 46)	S	R
Staph.epidermidis (A54)	S	R
Staph.epidermidis-(ATCC 35983)	S	R
E.coli (ATCC 25922)	S	R

S-Sensitive R-Resistant

PHOTOGRAPHIC PLATE NO III

ANTIBACTERIAL EFFECT OF SILVER INCORPORATED LATEX CATHETER MATERIAL

Bacteria used : *Staph. epidermidis* from UTI



Left : Cloxacillin used as control

Right : Silver incorporated latex

Below : Control latex without silver incorporation

Table 4.8

BACTERIAL STRAINS USED AND THE ANTIBACTERIAL EFFECTS OF PU MATERIALS TESTED.

Material No	Wood 46 (Mean±SD) mm	A 182 Mean±SD mm	A 313 Mean±SD mm	A 61 Mean ± SD mm	A 72 Mean±SD mm
1.	4.23 ± 0.153	3.00 ± 0.100	4.23 ± 0.058	6.60 ± 0.200	2.60 ± 0.264
2.	7.30 ± 0.300	4.30 ± 0.058	2.20 ± 0.264	7.00 ± 0.321	8.20 ± 0.321
3.	14.3 ± 0.360	13.3 ± 0.264	12.4 ±0.153	12.4 ± 0.208	12.9 ± 0.153
4.	16.4 ± 0.208	15.6 ± 0.376	9.00 ± 0.208	11.4 ± 0.200	12.7 ± 0.611
5.	10.3 ± 0.608	11.7 ± 0.208	8.70 ± 0.200	10.4 ±0.099	10.7 ±0.305

Each value represents the zone of inhibition Mean ± Standard deviation of three observations.

