"Comparison of Phenotypic and Genotypic methods for biofilm forming Staphylococcus epidermidis from various clinical samples- A cross sectional study."

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This is to certify that the dissertation entitled **"Comparison of Phenotypic and Genotypic methods for biofilm forming *Staphylococcus epidermidis* from various clinical samples- A cross sectional study"** is a bonafide and genuine research work carried out by **REG NO.BI0115002.**

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LIST OF ABBREVIATIONS

Bap	:	Biofilm associated protein
CFU	:	Colony Forming Units
CoNS	:	Coagulase Negative Staphylococcus
CSF	:	Cerebrospinal Fluid
CVC	:	Central venous catheter
ica	:	Intercellular adhesion operon
MCRA	:	Modified Congo Red agar
MR	:	Methicillin Resistant
MRCoNS	:	Methicillin Resistant Coagulase negative *Staphylococci*
MSSA	:	Methicillin Sensitive Coagulase negative *Staphylococci*
MTP	:	Microtitre Plate
NICU	:	Neonatal Intensive Care Unit
PBS	:	Phosphate Buffer Solution
PCR	:	Polymerase Chain Reaction
PIA	:	Polysaccharide Intercellular Adhesion
PVE	:	Prosthetic valve endocarditis
SBA	:	Sheep Blood Agar
TCP	:	Tissue Culture Plate
TSS	:	Toxic Shock Syndrome
SFP	:	Staphylococcal Food Poisoning
UTI	:	Urinary Tract Infection

ABSTRACT

Background

Coagulase Negative Staphylococci (CoNS), the indigenous flora of human skin and mucus membrane now represents one of the major nosocomial pathogen due to the patient and procedure related changes. Accurate species identification in clinically relevant framework helps in the diagnosis of the *CoNS* infection. Biofilm producing bacteria are responsible for several chronic infections. Biofilms protect microorganisms against both antibiotics used to treat infections and host immune system responses. The major virulence factor determining the pathogenicity of *Staphylococcus epidermidis* has now been well defined and found to be biofilm production.

Objectives

1. To compare Phenotypic and Genotypic methods for biofilm forming *Staphylococcus epidermidis* from various clinical samples
2. To isolate and identify all Coagulase negative *Staphylococci* isolates*(CoNS*) from various clinical samples in hospitalized patients .

Materials and method

The study was conducted in the Dept of Microbiology, Dr Prabhakar Kore hospital and MRC, KLE University Belagavi. A total of 86 clinical isolates of *CoNS* were initially identified by colony morphology, Gram staining, Catalase test, Slide and Tube coagulase test and mannitol fermentation.The simple, inexpensive test were selected from scheme of Kloos and Schleifer for speciation. A total of 25 clinical isolates of *S. epidermidis* were characterized and subjected to biofilm detection by tissue culture plate method (TCP), modified congo red agar method (MCRA) and PCR (polymerase chain reaction) for *icaA* gene detection.

Results:

Out of the toal 86 isolates *S.epidemidis* is the most frequent isolate 25 (29.07%), followed by *S. hemolyticus* 24 (27.9%), *S. hominis* 20 (23.25%), *S. kohnii* 5 (5.81%), *S. simulans* 3 (3.49%), *S. warneri* 3 (3.49 %), *S. schleiferi* 3 (3.49 %), *S. intermedius* 1 (1.16 %), *S. hyicus* 1 (1.16 %)and*S. Saprophyticus* 1 (1.16%). Biofilm formation by *Staphylococcus epidermidis* (25 isolates) was detected by phenotypic and genotypic methods. By MCRA method 10 (40%) *Staphylococcus epidermidis* isolates were defined as biofilm producers through their black colonies. By TCP method 14 isolates (56%) were found to be biofilm producers with different grades: 6 (24%) strong producers and 8(32%) moderate producers.The i*caA* gene was detected concomitantly in 17(68%) isolates. In comparison with the data of *icaA* gene detection MCRA had the sensitivity of 52.9% and specificity of 87.5% while TCP method represented 70.4% sensitivity and 75% sepecificity. It was also noticed that all strong biofilm producers by TCP method were positive for i*caA* genes.

Conclusion:

In this study, out of 86 *CoNS*, 52 (60.47 %) were in > 40 years age group. Isolations were more prevalent in males 55 (63.95%). The need for prevalence and identification of *CoNS* by simple and inexpensive methodology helps in identifying pathogenic *CoNS* involved in nosocomial infections. For optimum evaluation of biofilm production by *Staphylococcus epidermidis* both genotypic and phenotypic analysis are needed. Timely diagnosis and appropriate treatment helps in faster patient cure and invariably cuts down the hospital expenses.

Key words: Nosocomial infections, *icaA* gene, *Staphylococcus epidermidis*, Biofilm, TCP, MCRA.

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INTRODUCTION

Coagulase-negative *Staphylococci*, with over 40 recognized species and subspecies are ubiquitous colonizers that inhabit normal human skin and mucous membranes. They rarely cause primary invasive disease and are contaminants of microbiological cultures. However as there are recent changes in the practice of medicine and changes in underlying host populations coagulase-negative *Staphylococci* most notably *Staphylococcus epidermidis* have become brooding pathogens. This fact coupled with the emergence of these organisms as nosocomial pathogens complicates laboratory interpretation of their clinical significance. On isolation of these organisms from clinical specimens, every effort should be made to substantiate their clinical relevance in a particular patient.[1]

CoNS accounts for a large proportion of prosthetic device–related infections. These infections usually involve intravascular catheters, prosthetic valves, orthopedic devices, peritoneal catheters, pacemakers, left-ventricular-assist devices, vascular grafts and central nervous system (CNS) shunts.[2]

S. epidermidis, the most frequently encountered *CoNS* species owes its pathogenic success to two major features—its natural niche on human skin thus causing ready access to any device inserted or implanted across the skin, and its ability to adhere to biomaterials and form biofilms[3].Infections caused by *S. epidermidis* are often indolent and clinically difficult to diagnose.Treatment has become difficult due to increasing rates of antibiotic resistance in coagulase-negative *Staphylococci.* The effect of biofilms on host defense and antimicrobial susceptibility also plays a major role. Unfortunately infected prosthetic devices need to be removed

to exact cure. The use of indwelling medical devices is on an increase, so,it is anticipated that the clinical significance of coagulase-negative *Staphylococci* will also increase[4].

The ability of bacteria to aggregate and form biofilm is factually due to its capacity to produce an extracellular mucoid substance: the slime, whose main component is of polysaccharide nature consisting of glycosaminoglycans. The development of biofilm starts with the bacteria adhering to a surface mediated by a capsular antigen, namely capsulare polysaccharide/adhesin (PS/A) and then the bacteria multiply to form a multilayered biofilm, associated with production of polysaccharide intercellular adhesin (PIA) which mediates cell to cell adhesion.Biofilm development depends on many physical,chemical and biological factors.[5]

S. epidermidis contain the intercellular adhesion (*ica*) operon responsible for biofilm production. This operon contains the *icaADBC* genes, which exerts a regulatory function. Among *ica* genes, the *icaA* and *icaD* are known to a play a significant role in biofilm formation. The *icaA* gene encodes *N*-acetylglucosaminyl transferase, the enzyme which involves PIA synthesis[5].

Staphylococcal infections caused by *ica* carriers tend to be more problematic due to the presence of methicillin and mupirocin resistance genes . The rapid detection of the *ica* locus in hospital staphylococcal isolates, together with the simultaneous detection of antibiotic resistance genes (*mecA*) allows the development of prevention methods to reduce the bacterial capacity to invade the in-dwelling medical devices.[6]

The purpose of the present study was to determine the etiological role of various *CoNS* in causation of diseases,by characterization of *CoNS*. The biofilm producing ability and the presence of the *icaA* gene in *Staphylococcus epidermidis* was also tested. Early detection and management of biofilm-forming staphylococci can be one of the essential steps that prevents and manages device-associated nosocomial infections. Thus there is a need to evaluate a simple, cost effective and reliable phenotypic method to detect biofilm producers.

OBJECTIVES

1. To compare Phenotypic and Genotypic methods for biofilm forming *Staphylococcus epidermidis* from various clinical samples

2. To isolate and identify all Coagulase negative *Staphylococci* isolates *(CoNS*) from various clinical samples in hospitalized patients.

REVIEW OF LITERATURE

Staphylococci are ubiquitous gram positive cocci arranged in grape like clusters. Surgeon Sir Alexander Ogston from Scotland was the first person to describe *Staphylococcus aureus* from surgical abscess in a knee joint. On the basis of colour of colonies, Friedrich Julium Rosenbach distinguished *Staphylococcus aureus* (golden yellow colonies) from *Staphylococcus albus* (*now epidermidis*)(white colonies).[7,12]

Historically, the genera *Staphylococcus* and *Micrococcus* were placed together with genera *Stomatococcus* and *Planococcus* in family *Micrococcaceae,* which contained Gram positive, catalase- positive cocci[8]. Molecular, phylogenetic and chemotaxonomic analysis revealed that *Staphylococci* and *Micrococci* are not closely related. *Staphylococcus* now belongs to the Bacillus-Lactobacillus –Streptococcus cluster,which consists of Gram positive bacteria with DNA of a low G+C content.(30-39%).[9]

Staphylococci are gram positive nonmotile, non spore forming, spherical cells of 0.5-1.5**µ**m in diameter occurring as single cocci, in pairs, as tetrads, or as short chains,which divide in more than one plane to form irregular clusters like a bunch of grapes. The organisms are aerobic or facultative anaerobic with the exception of *Staphylococcus aureus* subspecies *anaerobius* and *S. Sacchrolyticus,* obligate anaerobes, and can be catalase negative. *Staphylococci* grow in the presence of 10% NaCl between 18°C-40°C.[10]

Whole genome sequences have been done for *Staphylococci aureus, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus lugdunensis.*[10]

As of January 2013, 45 species including 21 subspecies are recognized in the genus *Staphylococcus.*[5,10]

In 1925 the differentiation into coagulase–positive *Staphylococci aureus* group and *CoNS* based on detection of plasma coagulase was introduced. S. *lugdunensis* and *S. schleiferi* give a positive slide coagulase test. Later on subclassification of *CoNS* was done as novobiocin susceptible (*Staphylococcus epidermidis)* and novobiocin resistant (*S.saprophyticus*) species. Intrinsic resistance to novobiocin has been reported in *S. saprophyticus*, *S. cohnii* and *S. xylosus.* Despite limitations coagulase activity and novobiocin–susceptibility are still used for presumptive identification of clinical isolates[6,10].

In 1975,Kloos and Scleifer published a scheme for the phenotypic identification of *CoNS.*A large number of physiological and biochemical tests to differentiate coagulase –negative isolates were used. Modified conventional schemes have been published incorporating other tests.[11,16,49,60]

Automated identification of *CoNS* using commertial kits are 70% to 90% accurate. Automated identification systems available are Vitek2 gram positive (GP) Identification card, Microscan Panels, Sherlock microbial identification system, API Staph-Indent, BBL Crystal Gram positive(GP) IdentificationKit, Microbact Staphylococcal 12S Identification system(oxoid), RapiDEC Staph (bioMerieux).The available PNA FISH Culture Confirmation kit combines fluorescence in situ hybridization has shown very high sensitivity and sensitivity. MALDI TOF(Matrix assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry) is helpful for routine identification of staphylococcal isolates. It is faster and the results are equivalent to molecular defined reference methods. Majority of the studies reported

identification of staphylococcal isolates at the species level of >97%. PCR coupled with electrospray ionization- mass spectrometry (ESI-MS) gives excellent species identification and further molecular characterization is in parallel to it. Ramal spectroscopy and FTIR (Fourier Transform infrared) hold promising results for future[5,10].

Typing methods give information useful for outbreak investigations, surveillance purposes, detection of unknown / emerging strains, and prediction of clinical importance of a given isolate. Typing systems available for *CoNS* are[10,12]

1. Antibiotic resistance analysis
2. Phage typing
3. Slime production
4. Genotyping procedures (PFGE and ribotyping).

In near future whole genome sequencing may supersede the present methods.[17]

TAXONOMY OF CONS[9,10]

The Staphylococci belongs to the

Phylum- Firmicutes,

Order- Bacillales,

Class- Bacilli and

Family- Staphylococcaceae.

Genus- *Staphylococci*

Coagulase positive *Staphylococci (CoPS)* and

Coagulase negative *Staphylococci (CoNS)*

Coagulase-negative staphylococci (most commonly encountered in human diseases)[14]

Staphylococcus epidermis

Staphylococcus haemolyticus

Staphylococcus saprophyticus

Staphylococcus lugdunensis

Staphylococcus schleiferi

Coagulase-negative *Staphylococci* (Species Rarely Associated with Human Disease)[14]

Staphylococcus auricularis

Staphylococcus capitis

Staphylococcus caprae

Staphylococcus carnosus

Staphylococcus cohnii

Staphylococcus hominis

Staphylococcus Pasteur

Staphylococcus pettenkoferi

Staphylococcus pulvereri

Staphylococcus saccharolyticus

Staphylococcus simulans

Staphylococcus schleiferi

Staphylococcus warneri

Staphylococcus xylosus

Coagulase-negative staphylococci(found in Animals)[14]

Staphylococcus arlettae

Staphylococcus caseolyticus

Staphylococcus chromogenes

Staphylococcus condimenti

Staphylococcus delphini

Staphylococcus equorum

Staphylococcus felis

Staphylococcus fleurettii

Staphylococcus gallinarum

Staphylococcus hyicus

Staphylococcus intermedius

Staphylococcus kloosii

Staphylococcus lentus

Staphylococcus lutrae

Staphylococcus muscle

Staphylococcus nepalensis

Staphylococcus piscifermentans

Staphylococcus pseudintermedius

Staphylococcus sciuri

Staphylococcus simiae

Staphylococcus succinus

Staphylococcus vitulinus

Epidemiology of Coagulase-negative *Staphylococci* (*CoNS)*

The major habitats of most staphylococcal species are the skin and mucous membranes of mammals and birds.These species are found mainly as part of the resident microbiota.In humans, *S. epidermidis* usually colonizes the body surface[15]. It is invariably prevalent on moist areas, like anterior nares, axilla ,inguinal, perineal areas, and toe webs. *S. auricularis* colonizes in part of the healthy human external auditory canal [1]. *S. capitis* is found surrounding the sebaceous glands on the forehead and scalp following puberty[1,14]. *S. haemolyticus* and *S. hominis* are usually isolated from axilla and pubic areas. *S. saprophyticus* subspecies *saprophyticus* particularly colonizes the rectum and the genitourinary tract of young women[10].