PHOTOGRAPHIC PLATE NO IV

ADHERENCE OF *STAPH. EPIDERMIDIS* ON CONTROL AND SILVER INCORPORATED SILICONE MATERIALS

A. A_{54}^{*} adhered on control silicone material (cocci embedded in slime material).

B. A_{54}^{*} adhered on silver incorporated silicone material (markedly less adhered cocci).

C. $A_{332}^{\#}$ adhered on control silicone material.

D. D $A_{332}^{\#}$ adhered on silver incorporated silicone material.

E. A_{54}^{*} adhered on control silicone material (cluster of cocci embedded inside a slime mass).

F. A_{54}^{*} adhered on silver incorporated silicone showing less number of adherence of cocci.

* Strong slime positive

Weakly slime positive

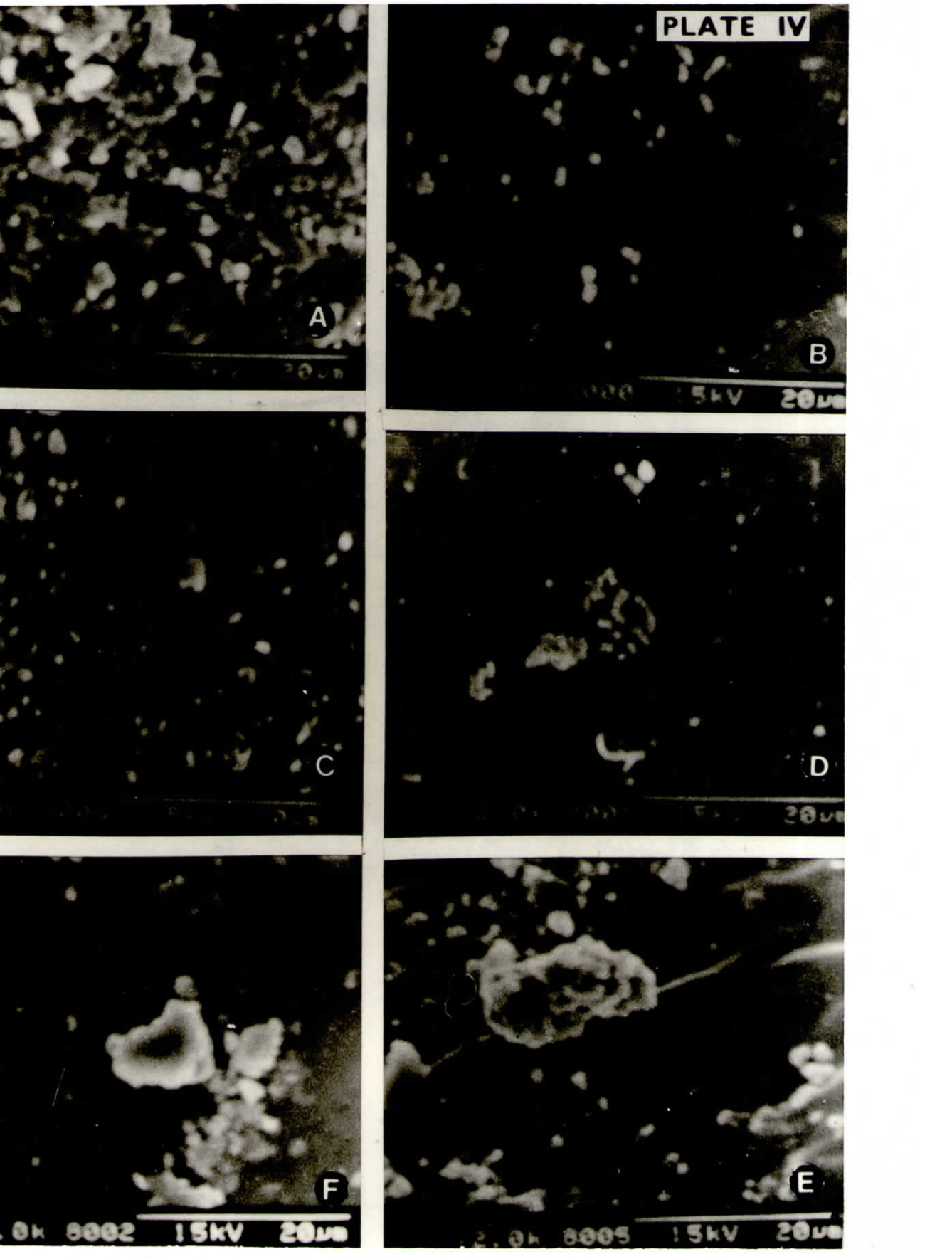


PLATE IV
A
B
15kV
C
D
F
15kV
E
15kV

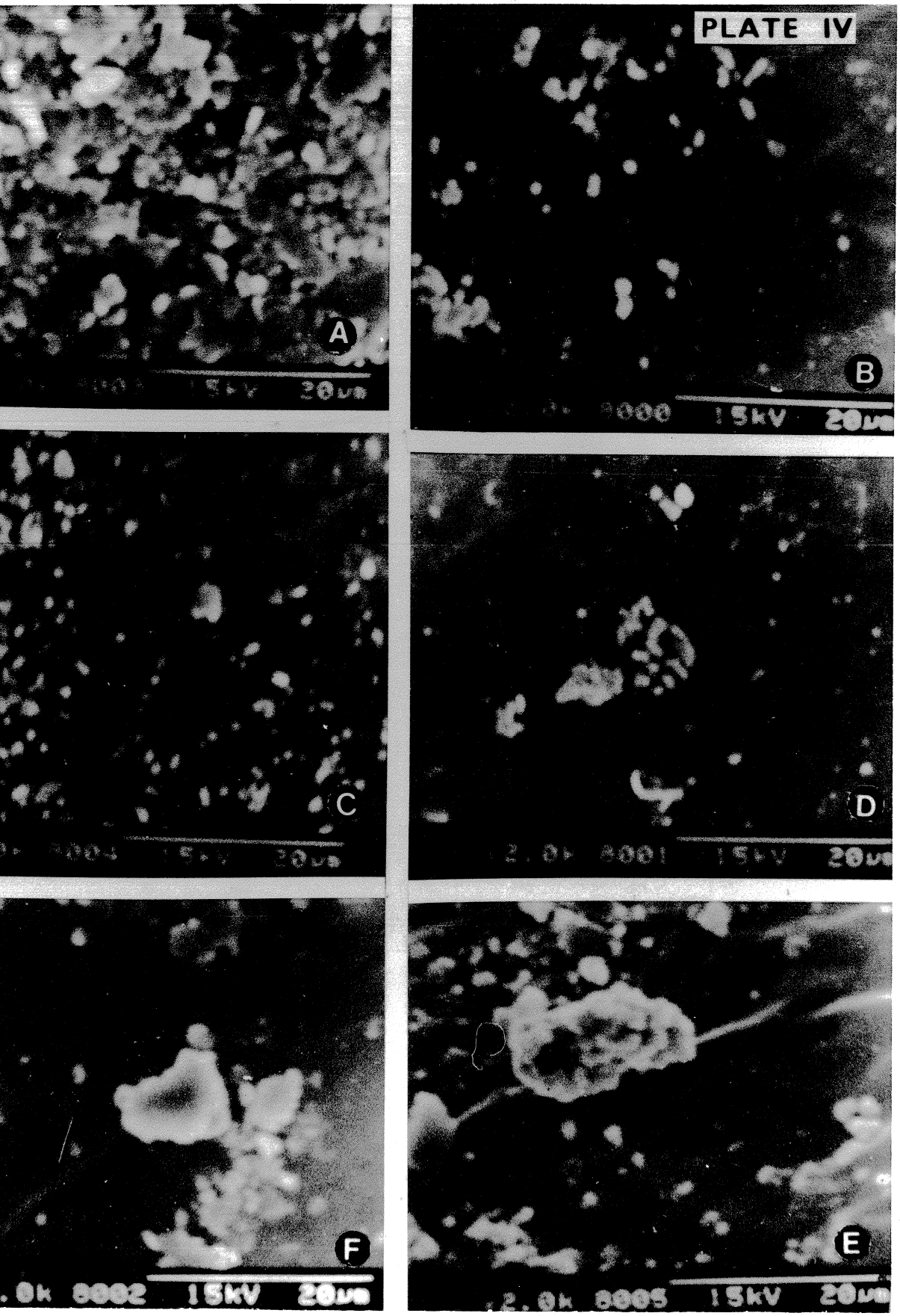


PLATE IV
A
B
C
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E

Table 4.9

MICROBIOLOGICAL MONITORING OF TISSUE CAGE FLUIDS

Group	No.of rabbits used	Dose of A 54 Strain	Dose of Cloxacillin given i.m rout	No.of CFU of A54 strain in tissue cage exudates on 16 th day
I Control (uninfected, untreated & unaspirated)	3	-	-	Nil
II Control (uninfected, untreated & aspirated).	3	-	-	Nil
III Test I (infected, untreated & aspirated).	3	10^4 CFU	-	$>10^{15}$ CFU
IV Test II (infected & treated & aspirated).	3	10^4 CFU	100mg/kg (for 4 days)	nil

A54: **Staph.epidermidis,** CFU: Colony forming units.

aspirated) showed no presence of bacteria in the exudate collected at the end of 16th day of observation.

The histopathological evaluation of tissue cages harvested at the end of experimental periods are shown in Table. 4.10. Control groups I and II showed no tissue inflammatory responses. However, infected, untreated and treated group revealed acute inflammatory responses without any chronic inflammatory reactions. Granulation tissue ingrowth into lumen of cages were observed in all the groups. (Photographic plate no. IX). It is observed that granulation tissue ingrowth also took place among the group of rabbits which were untreated, uninfected and unaspirated.

Table 4.10

HISTOPATHOLOGICAL EVALUATIONS OF TISSUE CAGES

Type of experiment	No.of rabbits used	Periodof observation (weeks)	Inflammatory responses	Fibrous encapsulation	Tissue ingrowth
Group I Control-uninfected, untreated & unaspirated	3	6	No acute chronic inflammation observed	Positive	Positive
Group II Control-uninfected, untreated & aspirated	3	6	No acute/chronic inflammation observed	Positive	Positive
Group III infected, Control-infected , untreated & aspirated	3	6	42% of animal showed acute inflammation. No chronic inflammation noted	Positive	Positive
Group IV Test-infected, treated & aspirated	3	6	50% of animals showed acute inflammatory response	Positive	Positive

PLATE IX

PHOTOGRAPHIC PLATE NO IX

HISTOPATHOLOGICAL RESPONSES OF RABBIT SUB-CUTANEOUS TISSUE TO IMPLANTED TISSUE CAGE (H&E STAINED AND MAGNIFICATION 60)

A. Infected, untreated and aspirated (group III)

B. Infected, treated and aspirated (group IV)

C. Uninfected, untreated and aspirated (group II)

D. Uninfected, untreated and unaspirated (group I)

↑ shows granulation tissue ingrowth.

M - Polymer Material.