S. lugdunensis is frequently isolated from the lower extremities and the groin. Some recently discovered staphylococcal species, such as *S.massiliensis*, *S. pettenkoferi*, and *S. petrasii*, are also likely to be part of the human skin microbiota. *S. sciuri* and *S. xylosus* are commensals of the skin and the mucous membranes of many animals and occasionally of humans[10]. Both species are also found in food. *S.xylosus* represents starter cultures used for meat fermentation, *S. sciuri* subsp. *carnaticus* is recovered from bovine hosts, and subsp. *rodentium* is found in rodents. *S. nepalensis* has been isolated from Himalayan goats. *S. musca*e has been described on flies. *S. rostri* was isolated from the noses of pigs, *S. devriese*i from bovine udder, *S. microti* from free living common voles, and *S. stepanovicii* from rodents and mammalian insectivores[10].

Clinical significance and infections of Coagulase-negative staphylococci (*CoNS*)

Drastic changes in patient populations—increased numbers of premature newborns and of elderly, multimorbid, chronically ill, and, often, immunocompromised patients—as well as the increasing use of inserted foreign bodies, led to an acknowledgment of the large variety of infections caused by *CoNS*. This was confirmed by many clinical studies that considered certain entities and patient groups[5,24]. As most studies address *CoNS* as a whole and fail to distinguish between different species so, the less frequently occurring species might go underreported. Despite all being commensals that colonize host or natural food surfaces, the ability of various staphylococci to cause infection differs[14,15]. *S. epidermidis* group, *S. lugdunensis,* and *S. saprophyticus* as typical examples—and relatively nonpathogenic. *Staphylococci*, represented by saprophytic species is usually associated with foods of plant and animal origin. Strain- specific features at the subspecies level and host-specific capabilities of a given staphylococcal species should also be considered. Thus, even less-virulent *CoNS* species may cause infections, particularly if cofactors are present that favor infections, such as foreign bodies and/or immunosuppression.

Overall, *S. epidermidis* is the most common species in (*CoNS)* Coagulase-Negative *Staphylococci* infections, followed by *S. hominis*, *S. haemolyticus,* and *S. capitis*[20]. In contrast in a global endocarditis study, *S. lugdunensis* was reported as the second most common *CoNS* pathogen .The most important clinical entity associated with *CoNS* is foreign body-related infections (FBRIs), also designated as device associated health care-associated infections (DA-HAIs). These comprise local and bloodstream-related entities which are associated with inserted or implanted medical

devices[26,38]. While *S. aureus* can cause superantigen and exfoliative toxin-mediated diseases, such as toxic shock syndrome (TSS), staphylococcal scalded skin syndrome (SSSS [dermatitis exfoliativa neonatorum Ritter von Rittershain disease]), and staphylococcal food poisoning(SFP), *S. epidermidis* strains recovered from patients with TSS symptoms produced no superantigens but could stimulate human monocytes to produce cytokines, which may have caused clinical symptoms[1]. It was found that *CoNS* species isolated from milk or dairy products have never been involved in any case of SFP following the ingestion of dairy products. But, *CoNS* may carry enterotoxin genes[10].

As the pathogenic potential of various species of *CoNS* is being increasingly recognised and also emergence of drug resistance amongst them denotes the need for better laboratory procedures to identify them along with continuous surveillance of their antimicrobial susceptibility pattern[16,17,49]. Early detection of resistance pattern is of vital importance to ensure appropriate antibiotic treatment in infected patients as well as control the spread of resistance in hospital environments. The usage of newer antimicrobial agents should be limited to treat resistant and life threatening *CoNS* infections.

Pathogenesis of Coagulase-negative staphylococci (*CoNS*)

There are few defined virulence factors in *S. epidermidis* and other coagulase-negative staphylococci. Significant advances have been made in the past ten years to define the pathogenesis of infections caused by *S. epidermidis.* The ability of *S. epidermidis* to adhere and form biofilm on the surface of biomaterials is thought to be the most significant virulence factor associated with this bacterium. However, other

factors, such as the secretion of poly-gamma-dl-glutamic acid (PGA) and phenol soluble modulins (PSMs), appear to complement and increase the virulence[5].

Virulence Factors[1,12]

Biofilm Formation[1]

Polysaccharide intercellular adhesin (PIA)

Accumulation associated protein (Aap)

Bap homologue protein (Bhp)

Extracellular DNA

Adhesin Molecules[1]

Autolysin, adhesin (Aae)

Autolysin (AtlE)

Bap homologue protein (Bhp)

Elastin binding protein (Ebp)

Extracellular matrix binding protein (EmbP)

Fibrinogen-binding protein (Fbe)

Glycerol ester hydrolase (GehD)

Staphylococcal conserved antigen (ScaA)

Staphylococcal conserved antigen (ScaB)

Serine aspartate repeat protein F (SdrF)

Serine aspartate repeat protein G (SdrG)

Staphylococcal surface protein 1 (Ssp-1)

Staphylococcal surface protein 2 (Ssp-2)

Teichoic acid

Other Putative Virulence Factors[1]

Peptidoglycan, lipoteichoic acid

Phenol-soluble modulins

Poly-d-glutamic acid

Delta toxin

Exoenzymes

Fatty acid–modifying enzyme (FAME)

Lipases

Proteases

Elastase

Lantibiotics

Epidermin, epilancin, epicidin, Pep5, K7

Clinical Syndromes by *S. epidermidis*

Bacteremia

Most of the *Staphylococcus epidermidis* blood stream infections are due to involvement of intravascular catheters[28] or other prosthetic medical devices[34]. Immunosuppressed patients[33], particularly those with severe neutropenia[38] are more prone to these infections. As *S. epidermidis,* occupy a prominent position in the commensal flora of human skin and mucous membranes, they are frequently encountered as culture contaminants. Approximately 1% to 6% of blood cultures are contaminated and coagulase-negative staphylococci are responsible in 70% to 80% of cases[1].

Typically rates of true bacteremia range from 10% to 25% when coagulase-negative staphylococci are isolated from blood cultures[1]. Factors helpful to distinguish between true-positive cultures with clinical impact and contaminated specimens taken from a patient with clinical features of infection include (i) isolation of a strain in pure culture from the infected site or body fluid and (ii) the repeated isolation of the same strain or combination of strains over the course of the infection. An algorithm to reduce misclassification of nosocomial bloodstream infections due to *CoNS* was defined as at least two blood cultures positive for *CoNS* within 5 days or one positive blood culture plus clinical evidence of infection[10]. Contaminants treated as true pathogens result in unnecessary use of laboratory resources, antibiotic treatment, emergence of antibiotic resistance, excessive,antibiotic-associated side effects, toxicity, and greater expense.

Methods that have been used to prevent blood culture contamination include use of effective skin antiseptics, phlebotomy teams, culture bottle preparation, blood culture kits, and double-needle bottle inoculation[1].

Intravascular Catheter Infections

For peripheral intravascular catheters and nontunneled central venous catheters (CVCs), infection most commonly results from coagulase-negative *Staphylococci* which enters via the cutaneous surface of the catheter to the bloodstream, for tunneled CVCs, hub colonization and passage of organisms along the lumen appears to be an important route of infection. The ability of coagulase-negative *Staphylococci* to adhere to the catheter and form biofilms are crucial traits that allow them to cause infection of vascular Catheters[5].

The diagnosis of coagulase-negative *Staphylococci* in CVC bloodstream infection is difficult because most infected CVCs have no obvious local evidence of inflammation or infection and, are most often clinically encountered as blood culture contaminants. Diagnosis of CVC-associated bloodstream infection was considered to require removal of the catheter and semiquantitative or quantitative culture of the catheter tip[35]. Automated, continuously monitored blood culture systems and use a 2-hour cutoff differential time helps clinicians to assess blood cultures drawn from the periphery and CVC. Short-term, nontunneled CVCs infected with coagulase negative *Staphylococci* should be removed. In patients with coagulase negative *Staphylococci*–infected tunneled CVCs who do not exhibit signs of severe sepsis, attempt to catheter salvage is advised. If the central venous catheter is retained, use of systemic antimicrobials through all the lumens of the catheter for 10 to 14 days facilitates quick recovery . If fever or bacteremia persists for more than 3 days after initiation of therapy, the CVC usually should be removed. Most health care–associated coagulase-negative *Staphylococci* are methicillin-resistant, and vancomycin is most frequently used to treat them[1,5].

Endocarditis

Prosthetic valve endocarditis (PVE), although rare, is caused by coagulase-negative *Staphylococci* in 15% to 40% of cases. The infection is usually health care–related (resulting from inoculation at the time of surgery) and manifests within 12 months of valve replacement. These isolates are usually methicillin-resistant[1]. An acute presentation is characterized by fever and physical evidence of valve dysfunction, whereas chronic presentation of endocarditis are more commonly observed in patients who exhibit a more indolent course.

The diagnosis is usually confirmed by documenting repeatedly positive blood cultures and vegetations on transesophageal echocardiography. Heart failure occurs in 54% of cases and more than 80% have complications, including prosthetic valve dysfunction and intracardiac abscesses[1,5]. Typically, antibiotic therapy for recovery of patients consists of vancomycin and rifampin for at least 6 weeks combined with gentamicin for the first 2 weeks. Isolates susceptible to penicillinase-stable penicillins should be treated with oxacillin or nafcillin instead of vancomycin. Vancomycin "MIC creep" is of major concern and clinicians have switched to daptomycin or other vancomycin alternatives. Valve replacement surgery usually becomes mandatory.[1]

The mortality caused by coagulase negative staphylococci PVE is high, inspite of proper treatment, about 25%. Unlike PVE, native valve endocarditis caused by coagulase-negative *Staphylococci* is unusual, accounting for only 5% to 8% of endocarditis cases.This infection is due to hematogenous seeding of previously damaged heart valves and endocardium.[1]

Orthopedic Prosthetic Device Infections

Coagulase-negative staphylococci, usually *S. epidermidis*, are responsible for 30% to 43% of cases, and are the most common cause of infection of prosthetic orthopedic devices[5,34].These organisms usually enter at the time of arthroplasty and, because of their relatively avirulent nature,are usually indolent in their clinical presentation. Risk factors include previous joint surgery, perioperative wound complications, and rheumatoid arthritis, history of malignancy, diabetes, corticosteroid use, obesity, age, nutritional status, infection at remote site at time of surgery, psoriasis, hemophilia, sickle cell anemia, dialysis, acquired immunodeficiency syndrome (AIDS), and solid organ transplantation[1,15,25].

Infections can be classified as early (within 3 months of surgery and are often caused by S. aureus); delayed (3 months to 2 years postoperatively; most frequently caused by coagulase-negative *Staphylococci);* and late (longer than 2 years after surgery; usually caused by hematogenous inoculation of organisms from some other source)[5,31]. Delayed infections caused by coagulase-negative *Staphylococci* are indolent and manifest as pain at the affected joint, without fever or other systemic manifestations. The recovery of organisms can be optimized by sonication of the prosthesis at the time of removal. Eradication of infection is best achieved by a two-stage exchange procedure combined with 6 weeks of antibiotic treatment. Vancomycin in combination with rifampin is most frequently used[5,12].

Cerebrospinal Fluid Shunt Infection

It is one of the most significant complications ranging in incidence between 1.5% and 38%, but more recently occurring in approximately 5% of patients[1]. Coagulase-negative *Staphylococci,* most predominantly *S. epidermidis*, are the most common cause and are responsible for approximately half of cases. Risk factors for infection include age younger than 6 months, shunt revision surgery, scalp dermatitis, duration of procedure, proficiency of the surgeon, and intraoperative use of a neuroendoscope[1,24].

Patients who receive a short-term ventriculostomy (external ventricular drain) are at substantial risk (approximately 10%) of developing ventriculitis or meningitis and coagulase-negative *Staphylococci* are the predominant pathogens. Although some reports have indicated a shift toward gram-negative pathogens. The duration of catheterization appears to be the major risk factor for infection. Signs and symptoms of shunt infection typically develop within 2 months of shunt insertion and should be

suspected in patients with local signs of inflammation, nausea, or vomiting, signs of increased intracranial pressure, or shunt malfunction[1].

The diagnosis is confirmed by isolation of coagulase-negative staphylococci from cerebrospinal fluid obtained from the shunt. Most infections are caused by methicillin-resistant strains of coagulase-negative *Staphylococci*, and combination therapy with vancomycin, gentamicin, and rifampin is a traditional regimen. Vancomycin and gentamicin are often delivered intraventricularly[1,29].

Vascular Grafts

Although infection is a relatively rare complication of arterial reconstruction (lower than 1%), coagulase-negative *Staphylococci* are one the most common causes (20% to 30%) of this feared entity. *S. epidermidis*, causing these infections are thought to be inoculated at the time of surgery from the patient's skin. Major risk factors include a groin incision, diabetes, emergency aortic aneurysm repair, steroid therapy, and remote infection. Most cases of vascular graft infection caused by coagulase-negative *Staphylococci* present in an indolent fashion, months to years after surgery and manifest as a false aneurysm, fistula or sinus tract formation, or hemorrhage at the anastomotic site[1].

Diagnosis is usually done based on local physical findings and is supported by radiographic modalities, such as computed tomography (CT), magnetic resonance imaging (MRI), or ultrasound. Blood cultures are often negative because infection may not extend to the graft lumen. Confirmation of the causative role of coagulase-negative *Staphylococci* is maximized by sonication of the explanted graft at the time of surgery to recover biofilm-associated organisms. Optimum treatment needs a

combined medical and surgical approach. Intensive and prolonged antibiotic therapy is important, but surgery is required for cure. Surgical strategy can be summarized as graft excision with extra-anatomic bypass or graft excision with in situ reconstruction using a prosthetic conduit, allograft, or autogenous tissue[5]

There is some support for the use of antibiotic-treated or silver-coated grafts in the treatment of vascular graft infection.[1]

Surgical Site Infections

Surgical site infections caused by the coagulase-negative *Staphylococci* occur frequently and are second only to *S. aureus* as a causative agent. Coagulase-negative S*taphylococci* are more often causative of superficial incisional infections rather than deep incisional infections and rarely cause organ or space infections[1,15]. A remarkable exception is mediastinitis after median sternotomy for cardiac surgery. In addition, coagulase-negative *Staphylococci* are more likely to cause infections involving clean procedures rather than those classified as contaminated (e.g., bowel, genitourinary).

Superficial incisional infections generally manifest within 5 to 10 days post-procedure and usually result from inoculation of organisms from the patient's endogenous flora or, less frequently, from the operating personnel or environment. Risk factors include duration of the surgical procedure, host factors (e.g., extremities of age, obesity, immunocomprised status, nutritional status), and experience of the surgeon and surgical staff. Signs and symptoms of a surgical site infection include pain, tenderness, swelling, warmth, erythema, drainage at the incisional site, leukocytosis, and fever.

The causative pathogen is confirmed by recovery of coagulase-negative *Staphylococci* from wound cultures. Generally, coagulase-negative *Staphylococci* are interpreted to be the causative agent of the infection if they are the predominant or only isolate from purulent drainage and/or are repeatedly cultured from the same source. Treatment depends on the severity of the infection and ranges from topical wound care alone to surgical débridement and parenteral antibiotics[5].

Peritoneal Dialysis Catheter-Associated Infections

Coagulase-negative *Staphylococci,* accounting for 25% to 50% of cases, are the most frequent cause of peritonitis in patients undergoing peritoneal dialysis. *S. epidermidis* is responsible for 50% to 80% of these infections . Coagulase-negative *Staphylococci* gain access to the peritoneum from the patient's skin via the intraluminal route or from the exit site via the periluminal route[1,26].

Diagnosis is confirmed by documenting more than 100 white blood cells/mL in dialysate fluid and recovery of coagulase-negative *Staphylococci* in cultures of the fluid. When culturing the dialysate, it is necessary to culture large volumes of fluid (more than 100mL) or to use a filter technique or broth enrichment to detect small numbers of organisms. However, extreme caution is required as it may be difficult to differentiate contaminants from causative pathogens. Although historically most of these infections were caused by methicillin susceptible strains, but as of now the antimicrobial susceptibility pattern has shifted and most causative strains are now methicillin-resistant[17].

Treatment with vancomycin via the dialysate fluid is a relatively convenient dosing method and is invariably successful. Refractory peritonitis is an indication for

catheter removal. Prevention of infection depends on proper catheter placement, exit site care, infusion with Y sets and twin bag systems, and careful training of patients regarding aseptic practice. Perioperative antibiotic prophylaxis significantly reduces the risk of early peritonitis but not exit site or tunnel infections[1,5].

Endophthalmitis

Coagulase-negative S*taphylococci* are recovered from conjunctival cultures of preophthalmologic surgery patients, and are the most frequent cause of postoperative endophthalmitis, responsible for 60% to 70% of cases[1,5,18]. Symptoms typically develop within 1 week of surgery and usually consist of pain, redness, and decreased visual acuity. Fever is generally absent and leukocyte count is normal. The physical examination reveals conjunctival injection and a hypopyon.

Optimal treatment consists of vitrectomy and intravitreal administration of antibiotics. Vancomycin is usually administered intravitreally and bactericidal concentrations usually persist for 2 to 3 days. Intraocular lens removal is generally not required. Although prognosis largely depends on presenting visual acuity[1,27,37].

Urinary Tract Infection

Urinary tract infections caused by the coagulase-negative *Staphylococci* fall into two major groups. The first is caused by *S. saprophyticus*[6]. The second group is uncommon and occurs almost exclusively in hospitalized patients with underlying urinary tract complications. Most of these patients have a urinary catheter in place and have recently undergone urinary tract surgery, kidney transplantation, experienced kidney stone disease, or have a neurogenic bladder or obstructive uropathy[1]. Coagulase-negative *Staphylococci* cause approximately 3% of nosocomial urinary

tract infections, with *S. epidermidis* responsible for 90% of these isolates. Additional risk factors include advanced age and extended length of hospital stay.

Approximately 1% of urinary tract infections in outpatients are caused by coagulase-negative *Staphylococci.* Infection with coagulase- negative *Staphylococci* is associated with a lesser degree of pyuria than infection with gram-negative bacilli (mean urine leukocyte count, of 39 vs. 121 white blood cells/mL) and most of these patients are asymptomatic. Coagulase-negative *Staphylococci* causing nosocomial urinary tract infections are usually methicillin-resistant and treatment should be based on the susceptibility profile of the organism[1,5].

Infections of Genitourinary Prosthesis

S. epidermidis is responsible for 35% to 60% of infections of synthetic urinary sphincters and penile prosthesis in which an overall infection rate of 2% to 4% is observed[1]. Coagulase-negative staphylococcal infections of penile prosthesis are often indolent and may take up to a year from the date of implantation to manifest clinically. Those with infected prostheses exhibit local pain, swelling, induration, and erythema of the penis. Occasionally, fistula formation is observed and malfunction or impairment of the device is frequent.

Rarely do systemic signs of infection occur. Diagnosis is made clinically and by culture of any drainage or of the device itself. Surgical removal of the device is required, accompanied by 10 to 14 days of systemic antibiotics for uncomplicated infection. In addition to fastidious surgical technique and antibiotic prophylaxis, the use of antibiotic-coated prosthesis appears promising as a means to prevent infection[1,5,10].

Infections of Breast Implants

Infection associated with breast implant surgery occurs in about 1% to 2% of patients and is usually caused by coagulase-negative *Staphylococci. S. epidermidis* inhabits the glandular tissue ducts of the breast and from there can gain access to the space surrounding the implant. Infection can present acutely or can be very indolent. Signs and symptoms are usually localized and include erythema, tenderness, pain, swelling, induration, and drainage. Acute infections are usually associated with systemic findings like fever and leukocytosis.

Diagnosis is confirmed by culture of the drainage or fluid surrounding the implant or of the implant itself. Treatment consists of antibiotic therapy and a two-stage replacement procedure.Capsular contracture remains the most common complication following breast augmentation. Although controversial, chronic low-grade or subclinical infection with coagulase-negative *Staphylococci* may be a cause for capsular contracture[1].

Patient Populations at Increased Risk of Infection by *S. epidermidis*

Solid organ or hematopoietic stem cell transplant patients are susceptible to coagulase-negative staphylococcal infections caused by immunosuppression, intravascular catheterization, and mucosal or skin breakdown. These infections most often manifest as a *S. epidermidis* bloodstream infection and are due to an infected intravenous catheter[1,38]. Mucositis and the breakdown of gastrointestinal mucosal integrity, related to cytotoxic chemotherapy or radiation therapy, may be an alternate source for coagulase-negative staphylococcal bacteremia. Interleukin-2 therapy has been associated with an increased risk of *S. epidermidis* bacteremia. Cardiac

transplant patients are at increased risk of sternal wound infections and mediastinitis caused by coagulase negative *Staphylococci.*[55]

Neonates

Approximately 20% of very low birth weight preterm infants (less than 1500Â€g) experience late-onset neonatal sepsis (more than 3 days after birth). Half of these infections are caused by coagulase-negative *Staphylococci* and are associated with a mortality rate of 9%. *S.epidermidis* accounts for 60% to 93% of the infections caused by coagulase negative *Staphylococci,* with lesser contributions by *S. haemolyticus, S. hominis, S. warneri, S. saprophyticus, S. cohnii*, and *S. capitis*. Neonates become colonized with coagulase-negative *Staphylococci* on their skin and in their nares, umbilicus, and pharynx within days of their admission to the neonatal intensive care unit (NICU) and, in most cases, these organisms do not originate from the mother but are acquired from the hospital environment and health care workers. In addition, endemic strains of coagulase-negative staphylococci can persist in the NICU for many years. Risk factors for developing coagulase-negative staphylococcal bacteremia include low birth weight, the presence and duration of use of CVCs and umbilical catheters, mechanical ventilation and total parenteral nutrition, especially with IV lipid emulsions[5,40]

Coagulase-negative staphylococcal bacteremia is often indolent and signs of infection may include abdominal distention, apnea, bradycardia, inability to maintain body temperature, feeding difficulties, lethargy, neutropenia, thrombocytopenia, hyperglycemia.etc. Differentiating true bacteremia from contamination is made even more difficult in neonates for several reasons, including the difficulty in obtaining blood from low-birth-weight infants, the small volume of blood generally obtained

(0.1 to 1mL), and the common practice of obtaining a single sample of blood for culture to preserve blood volume. It is usually necessary to correlate other laboratory findings along with the clinical presention.. Similarly, there are data associating delta toxin–producing *S. epidermidis* to neonatal necrotizing enterocolitis[1].

Prevention of coagulase-negative staphylococcal infection in neonates has largely concentrated on prevention of intravascular catheter– associated infection. Catheters should be inserted with meticulous attention to aseptic practices. Staff should adhere to appropriate protocol. Other preventive measures include use of prophylactic antibiotics or antimicrobial flush solutions[1,36].

Laboratory diagnosis of coagulase-negative *Staphylococci*

As coagulase-negative *Staphylococci* are ubiquitous colonizers, they are frequently found as contaminants in clinical specimens. The emergence of these organisms as nosocomial pathogens, complicates laboratory interpretation of their clinical significance. When these organisms are isolated from clinical specimens, every effort should be made to substantiate their clinical relevance [14].

Isolation

Routinely, isolation of *Staphylococci* from clinical specimens is done using Columbia or tryptic soy broth containing 5% sheep blood . Growth usually occurs within 18 to 24 hours but in case of Small colony variants (SCV) it may take 48 to 72 hrs of incubation[6].

Procedures for detection of Foreign body related infections

Catheter related- blood stream infections (CRBSIs).

With catheter removal[35]

a) Semi-quantitative- Maki roll-plate culture technique: the distal segment of the central venous catheter be cut and rolled across the surface of a Columbia blood agar plate at least four times. After overnight incubation, a colony count of >/=15 CFU/plate may indicate catheter colonization.
b) Sonication and/ or vortexing :detachment of biofilms from different implants can be improved by this method.

Without catheter removal[1,15]

a) Time to positivity: On examination of paired quantitative blood cultures are drawn simultaneously from the catheter and a peripheral vein, Central line associated blood stream infection (CLABSI) is probable if the catheter sample becomes positive first and the time difference between both samples samples is 2 hours.
b) Paired Quantitative culture: A difference in count of catheter and peripherally collected blood sample by > 5-10 indicates CLABSI.
c) Endoluminal brush: It can be used for in situ diagnosis of CRBSI and hence no line sacrifice is needed.

Implant associated infections: Sonication prior to culture increases chances of isolation, particularly in patients who have been on antibiotics 2 weeks prior to implant surgery.

Ocular examination and gram stain followed by culture of sample obtained by vitrectomy can be used for diagnosis of endophthalmitis.

Identification [7,53]

Direct examination: *CoNS* are Gram-positive, nonmotile, non-spore forming usually arranged singly, in pairs, tetrads, in irregular (grape –like) clusters, or in short chains of three or four cells.

Colony characteristics[14]: On Sheep blood agar colonies are usually nonpigmented, smooth, entire margins, glistening, and opaque. Some *CoNS* species display a hazy or distinct zone of beta-haemolysis around the colonies. SCVs of *CoNS* have pinpoint colonies reaching only 10% of the wide-type colony size.

Biochemical characterization[11,19,41]: Baird Parker, Kloos and Schleifer published schemes for the biochemical identification of *CoNS*. In these schemes, coagulase test and a variety of biochemical tests are used for differentiating the *CoNS* species. Although the tube coagulase test is still used, the usefulness of detecting clumping factor by the slide agglutination test has low sensitivity and specificity. These tests help in the speciation of *CoNS*, especially in laboratories where automated or molecular techniques are not available.

Automated biochemical test systems[1,10]:

These systems are still a cornerstone of many routine laboratories and comprise the following: *Staphylococcus*-specialized API Staph (bioMérieux, LaBalme LesGrottes, France) and the Rapidec Staph (bioMérieux)system,as well as more general systems, such as Vitek 2 (bioMérieux), Microscan panels (Siemens Healthcare Diagnostics, Deerfield, IL), the BBL Crystal identification system's Rapid Gram-Positive ID kit (BD Diagnostic Systems, Sparks, MD), the Phoenix automated microbiology system (BD Diagnostic Systems), and the Biolog systems (Biolog,

Hayward, CA). The Sherlock microbial identification system (MIDI, Newark, DE) combines cellular fatty acid analysis with computerized high-resolution gas chromatography.

Identification by nucleic acid-based approaches[5]

1) **Amplification-based assays:** Commonly used universal target genes for *CoNS* includes ribosomal genes (16S and 23SrRNA), glyceraldehyde- 3-phosphate dehydrogenase- gene (*gap*), RNA polymerase beta subunit gene (*rpoB*) and gyrase gene (*gyrA*). Several commercial tests are available for the detection of the *mecA* and toxin genes.
2) **Oligonucleotide microarrays:** Microarray-based diagnostics may combine the advantages of high-throughput screening with the possibility of the identification of different genes useful for species determination and, in parallel, the identification of a multitude of virulence,drug resistance,and subtype-determining signatures. These assays have been tested successfully on clinical isolates.
3) **Nucleic acid hybridization approaches (PNA FISH):** A qualitative nucleic acid hybridization assay targeting rRNA gene sequences, based on peptide nucleic acid fluorescence in situ hybridization (PNA FISH), has been developed for the rapid identification of S. aureus and several CoNS species in smears prepared from positive blood cultures

Spectroscopic and spectrometric methods for *CoNS* identification:

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is rapid, high throughput and most studies have demonstrated specificities of >97% for CoNS speciation.