B. Discussion

The number of coagulase-negative staphylococci strains prevalent among the isolates from different sources indicate minimum of 35 (6.3%) from hospital staff and maximum of 207 (37.2%) from hospitalised patients . The rate of CNS isolated from other sources are: non hospital staff 51(9.1%), non-hospital environment 121(21.8%) and hospital environment 141 (24.4%). 91.9% of CNS strains identified belonged to only three species namely **Staph.epidermidis** 34.2%, **Staph.saprophyticus**- 25.4% and **Staph.hominis** 31.3%, confirming the predominance of **Staph.epidermidis** over other CNS species. Among **CNS** isolated from hospitalised environment one each of **Staph.haemolyticus** and **Staph.xylosus** were also identified. **Staph.hominis** is found to be the leading species after **Staph.epidermidis**. According to Pfaller et al (1988) **Staph.hominis** is one of the variant species under **Staph.epidermidis** group of CNS having uncommon pathogenic significance. It is quite different from our earlier findings where **Staph.saprophyticus** was the second leading species after **Staph.epidermidis** species among CNS strainss isolated from different sources (Shanmugam et al 1994). The prevalence of **Staph.epidermidis** was the leading species found in our current study correlates well with the report of Thurn et al (1992) who found 89.1% belonging to this species. **Staph.epidermidis** was isolated more among the hospital personnel than the patients- 48.5% and 38.% respectively. Prevalence of more **Staph.epidermidis** isolated from hospital staff can be attributed to specific sites such as nose, skin and throat. These sites are known to be reservoir of

Staph.epidermidis as an autochthonus bacteria. Deighton et al (1988) demonstrated 63% of their CNS strains belonging to **Staph.epidermidis** which has been in quite contrast to our study of 38.6% of **Staph.epidermidis** among clinical isolates. Studies on prevalence of CNS species among entirely different sources was never before taken up and exploited as done in this study. An attempt made here towards this direction gives a lot of impetus for further research work on isolation, identification and speciation of CNS species so that infection due to interaction of CNS species especially **Staph.epidermidis** with biomaterial implants would be prevented and eradicated.

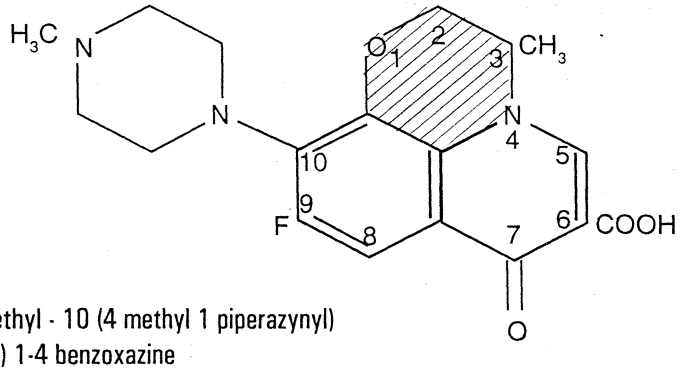
It has been well established that susceptibility of CNS species to antimicrobial agent is extremely variable. Although community acquired isolates are frequently belong to wide variety of bacterial strains, isolates from hospitalised patients (clinical specimens) have been noted to be resistant to an increasing number of antibiotics (Archer, 1978). Study using Beta-lactam antibiotics (Table 3.3) also confirms the earlier findings (Rathinam and Shanmugam, 1994) that CNS strains isolated from various sources are highly resistant to a variety of Beta-lactam antibiotics. Present study with **Staph.epidermidis** alone against Beta-lactam antibiotics showed susceptibility pattern (Graph 4.1) that Cloxacillin, Methicillin, Oxacillin and Ticarcillin are most effective antistaphylococcal agents other than Penicillin group of antibiotics. Vijayalakshmi Natesan et al (1980) demonstrated 100% susceptibility of **Staph.epidermidis** isolated from clinical specimens towards Cloxacillin. Our earlier study with CNS species showed 76.5% sensitivity to Oxacillin (Rathinam and Shanmugam 1994). Current study with Cloxacillin demonstrated 95

and 100% sensitivity of **Staph.epidermidis** isolated respectively from Clinical and aerial samples. Jayesh et al (1987) showed only 68.6% sensitivity of CNS strains against Cloxacillin.

Among the Fluoroquinolones tested Ofloxacin exhibited maximum (100%) killing effect against **Staph.epidermidis** strain isolated both from clinical and aerial sources. This is followed by Ciprofloxacin(clinical 93.7%, aerial 97.4%) and Norfloxacin (clinical 84.3%, aerial 76.9%). One of the groups of antimicrobial agents recommended recently for treatment of **Staph.epidermidis** infection is quinolones (Barry and Jones 1987). It is also quite interesting to note that more of the cyclic group attached to "N" atom of Fluoroquinolone possesses more antibacterial effect(Fig 4.1). It is proved by *in vitro* antimicrobial susceptibility study that **Staph.epidermidis** infection would be prevented/ eradicated by Oflaxacin and Ciprofloxacin rather than by Norfloxacin.

Low MIC value and Beta-lactamase production was exhibited by 84.61% and 20.6% respectively by aerial and clinical **Staph.epidermidis** strains. It is noted here that this correlation is very high (84.6%) among aerial **Staph.epidermidis** than clinical **Staph.epidermidis** (20.6%). This may be due to poor acquisition of antibiotic resistant pattern by aerial **Staph.epidermidis** showing low MIC values (0.125 to 50 units of Penicillin) whereas correlation between high MIC values and Beta-lactamase positivity showed higher value (79.3%) by clinical **Staph.epidermidis** strains. This may be due to prevalence of maximum resistant towards Penicillin. This is also true with the total number of Beta-lactamase producing

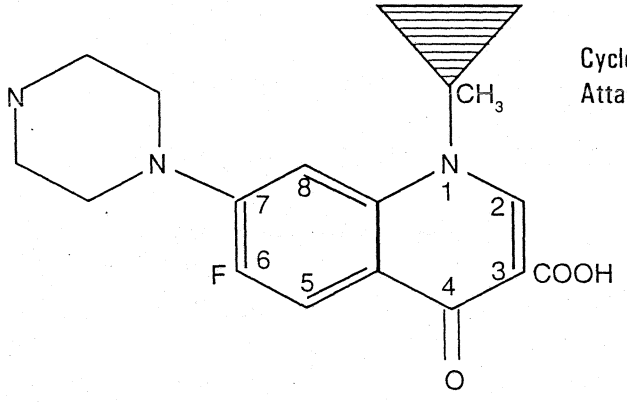
OFLOXACIN



Benzoxazine Cyclic Group
Attached to N-atom

6-Fluoro, 2-3 dihydro - 3 methyl - 10 (4 methyl 1 piperazynyl)
7-oxo-7H pyridol (1, 2, 3 de) 1-4 benzoxazine
6 - Carboxylic Acid.