Biofilms

Biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.[4]

Matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces with a self-produced extra-cellular matrix" J.W. Costerton et al., Annu. Rev. Microbiol, 1995 Biofilms have been described in many systems since Leuwenhoek, who described the animalcules in the plaque of his own teeth in the seventeenth century.In 1976 Marshall described the polymer fibrils that anchored bacteria to surfaces.In 1978 Consterton et al described communities of adhered bacteria enclosed in a glycocalyx matrix, polysaccharide in nature.In 1990 Characklis and Marshall described the biofilm in terms of spatial and tensorial heterogenicity and involvement of inorganic and abiotic substances in the formation of biofilms.Consterton and Lappin-scott stated that adhesion triggered gene expression that controlled the production of bacterial components needed for adhesion and biofilm formation[52,5]

Over 99% of microbes live in Biofilms: structured, surface associated communities. According to the CDC, approximately 60 % of all infectious diseases involve biofilms. Biofilms of natural body flora are one of the bodies principle protective mechanisms against exogenous infections. An understanding of biofilms and their residents has enabled the creation of new industries, from PCR to biobarriers and from probiotics to infection resistant prosthetics[5,52,54].

Why do organisms form biofilm[77]?

1. To protect themselves from antibiotics, toxins, immune cells, and bacteriophages
2. To Increase the exchange of genetic information- by-Conjugative plasmid transfer and Transduction
3. To increase the availability of nutrients-Mutualistic Symbiosis (Synthrophie, Commensalism)
4. To built a favorable microenvironment which is highly hydrated and with low oxygen fascilitating their growth.
5. For stability so that they can detach and leave.
6. Community is built which also fascilitates Gene transfer, signal transduction, quorum sensing.

Pathogenic bacteria known to reside in biofilms , but are not limited , include- *Borrelia burgdorferi* (Lyme bacteria), *Escherichia coli, Candida albicans* (yeast and fungal mutation), *Clostridium difficile, Clostridium perfringens, Helicobacter pylori, Klebsiella pneumoniae, Legionella pneumophila, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Staphylococcus epidermidis, and Vibrio cholerae.*[68].

Biofilms in Medicine

Predisposing factors-[52,77]

1. Infections **2. Implants**

1. Infections

- Caries
- Periondontosis

- Otitis media
- Heart valve infection
- Bone marrow infection
- Prostate infection
- Gall bladder infection
- Cystic fibrosis

2. Medical devices/Implants

- Contact lenses
- Bladder catheter
- Centralvenous catheter
- Sutures
- Arteriovenous shunts
- Artificial heart valves
- Blood vessel grafts
- Joint implants

Prolonged administration of broad spectrum antibiotics, prolonged hospital stay, immunosuppression can also predispose to biofilm formation

Formation of Biofilms-Mechanism[4,52]

Biofilm like other communities form gradually over time. There are five stages in universal growth cycle of a biofim.

i. Initial attachment
ii. Irreversible attachment
iii. Maturation I

iv. Maturation II

v. Dispersion

Stage I : Initial attachment

It can take only seconds to activate and is likely induced by environmental signals. These signals vary by organisms but they include changes in nutrients and nutrient concentrations, pH, temperature, oxygen concentration, osmolality and ion. The initial binding in stage I is reversible as some cells detach. During this stage yeast cells exhibit a logarithmic growth rate.

Stage II : Irreversible attachment

It is characterized as irreversible binding and begins minutes after stage I. After adhering to the epithelial surface, the bacteria begin to multiply while emitting chemical signals that "intercommunicate" the yeast cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide (EPS) production are activated traps nutrients and planktonic bacteria. During stage II cell aggregates are formed and motility is decreased.

Stage III : Maturation I

This occurs when cell aggregation becomes progressively layered with a thickness greater than 10μm.

Stage IV: Maturation II

When biofilms reach their ultimate thickness generally greater than 100 mm,it is stage IV

Stage V: Dispersion

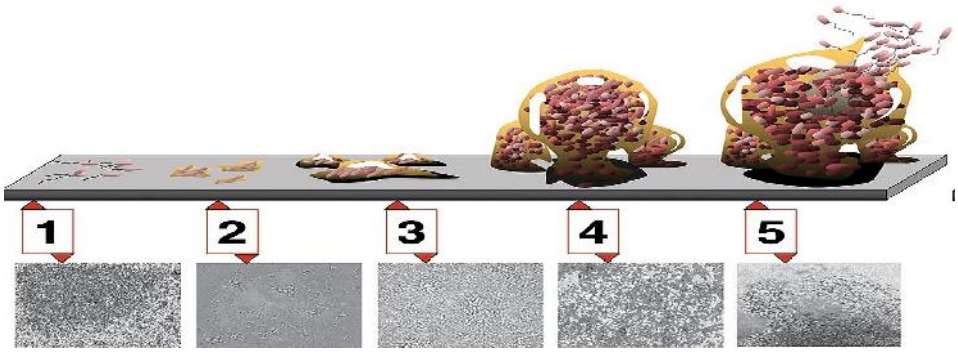


Photo 1 : Stages of biofilm development

PIA (Polysachharide Intracellular Adhesins)[42,43,77] production plays an important role to form biofilms. PIA synthesis is linked to biosynthetic enzyme-encoding genes located in the intercellular adhesion operon (ica ADBC)[23]. In addition *icaR* gene exerts a regulatory function and is transcribed in the opposite direction.PIA is synthesized from UDP-N-acetylglucosamine[48] by N-acetyl glucosaminyl transferase,which is encoded in *ica* locus particularly *icaA*. It has been reported that *aap* (accumulation-associated protein) and *bhp* [Bap (biofilm associated protein) homologue protein] are two factors that mediate the independence of staphylococcal biofilm formation from PIA[45,50,55,95].

Biofilm associated infections and their implication in health care.

The word BIOFILM made its first appearance in scientific literature in 1970s.According to a recent public announcement from the National Institutes of Health, more than 60% of all infections are caused by biofilms.

Christensen GD et al(1985)[72] have investigated visual assessment of bacterial adherence to culture tubes, microtitre plates, mouse and rat animal models for biofilm producing properties. They investigated sensitivity and specificity of different phenotypic methods for testing the biofilm producing properties of bacterial isolates

Stickler DJ et al (1996)[59], reported biofilm production and catheter blockage that caused cystitis and septicemia in patients with long term indwelling urinary catheter.

Panda PS[21] et al (2016) investigated bacterial biofilms on uropathogens.

Biofilms are highly resistant to antimicrobial treatment[22]. Antimicrobial resistance of biofilm producing bacteria including mechanisms like trapping of antibiotics in the exopolysaccharide matrix causing a diffusion barrier by restricting the rate of molecule transfer to the interior of the biofilm, escaping of bacteria within biofilms from host defence mechanisms[77], and alteration of metabolism and decrease in bacterial growth rate due to Quorum sensing and genotyping adaptations[46,51,56,57].

Various Methods for detection of biofilm[5,21,58,77]:

- Microtiter assay (biofilm development in static conditions)
- Tube method (TM)
- Congo red Agar method (CRA)
- Attenuated Total Reflecting Spectroscopy (ATR)
- Flow cells (biofilm development in flow conditions)
- Piezoelectric Sensors
- Confocal scanning laser microscopy (CSLM)
- Atomic force microscopy (AFM)
- Confocal Raman
- GFP/DsRed marked Pseudomonas putida
- Molecular characterization of biofilm bacteria using 16 S rDNA
- In situ hybridization with 16S rDNA probes
- Tagging of bacteria with gfp/DsRed marker genes

Detection of biofilms on implants removed from diseased areas of the body is now possible.

Microtiter assay a rapid method is a colorimetric assay in which a suspension of bacteria is prepared in a culture medium which is then placed in the wells of a microtiter plate or tubes. After incubation for 24 to 48 hours, the medium is removed from the wells and each well is rinsed several times to remove any bacteria not adhering to the sides. After rinsing, a stain, such as crystal violet or safranin, is added. After a specified time, the wells are rinsed several times, and then the wells are evaluated with a spectrophotometer for the presence of stain. The ability of the bacteria to form biofilms is determined by the degree of stain adhering to the bacteria in the wells or tubes after rinsing.This technique allows to study the effects, an antimicrobial agent might have on the formation or inhibition of the growth of a biofilm. In a similar method, bacteria are incubated in the presence of silicone disks, and the ability of the bacteria to adhere to the disks is determined by a colorimetric method. The main problem with assays for the detection of biofilms or the potential of bacteria to form biofilms is the lack of standardization, so analysis of data from these studies needs to include a critical assessment of the methods used.

Tube method is a qualitative assessment of biofilm formation where trypticase soy broth is used to grow microorganisms with 1% glucose in tubes for 24 hrs. The tubes are then decanted and washed with PBS (Phosphate Buffer Saline) and stained with crystal violet (0.1%). The tubes are washed and dried. Visible film lines on the wall and the bottom of the tube is considered positive for biofilm formation.

Congo Red Agar method is a method where microorganisms are grown on Brain Heart Infusion Agar with 5% sucrose and Congo Red. Black colonies with a dry crystalline consistency indicate a positive result.

Attenuated Total Reflecting Spectroscopy (ATR) has been used to monitor conditioning films that are an early harbinger of biofilm formation.

Piezoelectric Sensors such as quart with crystal microbalances monitor frequency shifts as mass accumulates on the sensor surface.

Techniques to evaluate biofilm formation by bacteria have become more available in the last decade. These techniques add to our knowledge about the organisms that form biofilms and allow us to study how to eliminate them or prevent them from initiating biofilm formation. Such methods are observations by microscopic techniques (e.g., epifluorescence, CLSM, transmission electron, scanning electron[30]) or enumeration of sessile bacteria after detachment from the surface by scraping, vortexing, sonication, or the use of beads. These methods allow us to obtain detailed information, but they are laborious and time consuming and thus do not lend themselves to large-scale screening assays.

This detection of biofilms is based on the use of combined methods, such as polymerase chain reaction assay and pathogen specific probes, along with special microscopic methods such as confocal laser scanning microscopic (CLSM) imaging. Although not done routinely in clinical microbiology laboratories, these techniques provide valuable information about the role of biofilms in the disease process and what interventions may be possible to eliminate or prevent biofilms.

Possible strategies to treat biofilm-associated infections[5,52,77].

- Use of substances that can destroy the biofilm matrix (e.g., dispersin B).
- Use of substances which destroy persister cells.
- Quorum-quenching enzymes.
- Substances which cause biofilm self-destruction.
- Strategies to boost antimicrobial agent action (e.g., electrical current).
- Removal of the device.

Antimicrobial susceptibility of Coagulase-negative *Staphylococci* (*CoNS)* infections

Treatment of *CoNS* infection is challenging given the frequency with which antimicrobial resistance has come up and frequent presence of foreign material[22].

In regard to resistance to antibiotics one can divide *CoNS* into 2 main groups

(i) "Those that haven't seen a hospital from inside" and are susceptible to the usually administered drugs

(ii) Those that have been exposed to antibiotic selection pressure in the healthcare environment. This holds true for animal adopted strains and their contact with veterinary medicine and abuse of antimicrobial agents in husbandry[5].

For clinical *CoNS* isolates, the sharpest and the most effective weapon, the bactericidal β-lactams have become progressively blunt. This deteriorating situation is further aggravated by phenomenon of multi drug resistance[16,17,39].

In previous decades loss of susceptibility towards most of the available antibiotics was recorded for *CoNS.*

Resistance to penicillins among *CoNS* approaches 90-95% due to β-lactamase production and resistance to methicillin can be as high as 60-80% for nosocomial isolates. These isolates are often resistant to multiple classes of antibiotics in addition

to β-lactams. Multi drug resistance includes resistance to aminoglycosides, phosphomycin. Fusidic acid, macrolides. Quinolones, tetracyclines, rifampin and trimethoprim-sulfamethoxazole. . In terms of glycopeptides *CoNS* are primarily resistant to teicoplanin. Plasmid mediated resistance to linezolid is due to acquisition of *cfr* gene. This gene *cfr* confers resistance to oxazolidinones, streptogramin A, lincosamides, phenicols and pleuromutilins too. An important exception is *Staphylococcus lugdunensis* which is unique as it is susceptible to wide range of antimicrobials. The genes responsible for resistance are found on plasmids and facilitate horizontal exchange of resistant genes among strains. The mecA gene encoding a low affinity penicillin binding protein (PBP2a) mediates methicillin or oxacillin resistance in *CoNS.* It is a part of mobile genetic element called SCCmec (Staphylococcal Cassette Chromosome). As this resistance is heterotypic and only a minority of bacterial population express resistant phenotype so the detection of resistance is very challenging. Highly penicillin-resistant *S. epidermidis* isolates, responsible for fatal subacute bacterial endocarditis,were being reported as early as 1949. This phenotype is caused by penicillinases, first described by Kirby in 1944.They represent a plasmid mediated staphylococcal β-lactamase encoded by the *blaZ* gene[5,6,10].

In comparing *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* publications of 1980s have reported high percentages of methicillin resistant isolates of both species. *S. haemolyticus* strains show highest resistance followed by *S. epidermidis,* and *S. hominis* strains. Indian studies have demonstrated a resistance of 20 - 66% and increasing trends of methicillin resistance coagulase negative *Staphylococci.* (*MRCoNS)* in neonatal septicaemia[10,36]. These were observed in South

India over a period of 3 years with prevalence of *MRCoNS* rising from 41.57% to 57.36%[10].

Majority of the clinically recovered *CoNS* strains are methicillin resistant vancomycin or other newer agents such as linezolid, daptomycin, telavancin, tigecycline and cephalosporins (ceftobirole and ceftaroline) are suitable options for empirical therapy of *MRCoNS* infections. Replacement of these by β-lactamase resistant penicillins is advisable for Methicillin susceptible isolates. When used simultaneously antibiotics with cell wall activities (β-lactams and vancomycin) combined with rifampin were shown to act synergistically. However, this combination is not recommended for catheter related blood stream infections. Foreign body blood stream infections caused by *CoNS* are a therapeutic challenge and frequently removal of device is required[5,10,12].

The antimicrobial susceptibility testing of *Staphylococci* may be performed conventionally by Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference methods.

Cefoxitin disk (30µ) is the disk diffusion method recommended by CLSI for testing *CoNS* infections for *mecA* mediated resistance (≥24mm).

For broth microdilution testing done for *CoNS* isolates, presence of *mecA* is predicted by applying lower oxacillin MIC breakpoints (resitance to oxacillin, MIC≥0.5µg/ml). Another alternative method to detect methicillin resistance is the use of antiPBP2a monoclonal antibodies available in a Latex Agglutination Asaay that can be performed on isolated colonies from a pure culture. The latex test if used for small cell variants (SCV) then the number of colonies must be increased to a hundred fold[5,10].

For definite verification of methicillin resistant *CoNS* isolate *mecA* gene is detected by molecular methods. To detect *mecC* and other *mecA* alleles specific oligonucleotide primers have been described and a microarray based approach has been published[5,10].

An algorithm to reduce misclassification of nosocomial bloodstream infections due to *CoNS* was defined as at least two blood cultures positive for *CoNS* within 5 days or one positive blood culture plus clinical evidence of infection.[10]

Contaminants and colonizing *CoNS* do not need susceptibility tests. All *CoNS* associated with true infections require susceptibility testing[10].

MATERIALS AND METHODS

The study was carried for a period of one year at Department of Microbiology, Dr Prabhakar Kore hospital and MRC, KLE University, Belagavi.

Source of data:

All *CoNS* isolated from different clinical samples received in the Department of Microbiology J.N.M.C, Belagavi, from January 2016 to December 2016

SAMPLE SIZE OF *CoNS*

Detection rate of *Staphylococcus epidermidis*=70%

P=70

$n = 4pq/d^2$

$q = 100 – p\%$

$n = 4pq/d^2$

$= 4 \times 70 \times 30/10^2$ (d = 10%)

= 84

Inclusion Criteria

All *CoNS* isolates from various clinical samples of hospitalized patients

Exclusion Criteria

All *CoNS* in which there is evidence of contamination.

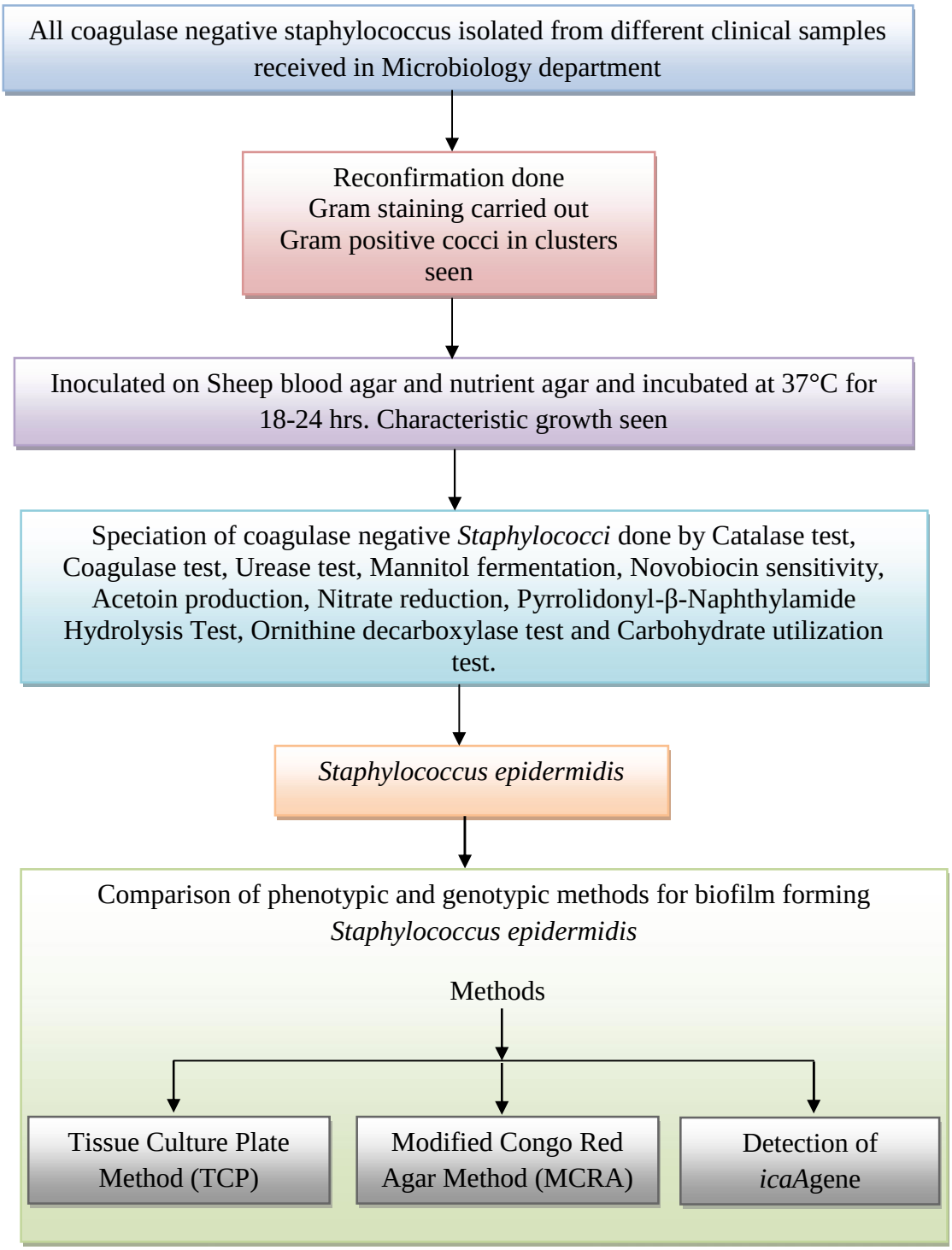
All *CoNS* from healthy individuals

A total of 86 consecutive non repeated clinically significant pure *CoNS* isolates were collected from various clinical samples like pus, sputum, urine, blood, fluid, ear swab, and throat swab.The isolates were identified as *CoNS* by colony morphology, Gram stain, catalase test and coagulase test (slide and tube coagulase). The simple, inexpensive test were selected from scheme of Kloos and Schleifer for speciation of *CoNS*. Bacitracin susceptibility was performed to exclude *Micrococci* and *Stomatococcus* species. Speciation of *CoNS* was done by Novobiocin resistance test, Urease activity, Ornithine decarboxylase and aerobic acid production from mannose. The Isolates were considered clinically significant when isolated in pure culture from infected site or body fluid. Identification of *S. epidermidis* was carried out using the standard biochemical tests including catalase, and coagulase production, growth and fermentation of mannitol on mannitol salt agar and susceptibility to novobiocin (≥ 16 mm) by the disc diffusion test. *S. epidermidis* were stored at -20 as nutrient agar stab cultures until further testing.The biofilm producing ability of *S. epidermidis* was detected by phenotypic methods(TCP method and MCRA method) and a genotypic method(*icaA* detection by PCR).

Criteria for pathogenic coagulase negative staphylococci(*CoNS*)

- *CoNS* seen in a Gram stained smear along with pus cells were included.
- *CoNS* strain repeatedly isolated from the same lesion were included.
- *CoNS* found relevant by critical appraisal of clinical picture were included.
- *CoNS* isolated with other bacteria (mixed culture)were excluded.
- *CoNS* isolates with no clinical correlation of symptoms were excluded.

The plan of investigation was as follows.



Identification and isolation of all coagulase negative staphylococci (*CoNS*) isolates by

1. Gram staining
2. Colony morphology
3. Catalase test
4. Coagulase test
5. Novobiocin sensitivity
6. Biochemical tests(Urease test, Oxidative and fermentative test, Furozolidine susceptibility test, Bacitracin susceptibility test, Novobiocin susceptibility test, Carbohydrate utilization tests)

Gram stain[14]:

Procedure: Huckers modification

1. A smear is prepared by emulsifying isolated colony in saline using a sterile loop and heat fixed on a microscope slide before staining.
2. Smear is covered with crystal violet, the primary stain,for 1 minute.
3. Then gently rinse off the stain with water.
4. The smear is covered with Gram's iodine,the mordant,for 1 minute.
5. The excess Gram's iodine is poured off
6. Then alcohol decolorizer is run off over the smear until the solution appears clear.
7. Slide is gently rinsed with water.
8. Finally the smear is covered with 1% safranin, the secondary or counterstain, for 1 minute and gently rinsed with water.

The blot dried slide is observed under oil immersion objective of the microscope for morphology of organisms.

Interpretation: *CoNS* are identified as gram positive cocci in clusters, pairs and singles.

Positive control: *Staphylococcus aureus* ATCC 25923

Negative control: *Escherichia coli* ATCC 25922

Colony morphology following culture[14]:

Samples were cultured on nutrient agar plates and blood agar plates and incubated for 24 hours at 37c The typical colony appearance of most *CoNS* species is nonpigmented, smooth, entire, glistening, and opaque. Rare strong biofilm producers display mucoid colony morphology. Colony diameters reach 3 to 6 mm after 3 days of incubation. Colonies of *S. lugdunensis, S. sciuri,, S. warneri,* and *S. xylosus* are found to be more or less regularly gray to yellow, yellow, or yellow to orange. Other *CoNS* species may show pigmentation that is usually yellowish.Colonies of *S, epidermidis* were white coloured. Colonies of *S. haemolyticus* showed beta- haemolysis on blood agar plates.

Catalase test[6]

This test differentiates catalase-positive micrococcal and staphylococcal species from catalase-negative streptococcal species.

Principle:

Aerobic and facultative anaerobic organisms produce two toxins during normal metabolism, hydrogen peroxide (H_2O_2) and superoxide radical (O_2-). These bacteria have two enzymes that detoxify the products of normal metabolism. One of these enzymes, catalase,is capable to convert hydrogen peroxide to water and oxygen.

The presence of the enzyme in a bacterial isolate is evidenced when a small inoculum introduced into hydrogen peroxide (3% for the slide test) causes rapid elaboration of oxygen bubbles. The lack of catalase is evident by a lack of or weak bubble production.

Procedure:

1. A platinum loop or sterile wooden stick is used to transfer a small amount of colony growth to the surface of a clean, dry glass slide
2. A drop of 3% hydrogen peroxide (H_2O_2)is added.
3. Evolution of oxygen bubbles is observed.

   Prescence of copious bubbles indicates a positive report. False positives may occur if the sample is contaminated with blood agar.

   Positive control: *Staphylococcus aureus*

   Negative control: *Streptococcus pyogenes*

Coagulase test[15]:

The test is used to differentiate *Staphylococcus aureus* (positive) from coagulase-negative *Staphylococci* (negative).

Principle

S. aureus produces two forms of coagulase, bound and free. Bound coagulase, or “clumping factor,” is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in precipitation of fibrinogen on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma. The presence of free coagulase,an extracellular protein enzyme forms clot when S. aureus colonies are incubated with plasma.The clotting mechanism involves activation of a

plasma coagulase-reacting factor (CRF) which is a modified or derived thrombin molecule, to forms a coagulase-CRF complex. This complex in turn reacts with fibrinogen to produce the fibrin clot.

Slide Test (Detects Bound Coagulase)

Procedure

1. A drop of coagulase plasma (preferably rabbit plasma with ethylenediaminetetraacetic acid [EDTA])is put on a clean, dry, glass slide
2. A drop of distilled water or saline is put next to the drop of plasma as a control.
3. With a platinum loop, straight wire, or wooden stick, a portion of the isolated colony being tested is emulsified in each drop, inoculating saline first . A smooth suspension is made and mixed properly.
4. Slide is gently rocked for 5 to 10 seconds.

Results:

Positive: Macroscopic clumping in 10 seconds or less in coagulated plasma drop and no clumping in saline.

Negative: No clumping in either drop. Note: All negative slide tests must be confirmed using the tube test

Clumping in both drops indicates that the organism autoagglutinates and is unsuitable for the slide coagulase test.

Tube Test (Detects Free Coagulase)

Procedure

1. Several colonies are emulsified in 0.5 mL of rabbit plasma (with EDTA) to give a milky suspension.
2. The tube is then incubated at 35°-37°C in ambient air for 4 hours.
3. Clot formation is looked for.

Results:

Positive: Clot of any size is tube coagulase positive test.

Test results can be positive at 4 hours and then revert to negative after 24 hours. If negative at 4 hours, incubate at room temperature overnight and check again for clot formation.

Positive control: *Staphylococcus aureus* (ATCC25923)

Negative control:*Staphylococcus epidermidis*(ATCC12228)

Modified oxidase test[6,14]

It determines the presence of enzyme oxidase, which catalyzes the transport of electrons between electron donors in the bacilli and the redox dye.The dye oxidizes to indophenol blue and deep purple colour is produced .It is used to differentiate Micrococcus and related organisms from most other aerobic Gram-positive cocci. Six percent TMPD (N,N,N tetramethyl-p-phenylenediamine dihydrochloride) dissolved in dimethyl sulfoxide is used as the reagent. The reagent should be kept away from light. A loopful of colonies from blood agar plates is smeared onto filter paper, and the reagent is dropped onto the bacterial growth. Development of a blue to purple-

blue color in 2 min indicates a positive reaction. *Staphylococci* give a negative color change, except for *S. sciuri, S. lentus,* and *S. vitulus.*

Positive control: *Pseudomonas aeruginosa* ATCC 27853

Negative control: *Escherichia coli* ATCC 25922

Furazolidone Disk Test[6]

Identification of staphylococci from micrococci can be done on the basis of susceptibility to furazolidone. *Staphylococci* are susceptible to this compound, whereas *Micrococci* are resistant. A suspension of the organism to be tested is prepared in sterile distilled water or broth. The suspension should be equivalent to a 0.5 McFarland turbidity standard. With a swab, organism suspension is spread onto one-half of a blood agar plate. Aseptically an FX(Furazolidone-100 mg) disk is placed in the center of the inoculated area, and gently tap the disk so that it adheres to the agar surface. The plate is then incubated at 35°C in an ambient-air incubator for 18–24 hours.

Micrococcus species are FX-resistant and will have zones of 6 mm (no zone)–9 mm. *Staphylococcus* species are FX-susceptible and will have zones of inhibition of 15 mm or larger.