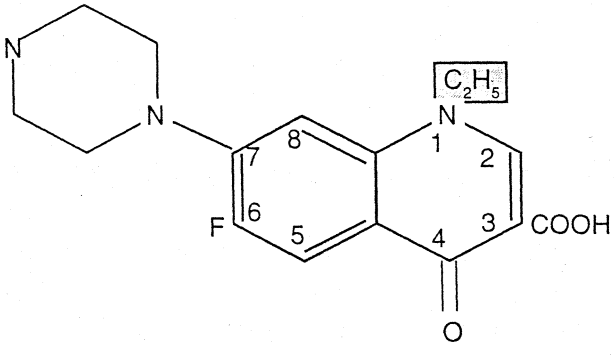
CIPROFLOXACIN



Cyclopropane Cyclic Group
Attached to N-Atom

1- Cyclo propane 6 - fluoro, 1-4- dihyro 4 oxo
7 (1 piperazynyl) 3 quinoline carboxylic acid

NORFLOXACIN



Ethyl Group
Attached to N-Atom

1-ethyl 6 fluoro, 1-4 dihydro 4 oxo - 7 (1 piperazynlyl).
3 quinoline carboxylic acid

Staph.epidermidis strains where 65.9% belong to clinical sources and only 35.9% to aerial sources. This particular finding is of great relevance to clinician who treat patients with implants associated infection due to **Staph.epidermidis** strains.

10.2% of clinical and 27% of aerial **Staph. Epidermidis are sensitive to Penicillin as** well as Beta-lactamase negative. It has already been demonstrated by many workers (Rathinam and Shanmugam 1994; Lowry and Hammer 1983) that Penicillin is the least effective antibiotic to treat **Staph.epidermidis**/CNS species. Penicillin resistant and Beta-lactamase positive **Staph.epidermidis** strain was found more among clinical **Staph.epidermidis** (48.9%) than among aerial Specimens (35.1%). This is due to higher production of Beta-lactamase by clinical isolates (Table 4.11) of **Staph.epidermidis** (Narayani et al 1989). When we compare our study with that of Rosenblatt and Newman (1978) we could established 48.9% correlation (Table 4.12)

It has already been demonstrated that maximum slime producing **Staph.epidermidis** strains were detected by Congo red agar(CRA) method (56.8%) followed up by test tube (38.9%) and microplate method (32.6%).In our previous study (Rathinam et al 1993) 52.4% of slime production by **Staph.epidermidis** isolated from different sources using test tube method was reported.

Detection of slime by CRA plate method (Freeman et al 1989) provided more percentage of slime positivity while compared to all other method. Here CRA technique yielded 18% and 24.2% additional positivity respectively over test tube and microplate technique (Christensen et al 1982) with all **Staph.epidermidis** strain

Table 4.11

CORRELATION BETWEEN BETA-LACTAMASE PRODUCTION & PENICILLIN SUSCEPTIBILITY PATTERN (Narayani et al,1989).

Susceptibility to Penicillin	Beta-lactamase production	Inference
Sensitive	Negative	Highly sensitive due to the absence of Beta-lactamase.
Resistant	Positive	Resistance due to Beta-lactamase production
Sensitive	Positive	Sensitive due to insufficient Beta-lactamase/intrinsic factor.
Resistant	Negative	Resistance is not due to Beta-lactamase (cell wall/intrinsic autolytic enzymes

Table 4.12

DATA ON COMPARATIVE STUDIES OF BETA-LACTAMASE PRODUCTION AND PENICILLIN RESISTANT STRAINS OF STAPH.EPIDERMIDIS ISOLATED FROM CLINICAL SOURCES.

Studies conducted by	No.of **Staph.epidermidis** tested	% Correlation
Rosenblatt & Neumann (1978)	15	0
Rathinam & Shanmugam (1995)	24	48.9

isolated from different sources. The comparison of these three techniques also revealed that by test tube and microplate techniques 10 to 20% false negative results can be obtained. This may be due to potential possibility of the removal of slime while emptying the culture medium initially or after staining and/or due to failure of slime sticking to wells or the wall surface of the test tube or floor surfaces of microwells. These potential drawbacks can not be expected with CRA plate technique. Here the results are very clear, different degrees of positivities can be recorded, contamination if any can easily be noticed and the reproducibility was far better than with other two techniques. This study further confirms that high prevalence of **Staph.epidermidis** among clinical, hospital environments and hospital personnel than non hospital aerial and non hospital staff and enlighten the supremacy of CRA technique over other two tests for slime detection.

Staph.epidermidis isolated from clinical specimens showed more hydrophobicity (56.9%) than hydrophilicity (43.0%). However aerial **Staph.epidermidis** revealed more hydrophilicity (89.6%) than hydrophobicity 10.4%. Since hydrophobicity of the bacterial cell surface is considered to be relevant property for the adherence of **Staph.epidermidis** to polymer surfaces and cause subsequent infection and assessment of bacterial surface property of

Staph.epidermidis isolated from different samples become meaningful . Among various tests such as;

A. Salt aggregation test (SAT),

B. Contact angle measurement,

C. Hydrophobic interaction chromatography and

D. Adherence to xylene, available, the adherence of **Staph.epidermidis** to p-xylene was chosen because of ease of the experiment and high reproducibility. A comparative analysis of slime production (Test tube method) by **Staph.epidermidis** and their hydrophobicity was made among clinical samples. It was found that only 20% correlation could be established (Table 4.13) between these two surface properties.

Silver incorporated Latex catheter material showed marked antibacterial effects against **Staph.aureus** (Wood 46), **Staph.epidermidis** (ATCC 35983 and A54), **E.coli** (ATCC 25922) and **Staph.epidermidis** (clinical isolate from a patient). The control Latex (without silver ion incorporation) did not show bactericidal effect against all the bacteria tested. Photographic plate no III clearly depicts the antibacterial effects of silver incorporated Latex against **Staph.epidermidis** isolated from urine of a patient. Table 4.14 illustrates the different studies carried out with silver incorporated polymeric materials against coagulase-negative Staphylococci.

The silver incorporated catheter materials appears to be significant technologic advance for prevention of catheter related infection. Binding or

Table 4.13

CORRELATION BETWEEN STRONG SLIME PRODUCING STAPH.EPIDERMIDIS AND THEIR HYDROPHOBICITY

No.of Staph.epidermidis tested	No.of Staph.epidermidis strains showed strong slime positive by test tube method	No.of Staph. epidermidis showed hydrophobicity	Positive correlation between slime positive & Hydrophobicity
50 (clinical)	32 (64.0%)	28 (56.0%)	12 (20.0%)

Table 4.14

ANTIBACTERIAL STUDIES CARRIED OUT WITH SILVER COATED CATHETER MATERIALS

Sl. No	Conclusion of the study	References
1.	Silver-coated Polyurethane catheter showed good ability to prevent microbial colonization by **Staph.epidermidis, E.coli** and **P.aeroginosa.**	Jansen et al 1994
2.	Silver incorporated attachable cuff can substantially reduce the incidence of catheter-related infection with most percutaneously inserted central venous catheter.	Maki et al 1988
3.	Silver incorporated latex catheter material showed antibacterial effect against **Staph.epidermidis, Staph.aureus** and **E.coli.**	Rathinam et al 1995

incorporating non toxic antiseptic and antimicrobial agent such as Silver salts to the catheter material itself may ultimately prove to be the most effective technologic innovation for reducing the risk of device related infection. Development of such material is the need of the hour in our country where over six million urinary catheters are used annually and 20% of the patients with the device developed catheter associated urinary tract infection(UTI).