Novobiocin Disk Test[15]

Coagulase-negative *Staphylococci* can be divided into novobiocin-susceptible and novobiocin-resistant species. Among the novobiocin-resistant species, *S. saprophyticus* is the one usually recovered from humans as it causes urinary tract infections. So screening coagulase-negative *Staphylococci* isolated from quantitative urine cultures for susceptibility to novobiocin provides a reliable presumptive

identification of this species. A suspension of the organism to be identified is prepared in sterile distilled water or broth. The suspension is equivalent in turbidity to a 0.5 McFarland standard. Some of the suspension is spread with the sterile swab over half of a blood agar plate. Aseptically novobiocin disk is placed on the inoculated area. Susceptibility to furazolidone is assessed on the same plate by placing the disks about 4 cm apart on the inoculated area. Disk(s) are tapped gently with sterile forceps to assure contact with the agar surface. The plate is aerobically incubated for 18–24 hours at 35°C. *S. saprophyticus* are novobiocin-resistant and show zones of inhibition of 6 mm (no zone)–12 mm. Other coagulase-negative *Staphylococci* and *S. aureus* are novobiocin-susceptible and will show zones of 16 mm or larger

Urease Test (Christensen's Method)[6,10]

This test is used to determine an organism's ability to produce the enzyme urease, which hydrolyzes urea. Ammonia is produced, which causes a rise in pH that is detected by a change in color of the indicator phenol red to pink under alkaline conditions (pH 8.4). Bacteria are cultured on a medium containing urea, e.g., Christensen urea agar.

Positive control: *Proteus* species

Negative control: *Escherichia coli*

Methyl Red Test[6]

Methyl red is a pH indicator, with a range between 6.0 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, color change is produced when the test organism produces large quantities of acid from the carbohydrate substrate

being used. Bacteria are incubated in a broth medium containing glucose. The development of a stable red color in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. organisms may produce smaller quantities of acid from the test substrate, an intermediate orange color between yellow and red may develop. This does not indicate a positive test.

Positive control:*Escherichia coli*.

Negative control:*Enterobacter aerogenes*.

Voges-Proskauer (VP) test (α-naphthol/KOH)[19]

The VP test is used to detect acetoin (acetyl-methylcarbinol), which is produced by certain bacteria during growth in a buffered peptone-glucose broth(methylred-VP broth). The test uses 5% α-naphthol, which is prepared by dissolving 5 g of α-naphthol in 100 ml of absolute ethanol, and 40% KOH, which is prepared by dissolving 40 g of potassium hydroxide in 100 ml of distilled water. The test is performed using methyl red-VP broth which is inoculated and incubated (24 hours) until good growth is obtained. Then 0.6 ml of the α-naphthol solution and 0.2 ml of the 40% KOH are added to 2.5 ml of culture broth. A positive reaction is indicated by the formation of a pink-red product within 5 min. However, 15 min for color development is allowed before considering the test negative.

Positive control: *Enterobacter aerogenes*

Negative control: *Escherichia coli*

Nitrate reduction test (N,N-dimethylnaphthylamine and sulfanilic acid)[19]

The nitrate reduction test is used to determine the ability to reduce nitrate to nitrite or free nitrogen gas. This test involves the use of two reagents. Reagent A is prepared by adding 0.8 g of sulfanilic acid to 100 ml of 5 N acetic acid. Reagent B is prepared by adding 0.6 ml of N,N-dimethyl-naphthylamine to 100 ml of 5 N (30%) acetic acid. These reagents are stored in the dark under refrigeration. The test is performed by adding 0.05 ml of reagent A to 10 drops of an overnight growth from the nitrate broth culture and then incubated for 5 to 10 min. Then 0.05 ml of reagent B is added and incubated for an additional 5 to 10 min. Incubation should be in the dark. (Note: reagents A and B may be mixed and added together but this lowers the sensitivity of the test.)

An organism is reported as nitrate positive if a red or purple-magenta color develops in the medium within a few minutes after nitrate reagents A and B are added to the medium, indicating that the organism has reduced nitrate to nitrite. The absence of a red-purple color after the addition of both reagents does not automatically mean that the organism is unable to reduce nitrate. Strains reduce the nitrate to nitrite and then reduce the nitrite completely to nitrogenous gases that are not detected when nitrate reagents A and B are added to the medium. If the medium doesnot change color after the addition of sulfanilic acid and α-naphthylamine, a small amount ("knife point") of zinc dust is added to the incubated medium. The zinc dust will catalyze the reduction of nitrate to nitrite chemically. Thus, if the nitrate has not been reduced by the organisms, i.e., they are nitrate negative, it will be reduced by the zinc dust and a red color will develop in the incubated medium within 15min.If no color develops in the incubated medium after the addition of zinc dust, the organisms not only have

reduced nitrate to nitrite but have reduced nitrite to nitrogenous gases; these organisms are also nitrate positive.

Positive control: *Escherichia coli*

Negative control: *Acinetobacter baumannii*

Bacitracin Susceptibility Test[6]

Susceptibility to low concentrations of the polypeptide antibiotic bacitracin provides an easy and inexpensive method for the presumptive identification of staphylococcus species. Bacitracin differential disks (0.04 units/disk) are used. three to four isolated colonies are picked and the inoculum is streaked down the center of half of a blood agar plate. The inoculum is spread as a lawn over the entire half of the plate. Bacitracin disk is placed evenly on the inoculated area. Using flamed forceps, the disks are gently tapped down so that they adhere to the agar surface. The plate is incubated in ambient air at 35°C for 18 to 24 hours.*Staphylococcus* species are bacitracin resistant(10mm/no zone) while *Micrococcus* are bacitracin susceptible(10 to 20 mm zone size).

PYR (Pyrrolidonyl Aminopeptidase) Test[6]

It is used for the detection of pyrolidonyl arylamidase (also called pyrrolidonyl aminopeptidase) The enzyme *L*-pyrrolidonyl arylamidase hydrolyzes the L-pyrrolidonyl- β-naphthylamide substrate to produce a β-naphthylamine. The β-naphthylamine can be detected in the presence of N,N-methylaminocinnamaldehyde reagent by the production of a bright red precipitate. Following hydrolysis of the substrate by the peptidase, the resulting b-naphthylamide produces a red color upon the addition of 0.01% cinnamaldehyde reagent. When a visible inoculum of

microorganism is rubbed onto a small area of a disk impregnated with the substrate, the hydrolysis occurs within 2 min, at which time the cinnamaldehyde reagent is added to detect the reaction by a color change to purple.It differentiates *Staphylococcus haemolyticus* (positive) from *S. auricularis*(negative).

Positive Control: *Enterococcus faecalis*

Negative Control: *Streptococcus agalactiae*

Ormithine Decarboxylase Test[6,19]

Decarboxylases are a group of substrate specific enzymes that are capable of reacting with carboxyl (COOH) portion of amino acids, to form alkaline reacting amines. This reaction called as decarboxylation, forms CO_2 as a secondary product. Ornithine decarboxylase enzyme is specific for amino acid ornithine. Ornithine undergo decarboxylation to form putrescine. Moeller Decarboxylase medium is the base used to determine the decarboxylase capabilities. Ornithine is added to this base before the test organism is inoculated. A control tube consisting of only the base without ornithine is set up in parallel. Both tubes are anaerobically incubated at 35°C for 18-24 hrs by overlaying with mineral oil. During initial stages of incubation, both tubes turn yellow due to the fermentation of small amount of glucose in the medium.

If ornithine is decarboxylated, alkaline amine putrescine is formed and medium reverts to its original purple colour.

Positive control: *Enterobacter cloacae*

Negative control: *Klebsiella pneumoniae*

Phosphatase test[6]

The test determines the ability of the organism to produce free phosphates formed on phenolphthalein phosphate agar (PPA). Free phosphates are produced by

some CONS with addition of ammonia vapour and is indicated by the appearance of pink colour of the colony. Growth of the isolate from the pure culture plate is streaked on PPA plate and incubated aerobically at °C for 18-24 hrs. 0.11 ml of ammonia solution placed in the lid of petridish and is covered with the inoculated PPA plate. Pink colour colonies within few minutes indicated the production of phosphate. It is used to differentiate Staphylococcus epidermidis/ Staphylococcus aureus(positive) from Staphylococcus saprophyticus/ staphylococcus hominis (Negative).

Positive control: *Staphylococcus aureus*

Negative control: *Staphylococcus saprophyticus*

Oxidation-Fermentation (OF) test (modified Hugh and Leifson test)[6,10]

This test determines whether an organism uses carbohydrate substrate to produce acid bye products either oxidatively or fermentatively. OF medium contains 0.3% agar, increased sugar concentration that is 1% and decreased peptone concentrations.The organism is inoculated in OF medium in duplicate and one of the tubes is covered with 1cm layer of liquid paraffin to create anaerobic environment. Acid production changes the color of the medium from green to yellow. If the sugar is fermentatively utilized, acid is produced in both tubes, where as non fermenters utilize sugar only oxidatively only in the tube without liquid paraffin overlay.*Staphylococcus* shows fermentative pattern whereas *Micrococci* shows oxidative pattern.

Carbohydrate utilization test[6,15]

These are used to determine the ability of an organism to ferment a specific carbohydrate that is incorporated in a basal medium thereby producing acid with or without visible gas. The test was performed on conventional culture media with test

sugar. Sugars tested were maltose, mannitol ,mannose, trehalose, xylose, sucrose and lactose, all with concentration of 1%. Each tube with culture media inoculated with 1 drop of an 18 to 24 hour broth culture incubated at 37°C for 1 to 2 days and then examined for acid production indicated by colour change. Pink colour indicates acid production.

Detection of biofilm

All 25 isolates of *Staphylococcus epidermidis* were tested for biofilm formation by using phenotypic and genotypic methods:

- Tissue Culture Plate Method (TCP) (Phenotypic method).
- Modified Congo Red Agar Method (Phenotypic method).
- Detection of *icaA* gene (Genotypic method).
- **Tissue Culture Plate Method(TCP)[72,85]**

The capacity to form biofilms was assayed in 96 well flat bottomed microtiter polystyrene plates.10ml of Tripticase soy broth with 1% glucose was inoculated with loopful of test organisms from overnight culture on nutrient agar. The broth was incubated at 37°C for 24 hours..Further the culture was diluted 1:100 with fresh medium.96 wells flat bottom tissue culture plates were filled with 0.2ml of dilute cultures individually and only broth served as a control to check sterility and nonspecific binding of the medium. All 3 controls and blanks were put in tissue culture plates. After incubation for 24 h at 37°C, gentle taping of plates was done. Wells were washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free floating “planktonic” bacteria The plates were then vigorously shaken in

order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 2%sodium acetate and stained with 0.1% crystal violet for 20 minutes. The excess crystal violet dye was washed by rinsing the wells with deionized water, the amount of biofilm formed was estimated by solubilization of the dye with 95% ethanol, and the optical density (OD) was determined at 570 nm using a microplate reader. Experiment was performed in triplicate. Average of OD values was calculated and substracted from all test values. (TABLE 7 and 8)

Modified Congo Red Agar Method(MCRA)[21,97]

Congo red agar method (CRA): A simple qualitative method for detection of biofilm production was described by Freeman et al[73]. In the MCRA the CRA was modified by changing the concentration of Congo red dye and sucrose, omission of glucose, replacement of BHI Agar by an alternative agar, that is, Blood Base Agar.

The composition of modified Congo red agar used in this study was—
Composition/litre

- Congo red dye 0.4g
- Glucose 10g
- Blood agar base 40g
- Water 1000ml

Modified Congo red agar was prepared as concentrated aqueous solution and autoclaved at 121 for 15 minutes separately from other medium constituents.It was then added when the agar had cooled to 55°C. Then plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.The experiment was performed in triplicate. Positive results were indicated by dark black colonies. Crusty black

colonies with dry filamentous appearance were recorded as biofilm producers and smooth pink colonies as nonbiofilm producers.

Positive control : *Staphylococcus epidermidis* ATCC 35984.

Negative control :*Staphylococcus epidermidis* ATCC 12228

PCR for detection of icaA gene in *S.epidermidis* isolates.

Preparation of samples for PCR amplification

MATERIALS:

SI No	Reagents	Brand
DNA Extraction		
1	Tris Base	Fisher Scientific (Qualigens)
2	EDTA	Fisher Scientific (Qualigens)
3	Triton X	Fisher Scientific (Qualigens)
PCR Amplification		
4	Red Taq Master Mix	Ampliqon
5	Oligonucleotide Primers	Bioserve India pvt Ltd
Agarose Electrophoresis		
6	Agarose Mol. Biology	Invitrogen
7	Glacial acetic acid	Fisher Scientific (Qualigens)
8	Ethidium Bromide	Bangalore Genei
10	100 Base Ladder	Invitrogen

METHODOLOGY:

DNA extraction

1. Vortex the sample to dislodge into the TE buffer medium and Centrifuge at 5,000 rpm for 5 min.
2. Discard the supernatant. Add 500µLfresh T.E. buffer centrifuge for 3-4 minutes
3. Repeat above procedure for 2-3 times with fresh T.E. buffer.
4. Discard supernatant add 50 µl lysis buffer I, Vortex it and keep for 5 min,
5. Add 50 µl Lysis buffer II and 10 µl Proteinase-K (100ug/ml), vortex vigorously. Keep it in water bath for 2 hrs then keep in boiling water bath for 10 minutes to deactivate the enzyme.
6. Store the DNA at -20^{0}C.

Virulence marker detection (*icaA* gene)

DNA was extracted from the bacterial growth using the same procedure mentioned above as per above standard method. The extracted DNA from bacterial growth was subjected to PCR for the presence of *cagA*.

Primers

***icaA*: Forward: 5′-TCT CTT GCA GGA GCA ATC AA**

***icaA*: Reverse: 5′--TCA GGC ACT AAC ATC CAG CA**

Reference article-

Petrelli, D., Zampaloni, C., D'ercole, S., Prenna, M., Ballarini, P., Ripa, S., & Vitali, L. A. (2006). Analysis of different genetic traits and their association with biofilm formation in Staphylococcus epidermidis isolates from central venous catheter infections. *European Journal of Clinical Microbiology and Infectious Diseases*, *25*(12), 773-781.