As antibiotics carry the risk of developing bacterial resistance, the application of heavy metals such as copper and silver was investigated. Silver is well known antimicrobial agent with strong oligodynamic activity and has been found to be effective in urinary tract infection (Liedberg and Lundberg, 1990) and in this context our present study with silver incorporated latex material is of great relevance and importance in the development of urinary catheter so that UTI is avoided in clinical settings.

Bioimplants incorporated with antibacterial agents are proven to be effective in controlling gruesome foreign body associated infection. Here attempts are made to develop five different types of polyurethane (Angioflex) with different surface treatment. Chlorhexidine digluconate is one of the antimicrobial agents against gram positive cocci especially **Staph.epidermidis** and **Staph.aureus**. *In vitro* anti bacterial tests of these materials emphasises the fact that single coating of Chlorhexidine digluconate on PU material is sufficient enough to repel bacterial adherence. Glow discharge and double coating with Chlorhexidine digluconate did not afford any increase (synergistic and additive) antibacterial effect over single coated material. This finding is of particular importance in optimising the amount of

coating material used and cost of bacterial resistant PU material. Scanning electron microscopic studies of silver incorporated Silicone material and its surface adherence property towards **Staph.epidermidis**.

Scanning electron microscopic studies of any material provide its surface property and its interaction with any exogenous substances that come in contact with it. Here SEM studies of silver incorporated Silicone material (Proven to be bacterial resistant by *in vitro* antibacterial test) and its repellent effects towards strong slime positive **Staph.epidermidis** strain (A54), and weak slime **Staph.epidermidis** strain (A313). It is surprising to find out that silver incorporated Silicone material did not permit the adherence of **Staph.epidermidis** to a greater extend. Photographic plate no IV vividly demonstrate these inhibition of adherence by interacting bacteria on the surface.

G.pigs implanted subcutaneously with tissue cages do not seem to be an ideal animal model under our experimental condition of testing the short term efficacy of an antistyphylococcal agent namely Cloxacillin. Phenotypic changes of injected **Staph.epidermidis** (A54) was observed with its susceptibility pattern towards Cloxacillin (Photographic plate no VII) It is observed that aspirated **Staph.epidermidis** tested for antibiotic susceptibility test showed resistant pattern to Cloxacillin. This indicate that **Staph.epidermidis** injected into tissue cages acquired some phenotypic changes. The mortality among TC implanted G.pigs was more than 90%, the failure of this animal model was found to be due to a protrusion of implanted TC through the skin of this animal, the severe adhesion with subcutaneous tissue and tissue inflammatory

PHOTOGRAPHIC PLATE NO VII

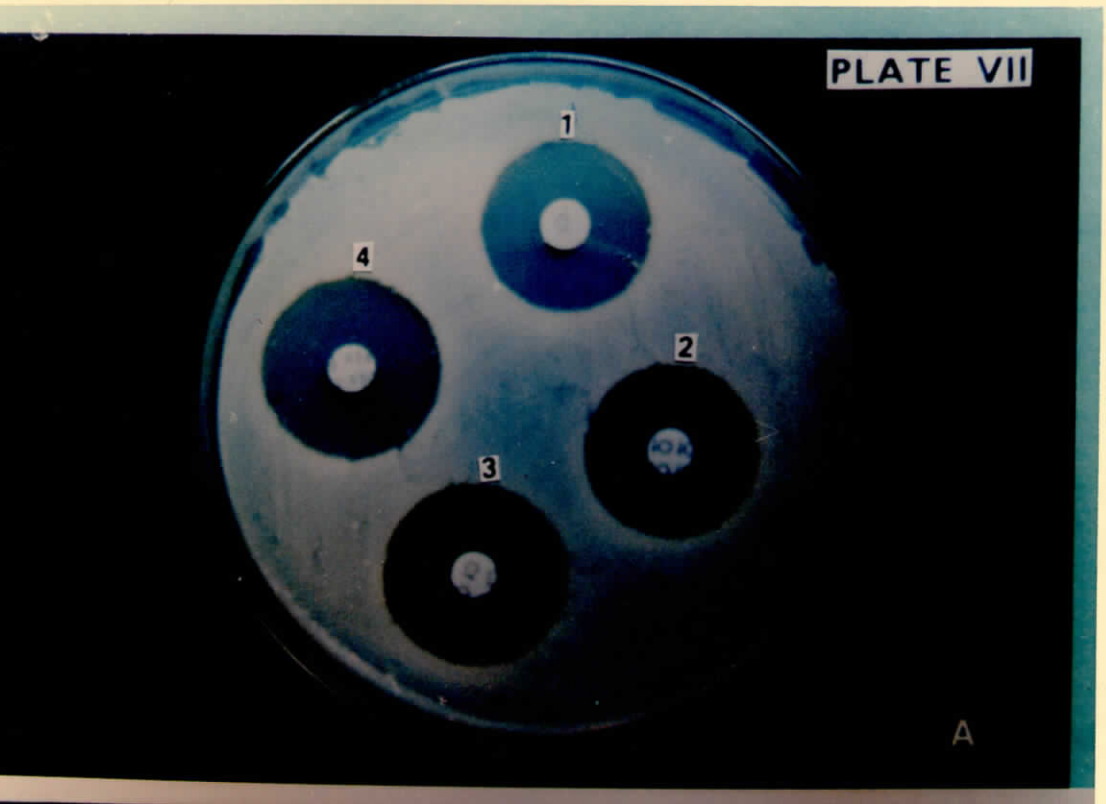
DEMONSTRATION OF PHENOTYPIC CHANGE (ANTIBIOTIC SUSCEPTIBILITY) OF A54 IN GUINEA PIG.