Product size-188bp

PCR Amplification

PCR Reaction Mix (25µL volume)

Reagents	**Amount added IX**
Master Mix	12.5 µL
Forward Primer (10 picomol)	0.5 µL
Reverse primer(10 picomol)	0.5 µL
Nuclear free water to a final volume upto	9.5 µL
Template DNA	2µL

Procedure:

1. Combine the components as listed in Table2, except DNA to prepare a master mix in a sterile micro centrifuge.
2. Add the master mix in the PCR tubes and Add1.5µl DNA mix it well and centrifuge for 5 sec.

3. Place the reaction mixture in a thermal cycler. Start the thermal cycling program.
4. Cycling program is given in the table 3. Each primer/ target combination requires optimization.
5. Store the PCR product at -20^{0}C until needed**.**

PCR Programming

Steps	Temperature	Time	Number of cycles
Initial Denaturation	95 °C	3 minutes	1 cycle
Final Denaturation	95 °C	30 seconds	50 cycles
Annealing	58 °C	30 seconds	
Extension	72 °C	30 seconds	
Final Extension	72 °C	1 minutes	1 cycle
Soak	4 °C	∞	

Agarose Electrophoresis

Preparation and Running of Agarose Gel

1. Agarose Powder mixed with 1X electrophoresis buffer is heated in a microwave oven until completely melted. Cool the solution to about 50^{0}C and add 1µL the ethidium Bromide (0.5 µg/ml) pour in to casting tray and allow solidifying at room temperature.
2. After the Gel solidifies, remove the comb carefully, mount the gel in the electrophoresis tank, and cover with electrophoresis buffer.
3. Load the 10µL-amplified product into wells and load 100bp ladder as marker.

4. Apply the current with fixed voltage (100-150V)
5. The distance DNA has migrated be judged by migration of tracking dyes. Bromophenol blue migrates through agarose gels approximately 2.2 fold faster than xylene cyanol independent of agarose concentration.
6. After electrophoresis DNA can be visualized by placing the gel in Gel Documentation system. The gel was visualized using a UV trans illuminator and take the photo for further analysis.
7. The amplified PCR products and 100 base pair DNA molecular markers were seen as bright fluorescent bands.
8. Linear DNA migrates through agarose gel with a mobility that is inversely proportional to the log10 of their molecular weight. Different concentration of agarose can resolve different sizes of DNA fragments.

All the PCR reactions were performed using Veriti (ABI) thermocycler. The amplified products were electrophoresed in 1.5% agarose along with standard molecular weight markers, stained with 0.5 µg/ml ethidium bromide solution, visualized by placing the gel in gel documentation system (Syngene).

RECIPES

TE Buffer

1M Tris Buffer : 0.5ml

0.5M EDTA : 100µl

Distilled water : made to 50ml.

50X TAE (pH 8.0)

1XTAE Buffer has the following composition:

1.Acetic acid : 20mM 2. EDTA : 1m M

PHOTOGRAPHS

Photo 2 : Gram stain showing Gram positive cocci in clusters

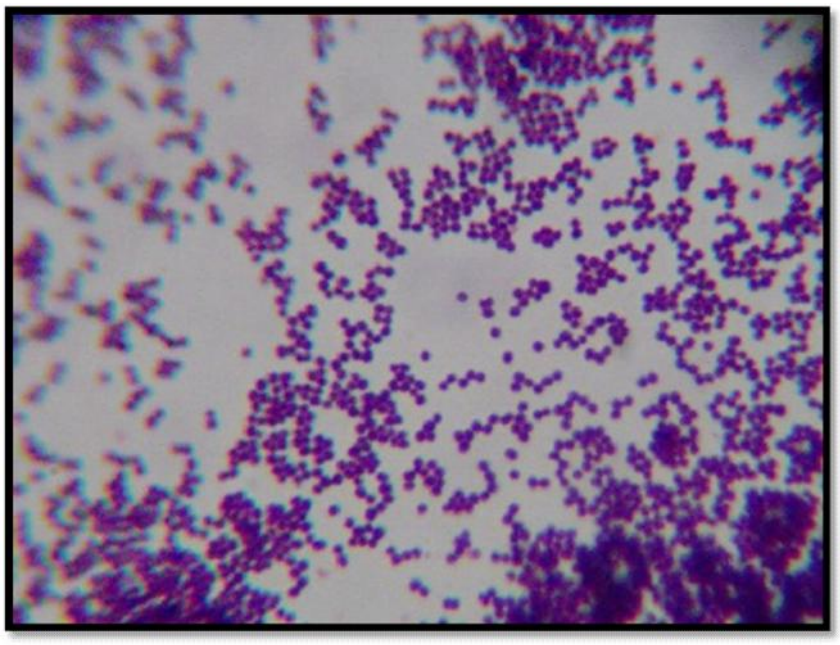


Photo 3 : Sheep Blood agar showing colonies of *Staphylococcus epidermids*

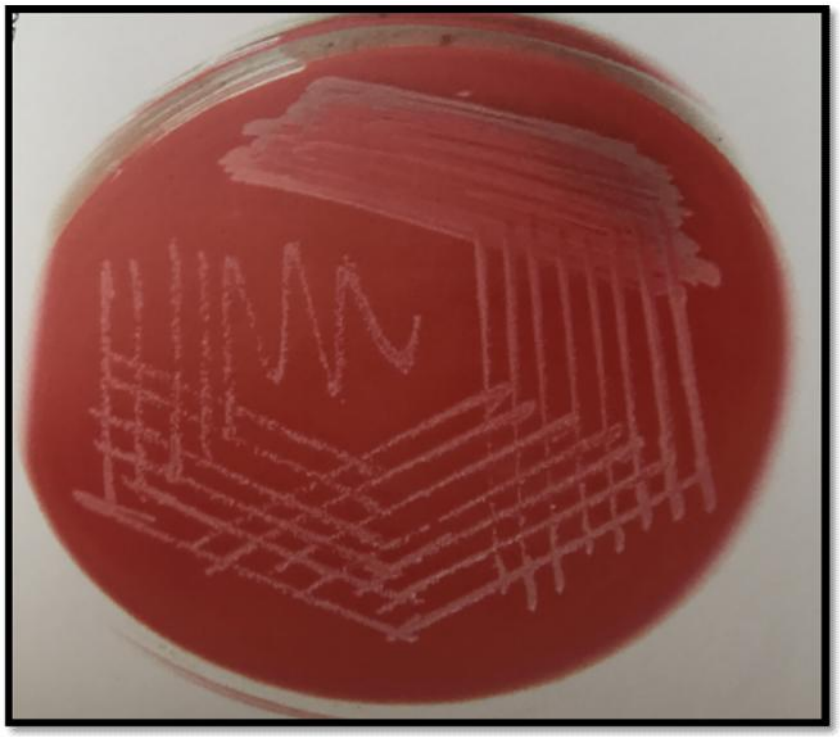


Photo 4 : Sheep Blood agar showing beta haemolysis by *Staphylococcus haemolyticus*

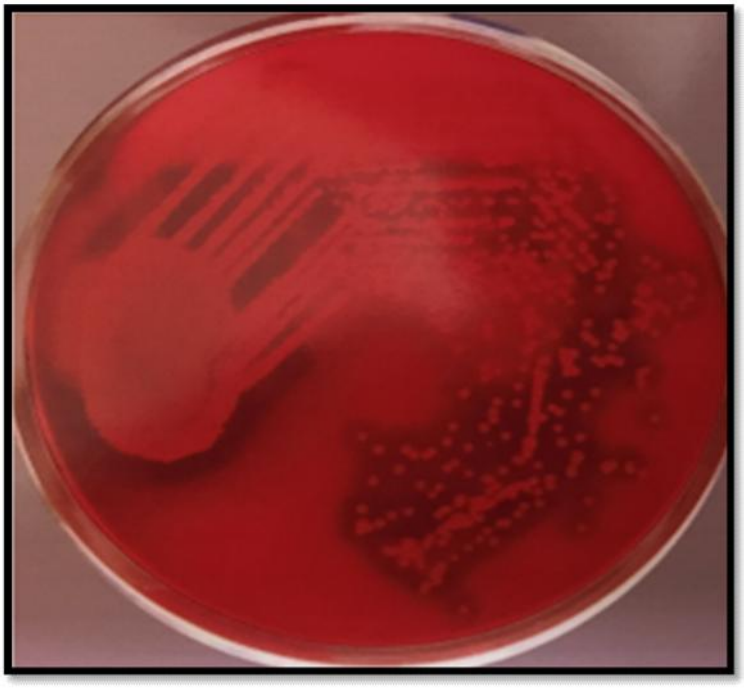


Photo 5 : *Staphylococcus epidermidis* on Sheep Blood Agar showing resistance to bacitracin

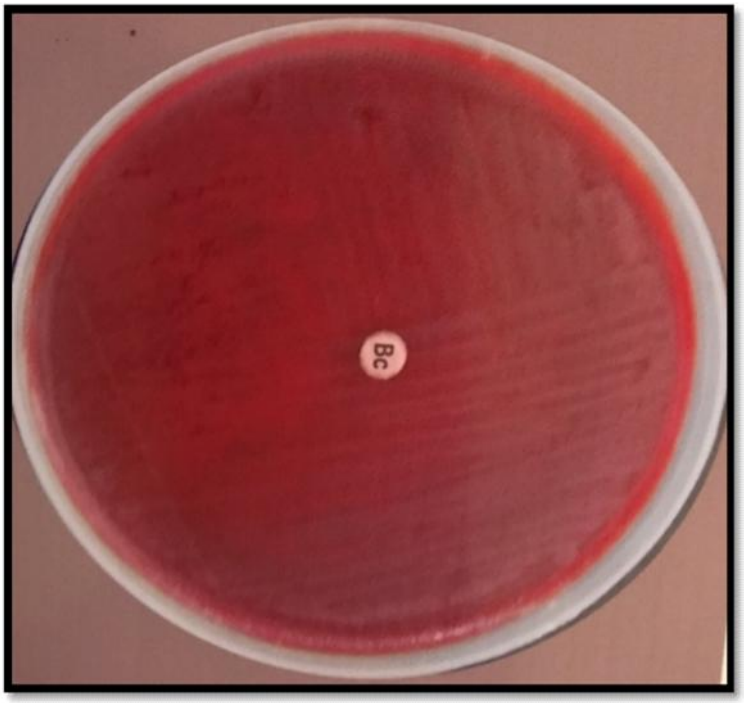


Photo 6 : Biochemical reactions of *Staphylococcus epidermidis*

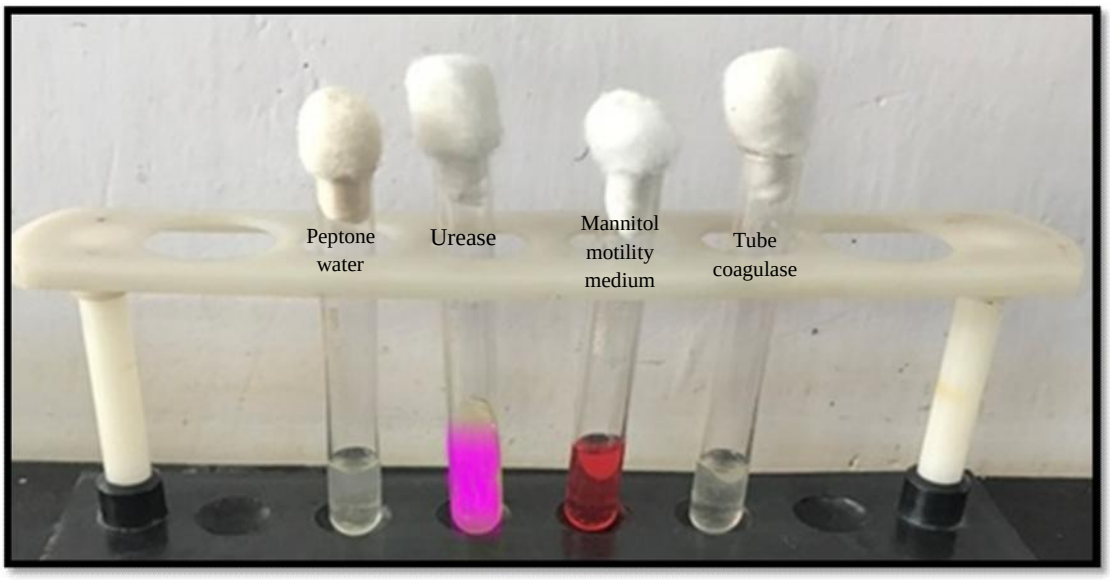


Photo 7 : *Staphylococcus epidermidis* on Nutrient agar showing sensitivity to Novobiocin