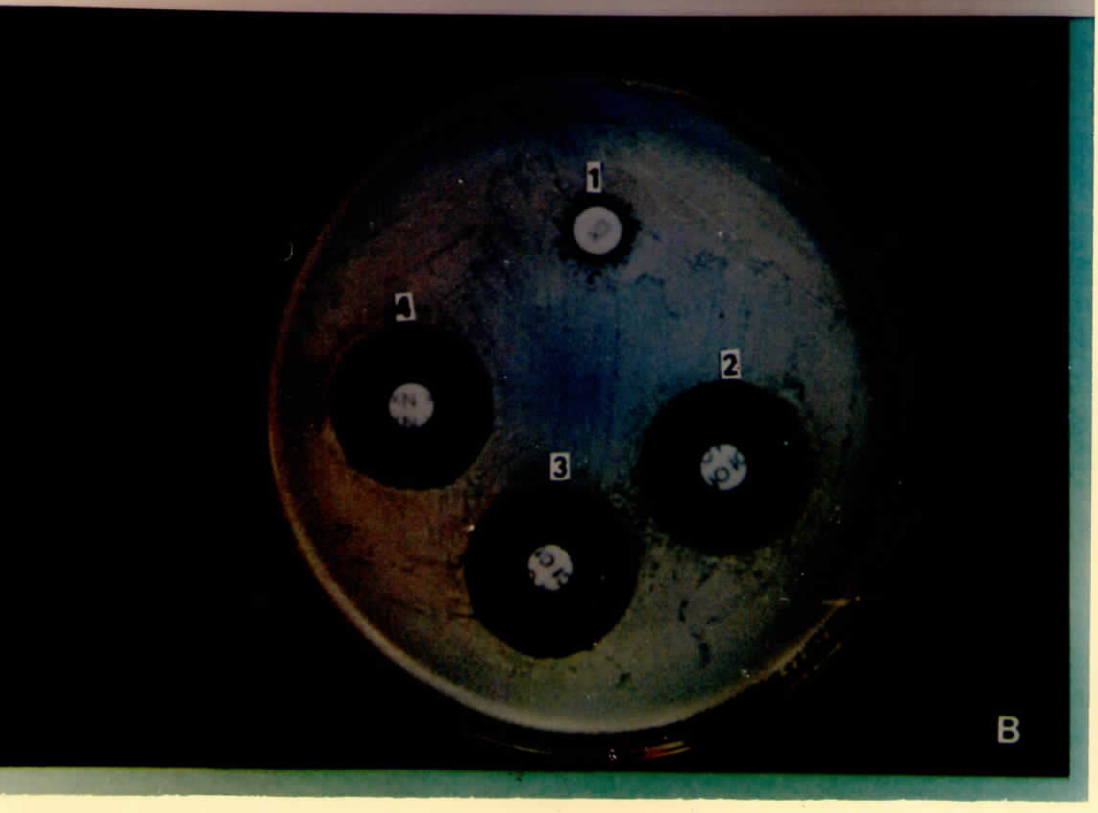
A. Antibiotic susceptibility pattern of A54 before injection.

B. Antibiotic susceptibility pattern of A54 after aspiration from injected Tissue Cage showing phenotypic change (Cloxacillin resistant).

1. Cloxacillin 2. Ofloxacin 3. Ciprofloxacin 4. Norfloxacin

PLATE VII





reactions. However in the case of rabbit model TC implantations were devoid of such undesirable phenomena (Photographic plate no X). It was observed no mortality of rabbits occurred during the period of experimentation and making this model as an ideal animal model for TC experiments. It has already been established that wound healing property is shorter in the case of rabbit than G.pigs (Jayashree et al, 1995)

Rabbit was also used as animal of our choice while studying the cellular responses to subcutaneously implanted material kept inside the stainless steel wire mesh(Jayabalan et al, 1991). It has been proved that TC experiment is an ideal tool to study the antibiotic prophylactic property of an agent in an *in vivo* condition that simulating clinical use (Zimmerli et al 1985). Hereby our experiment with infected TC's with **Staph.epidermidis** (A54) and treatment with Cloxacillin (one of the Beta-lactam antibiotic), we have established the fact of using tissue cages model for studying antibiotic prophylaxis assessment of any antimicrobial agent/ antibiotic. Widmer et al (1990) also used TC experiment to study antibacterial efficacy of antibiotics such as Vancomycin, Ciprofloxacin and Rifampin against experimental infection induced by **Staph.epidermidis.** Tshefu et al (1983) used TC's for studying the antimicrobial efficacy of short term administration of Rifampin in the prevention or eradication of infection caused by 10^3 CFU of **Staph.aureus** (Wood 46). The different studies carried out using tissue cages for studying the antibacterial efficacies of various antibiotics/antimicrobial agents is given in the Table 4.15. Another important finding derived from the present TC experiments was that tissue ingrowth of granulation tissue into the lumen of TC's were observed among all group

PHOTOGRAPHIC PLATE NO X

COMPARISON OF TISSUE CAGE EXPERIMENTS IN GUINEA PIG AND RABBIT

A. Protrusion of tissue cage through the skin of a guinea pig.

B. Showing severe haemorrhage, tissue adhesion, thick encapsulation of tissue cage in Guinea Pig experiment.

C. Rabbit carrying intact tissue cage (no protrusion observed).

D. Showing no sub-cutaneous adverse tissue response and having very thin encapsulation around the implanted tissue cage in rabbit.

↑ shows the site of Tissue Cage implants.

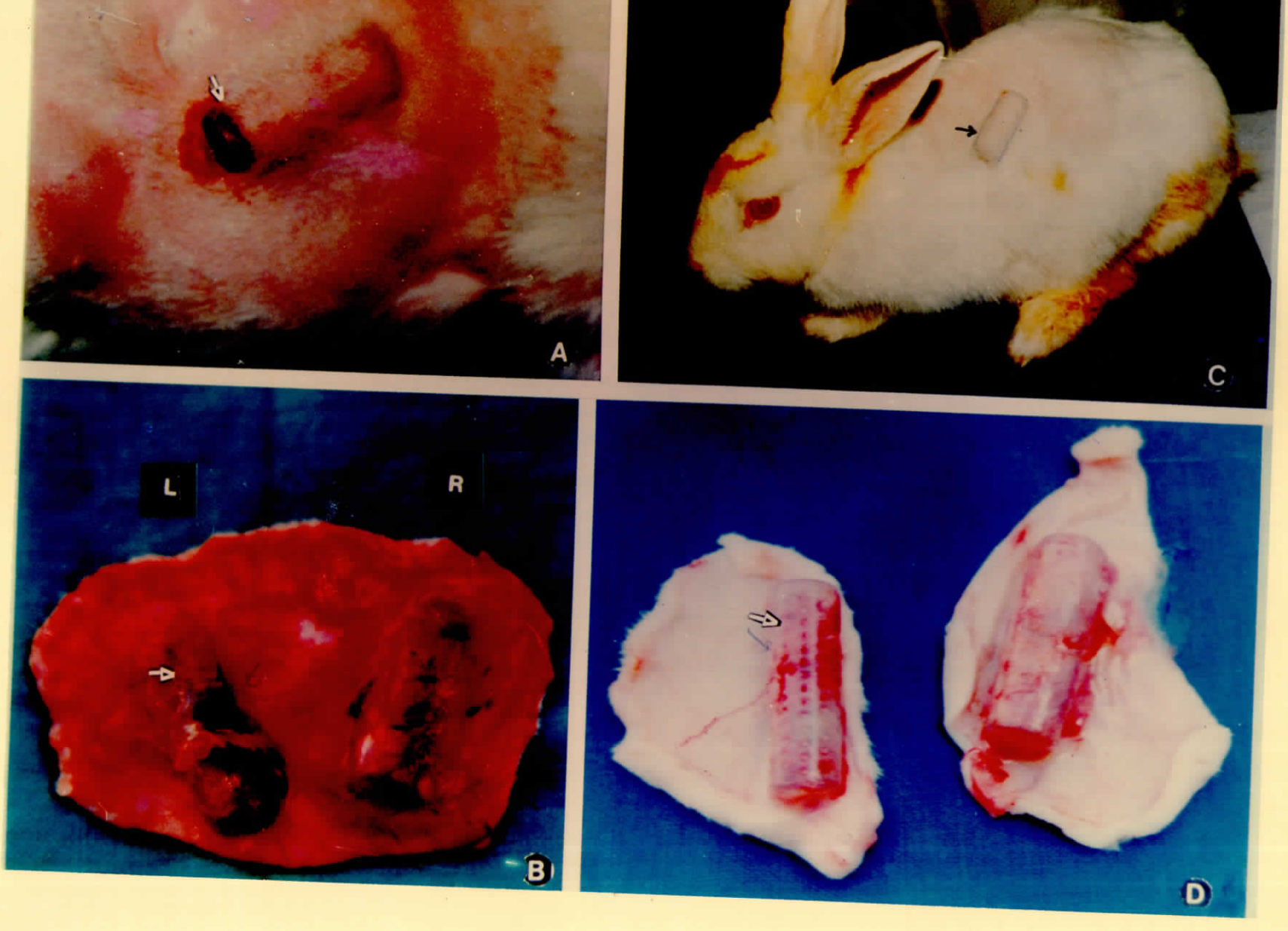

A
C
L
R
B
D

Table 4.15

DIFFERENT STUDIES CARRIED OUT USING TISSUE CAGE

Sl No	Animal Model	Conclusion derived	Reference
1.	Guinea pig	Proved that Rifampin could be used to prevent or eradicate infection caused by **Staph.aureus**	Tshefu et al 1983
2.	Guinea pig	Highlighting the tissue cage model for studying antibiotic prophylaxis in *in vivo* condition	Zimmerli et al, 1985
3.	Guinea pig	Drug efficacy in the treatment of device related **Staph.epidermidis** infection may be predicted by tissue cage experiment	Widmer et al 1990
4.	Rabbit	Anti staphylococcal effect of Cloxacillin has been demonstrated.	Rathinam et al, 1995 (Thesis work)

of animals carrying subcutaneous implants. This finding is a sharp contrast to the observation of Zimmerli(1986) and Eickenberg(1978) who hypothesized that the diameter of the holes in the tissue cages has to be less than 2mm. to prevent ingrowth of granulation tissues in to the cages. The hole diameter of the tissue cages used in this study is approximately 0.8mm diameter. The period of observation for these experiments were between 4 and 6 weeks.

Another intriguing findings of this experiments were presence of inflammatory response among infected -treated and infected -untreated groups of animals (Table 4.10). Histopathological evaluation did not reveal the presence of infected cocci in the case of group III, however while microbiological monitoring of the tissue cage fluid showed enormous number of gram positive cocci in bunches (Table 4.9). The absence of injected bacteria in histopathological specimen may be attributed to preparatory artifacts. The *in vivo* antibacterial efficacy of Cloxacillin demonstrated in this study against **Staph.epidermidis** (A54 strain) further reinforce our own *in vitro* antibiotic susceptibility pattern study performed using 10 different types of Beta-lactam antibiotics. While considering the treatment with semi synthetic Penicillinase resistant Penicillin against **Staph.epidermidis** infection, the isoxazolyl Penicillin such as Oxacillin, Cloxacillin and Dicloxacillin are recommended (Neu 1982). Here selection of Cloxacilin as drug of choice to treat experimental **Staph.epidermidis** induced infection in rabbits is very appropriate and also provides further evidence for treating such foreign body associated infection. Our studies on antibiotic susceptibility pattern of CNS strains also showed 91.5% of antibacterial effect against these strains.

" Perhaps it may turn out a sang, perhaps turn out a sermon. "

-Robert Burns

Summary, Conclusions and Recommendations

CHAPTER V

SUMMARY

The major aim of this study was to isolate, identify, speciate and to characterise the coagulase-negative staphylococci from entirely different sources so that their eventual influence in the Bacterial Biomaterial interaction could be forecasted. Results of these efforts bring many encouraging facts such as **Staph.epidermidis** could be isolated from these sources. **Staph.epidermidis** is considered to be causative bacteria in most of the foreign body associated infections.

While antibiotic susceptibility pattern of **Staph.epidermidis** is studied, Beta-lactam antibiotics such as Cloxacillin, Methicillin, Oxacillin and Ticarcillin were highly effective against these organisms in *in vitro* experiments. Among Fluoroquinolones tested, Ofloxacin was found to be the most effective anti-microbial agent against **Staph.epidermidis**

Higher minimum inhibitory concentration values of Penicillin were observed with clinical isolates of **Staph.epidermidis**. Positive correlation between higher M.I.C values and Beta-lactamase production was established (79.3%) among clinical Staph.epidermidis. When Beta-lactamase production was investigated, the clinical isolates of **Staph.epidermidis** showed 65.9% positivity while the aerial isolates of **Staph.epidermidis** produced only 35.9%.

Slime production by **Staph.epidermidis** was highest (58.8%) by Congo red agar method, followed up by Test tube method (38.9%) and least by Microplate method (32.6%).

Hydrophobicity /Hydrophilicity studies revealed that maximum (56.9%) Hydrophobicity was found in clinical isolates of **Staph.epidermidis**. Aerial isolates of Staph.epidermidis showed 89.6% of Hydrophilicity. Silver incorporated Latex rubber material was found to have bacterial repellent property against wide variety of bacteria such as **Staph.epidermidis**, **Staph.aureus** and **E.coli**.

Chlorhexidine digluconate coated silastic Polyurethane materials of different surface treatment proved that single coating of chlorhexidine digluconate was adequate enough to exhibit predictable antibacterial effects. Glow discharge treatment and double coating with chlorhexdine digluconate did not provide any further synergistic or additive anti-bacterial potentials to the materials.

Scanning electron microscopic study of silver incorporated silicone material afforded anti adhesive property towards **Staph.epidermidis** from different sources and different slime producing strains.

In vivo experiment with Guinea pigs and rabbits implanted with tissue cages revealed that G.pig was not an ideal animal model for such an experiment due to;

(A). Higher percentage of mortality,

(B). Protrusion of implanted tissue cage through the subcutaneous tissue resulting in trauma and infection.

(C). Severe inflammatory reaction induced by tissue cages and

(D). Severe adhesion of tissue cages with subcutaneous tissue.

Phenotypic changes (Antibiotic susceptibility of **Staph.epidermidis** (A54) towards Beta-lactam antibiotic Cloxacillin was demonstrated.

Subcutaneous implantation of tissue cages in rabbit provided enough experimental evidences in favour of using Rabbit for such experiments since it showed no tissue adhesion, no protrusion of tissue cages through the skin and quicker wound healing time.

Despite the diameter of holes made in tissue cages is approximately 0.8mm (less than 2.0mm) granulation tissue ingrowth was demonstrated, into the lumen of tissue cages. This observation is of particular importance, since earlier reports showed that granulation tissue ingrowth would not take place if the diameter of the holes is less than 2.0mm.

CONCLUSION

Prevalence of **Staph.epidermidis** from different sources such as patients, healthy carriers in hospital and non-hospital environments, was demonstrated. Cloxacillin among Beta-lactam antibiotics and Ofloxacin among Fluoroquinolones were found to be highly effective anti-bacterial agents against infection caused **Staph.epidermidis** irrespective of their source of isolation.

Minimum inhibitory concentration of Penicillin was found to be high with **Staph.epidermidis** isolated from clinical specimen. Whereas it was low with aerial strains of **Staph.epidermidis**. Strong Beta-lactamase positive **Staph.epidermidis** strains with Penicillin resistant property were found more among clinical isolates.

Slime detection by Congo red agar by in vitro method has been appraised in this study.

Bacterial surface hydrophobicity was demonstrated to be not an index of virulence of the bacteria or adherence factor since no correlation could be established between slime production and hydrophobicity.

Bacterial resistant property of silver incorporated latex catheter material was demonstrated against pathogenic bacteria causing urinary tract infection.

Single coating of silastic Polyurethane material (Angioflex) by Chlorhexidine digluconate was proved to be adequate enough to produce desired

anti-bacterial effects rather than other surface treatments such as low discharge and double coating with Chlorhexidine digluconate.

S.E.M study demonstrated the bacterial repellent surface property of silver incorporated silicone rubber material.

In vivo Tissue cage experiments with subcutaneous implantation in Guinea pigs was found to be less ideal than rabbit animal model.

Granulation tissue ingrowth through Tissue cage holes of lesser than 0.8mm was demonstrated in this study.

RECOMMENDATIONS

Clinical epidemiological studies become mandatory in understanding the incidence/prevalence of **Staph.epidermidis** among foreign body associated infections as a result of Biomaterial/Bacterial interactions in the host.

An efficacious and safe antibiotics such as Beta-lactam groups of antibiotics, Fluoroquinolones must further be exploited so that highly sensitive and safe anti-infective agent against the infection due to **Staph.epidermidis**, is obtained.

Among the coagulase-negative Staphylococci especially **Staph.epidermidis**, detection of slime by most reliable method using Congo red agar plate may be instituted as a routine test for assessing the possible virulence/ pathogenicity of a particular strain at the clinical settings.

Since Hydrophobicity/ Hydrophilicity surface characterization of Bacteria cause a variety of infection problems, a further in-depth study in this direction would certainly enlighten the present hypothesis that Hydrophobicity is a function of adherence and augment the virulence of a pathogenic bacteria.

Beside silver incorporated latex rubber or Chlorhexidine coated polyurethane materials for consideration of using bacterial resistant implants, further efforts be made to incorporate new antimicrobial/ antibiotic agents with Biomaterials so that incidence of implant associated infection would significantly be reduced.

The *in vivo* study carried out proves that rabbit is an ideal animal model to screen the antibiotic property of a new agents in *in vivo* condition, to understand the pathogenic character of a particular bacteria/ other causative organisms, to understand the *in vivo* tissue response towards invading infectious agents. Hence rabbit model can be used for studies related to foreign body associated infection.

Studies carried out in connection with this thesis work recommend strongly for interdisciplinary research activities calling for systematic study of biomaterial, implant and prosthesis associated infections involving the expertise of Clinicians, Microbiologists and Biomaterial scientists.

" In science, read by preference the newest work ; in literature, the oldest, the classic literature is always modern. "

-Edward Bulwer Lytton

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Appendix A

List of papers published

Sl.No	Authors	Title of paper	Name of the journal Vol.No. Pages and Year
1.	K. Rathinam , J Shanmugam & D. Rout	Slime Production by coagulase-negative staphylococcal species isolated from hospitalised patients, healthy carriers and environments.	Ind. J. Med. Micorbiol, 17: 149-152 1993
2.	K. Rathinam , J Shanmugam & D. Rout	Predominance of ***S. epidermidis, S.hominis, S.saprophyticus*** and the isolates of coagulase- negative staphylococci.	Biomedicine 14: 33-37, 1994
3.	K. Rathinam & J.Shanmugam	Antibiotic susceptibility pasttern of coagulase- negative staphylococcus strains against ten different penicillins.	JIMSA -IMSACON 1994 Spl. issue 42-44

APPENDIX B

List of papers presented

Sl.No	Authors	Title of paper presented	Name of the conference, venue, date and year
1.	K. Rathinam & J. Shanmugam	Foreign body associated infections- current status.	International Medical Sciences Academy, Kerala Chapter meeting, Trivandrum, 28.4.1991
2.	K. Rathinam & J. Shanmugam	Prevalence and characterisation of coagulase-negative staphylococci isolated from environments, Hospital personnel & surgical patients.	XIII Annual Conference of the Indian Association of Biomedical Scientists, Trivandrum. Sept. 19& 20 1992.
3.	K. Rathinam & J.Shanmugam	Predominance of **S.epidermidis, S. hominis, and S.saprophyticus** among isolates of coagulase-negative staphylococci	XVII National Congress of the Indian Association of Medical Microbiologists, Calcutta Dec. 18- 20,1993.
4.	K. Rathinam & J.Shanmugam	Antibiotic susceptibility pattern of coagulase-negative staphylococcus strains against ten different penicillin	CME Programme on "An update on Antibiotics", Trivandrum 12.3.94, sponsored by IMSA- Kerala Chapter
5.	L. Rowsen Moses, K. Sreenivasan, K. Rathinam, & R. Sivakumar	On the development of infection resistant catheters☆	MRSI, Annual general body meeting, IIT, Kharagpur. 8-10 Feb.1995
6.	K. Rathinam, & J.Shanmugam	Increased MIC values of Penicillin -G against **Staph.epidermidis** after contact with Intravenous catheters	XIX National Congress of the Indian Association of Medical Microbiologists, Pondicherry Oct. 6-8 ,1995.

☆ Received best poster presentation award