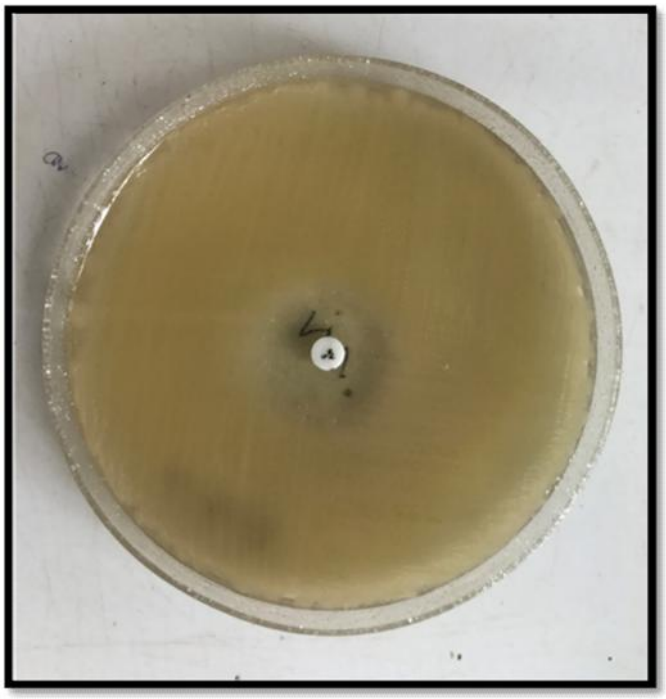


Photo 8 : Biofilm detection by Tissue Culture Plate Method

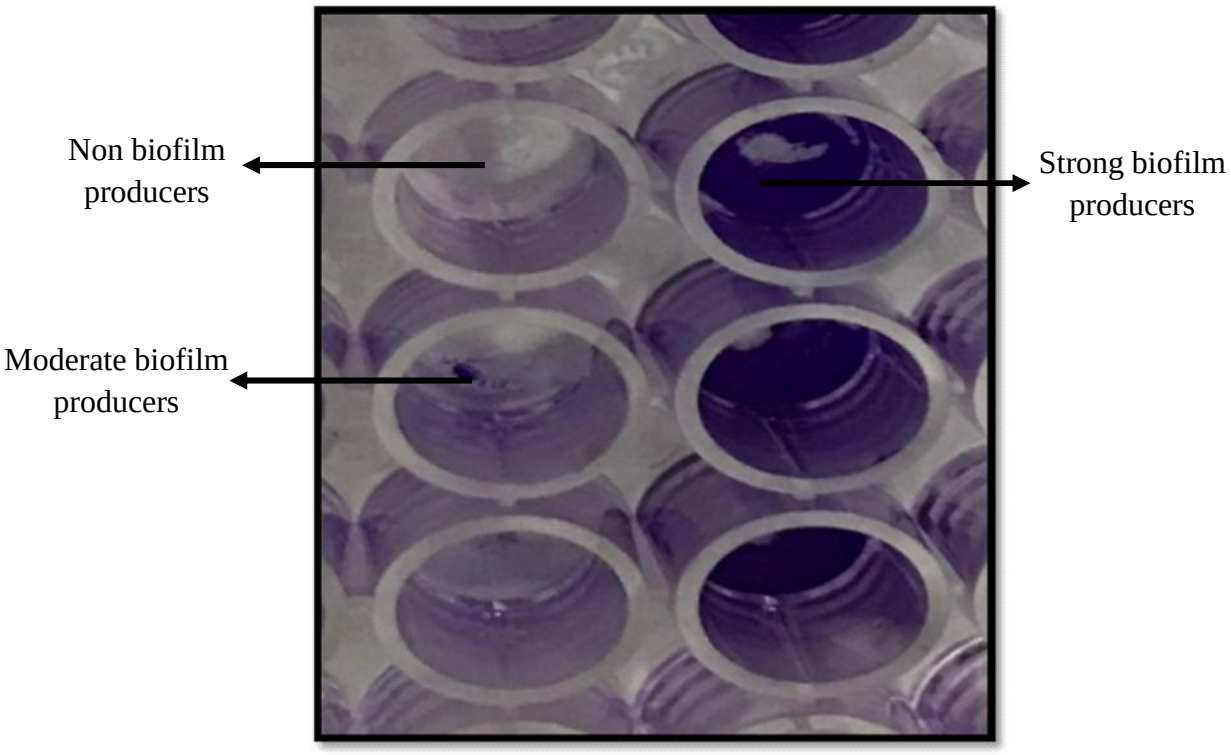


Photo 9 : Biofilm detection by modified Congo Red Agar Method

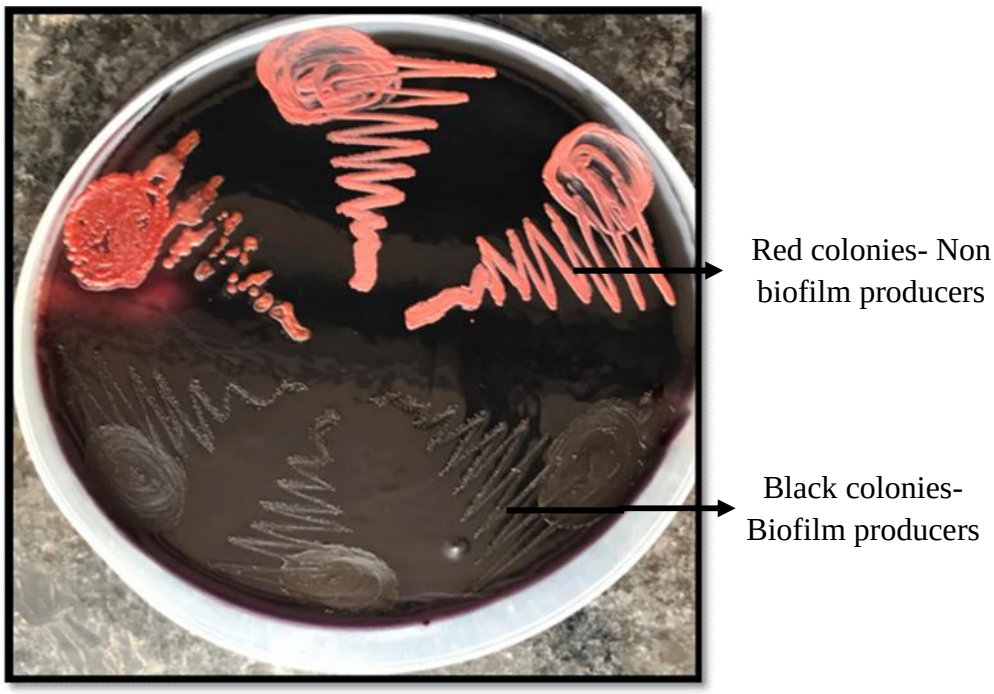
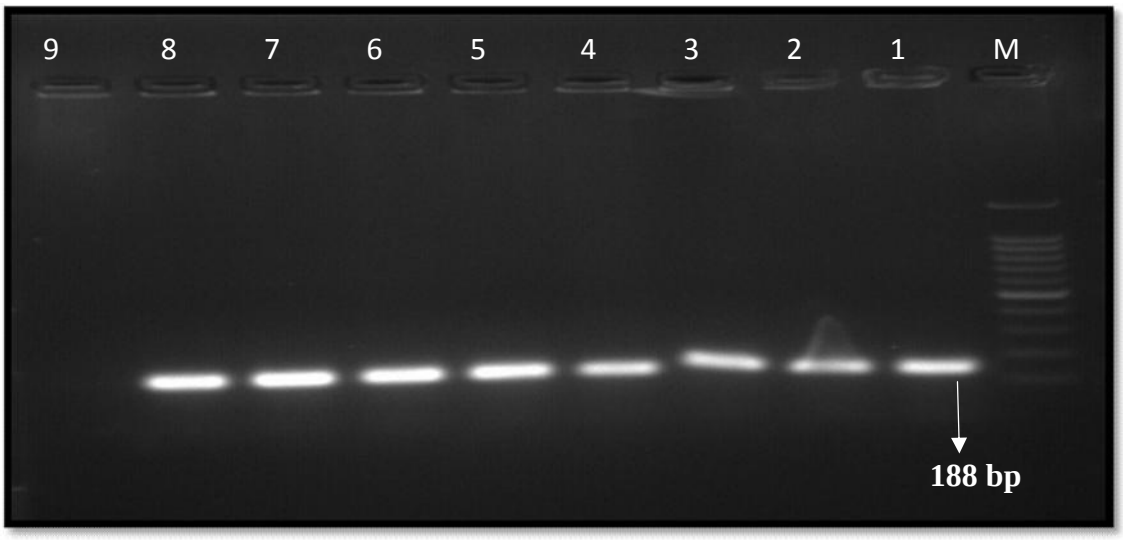


Photo 10 & 11: Result of PCR for ica A gene (188 bp product)

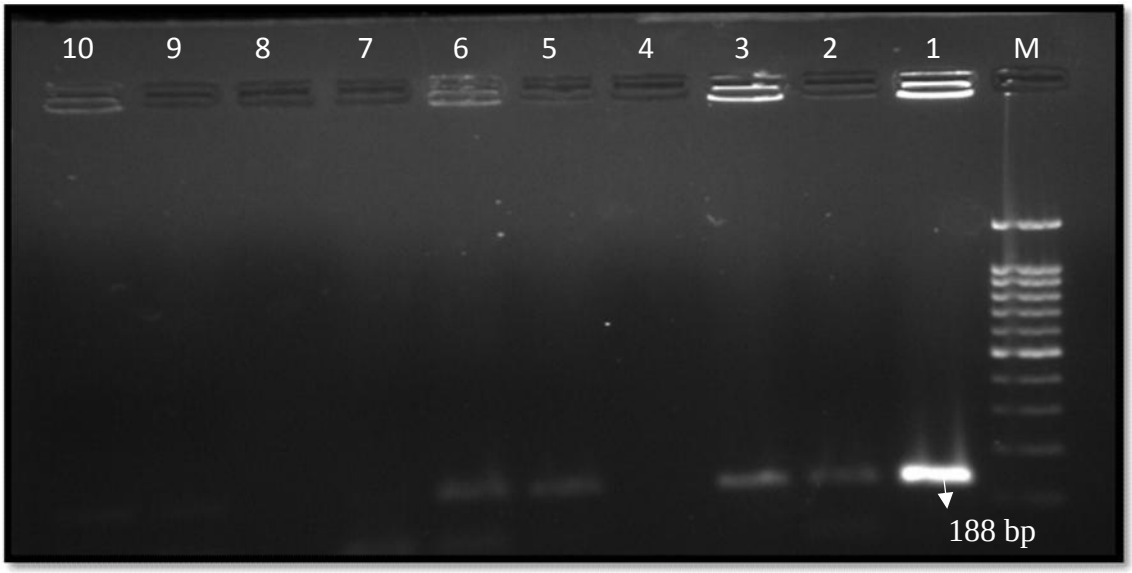


Lane M-100bp DNA ladder

Lane 1 Positive control - *S.aureus* ATCC 35556

Lanes 2-8 Positive samples

Lane 9 – Negative control- *S.epidermidis* ATCC12228



Lane M-100bp DNA ladder

Lane 1 Positive control - *S.aureus* ATCC 35556

Lanes 2, 3, 5, 6: Positive samples

Lane 4,7, 8, 9: Negative samples

Lane 10 – Negative control- *S.epidermidis* ATCC12228

RESULTS

A total of 86 isolates of Coagulase negative *Staphylococci* isolated from different clinical samples (pus/wound swab, blood, endotracheal tube, urine) were identified to the species level by using conventional biochemical tests.

Out of the total 86 *CoNS* isolates *S.epidemidis* was the most frequent isolate 25(29.07%), followed by *S. hemolyticus* 24 (27.9%), *S. hominis* 20 (23.25%), *S. cohnii* 5 (5.81%), *S. simulans* 3 (3.49%), *S. warneri* 3 (3.49 %), *S. schleiferi* 3 (3.49 %), *S. intermedius* 1 (1.16%), *S. hyicus* 1(1.16 %)and *S. saprophyticus* 1 (1.16 %). (Table 2)

S. epidermidis was isolated from 11 pus samples, 11 blood samples and 3 endotracheal tubes. *S. haemolyticus* on the other hand was isolated mainly from blood that is 21 samples followed by 3 samples from pus. *S. hominis* was isolated from 18 blood samples, and 1 sample each of pus and endotracheal tube. Other *CoNS* like *S. cohnii, S. simulans, S. warneri. S. schleiferi, S. intermedius, S. hyicus, S. saprophyticus* were less prevelant in the above clinical samples.(Table 3)

Majority of the isolates were obtained from male patients more than 60 years of age. (Table 4 and Table 5)

In the present study with the available history of patients out of 86 *CoNS* isolates, 27 strains were isolated from diabetic patients who had a history of catheters or intravenous devices or had abscesses. Eleven strains were isolated from patients suffering from chronic kidney disease, 8 strains were from post operative patients, 10 from neonates, 4 from cancer patients and 25 from other conditions like abscesses, COPD, pneumonia, cardiac diseases and liver diseases. One patient was on ventilator.

Biofilm production ability of 25 *S. epidermidis* isolates was detected by phenotypic (TCP method and MCRA method) and genotypic (PCR-*icaA* gene detection) methods. Biofilm formation by *Staphylococcus epidermidis* (25 isolates) was detected by phenotypic and genotypic methods. By MCRA method 10 (40%) *S. epidermidis* isolates were defined as biofilm producers through their black colonies. By TCP method 14 isolates (56%) were found to be biofilm producers with different grades: 6 (24%) strong producers and 8(32%) moderate producers. i*caA* gene was detected concomitantly in 17(68%) isolates in comparison with the data of *icaA* gene detection.(Table 6, Table 7, and Table 10)

As detected by TCP method biofilms were produced by 4 isolates obtained from pus/wound swab, 9 from blood and 1 from endotracheal tube. By MCRA method , biofilms were produced by 2 isolates from pus/wound swab, 7 from blood and 1 from endotracheal tube. The *icaA* gene was detected in 7 isolates from pus/wound swab, 7 from blood and 3 from endotracheal tube. (Table 9, Table 11, Table 12)

Statistical analysis

The data was tabulated. Inter-group comparison of categorical data was performed using chi square test (x^2-value).

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MCRA and TCP were calculated. P value <0.05 was considered statistically significant. Sensitivity: proportion of PCR-positive isolates that tested positive by the other phenotypic methods; Specificity: proportion of PCR-negative isolates that tested negative by the phenotypic methods.

Table 1:Biochemical characters of Coagulase Negative Staphylococcus(*CoNS*)

Test	*S. epidermidis*	*S. haemolyticus*	*S. hominis*	*S. cohnii*	*S. saprophyticus*	*S. schleiferi*	*S. simulans*	*S. warneri*	*S. hyicus*	*S. intermedius*
Colony pigment	-	d	d	-	d	-	-	d	-	-
Bound coagulase	-	-	-	-	-	-	-	-	d	+
Clumping factor	-	-	-	-	-	+	-	-	-	d
Alkaline phosphatase	+	-	-	-	-	+	(d)	-	+	+
PYR	-	+	-	-	-	+	+	-	-	+
Ornithine decarboxylase	(d)	-	-	-	-	-	-	-	-	-
Urease	+	-	+	-	+	-	+	+	d	+
Acetoin production	+	+	d	d	+	+	d	+	-	-
Novobiocin Resistance	S	S	S	R	R	S	S	S	S	S
Nutrate reduction	+	+	d	-	-	+	+	d	+	+
Acid (aerobically from)										
Trehalose	-	+	d	+	+	d	d	+	+	+
Mannitol	-	-	-	(d)	d	-	+	d	-	(d)
Mannose	+	+	-	(d)	-	+	d	-	+	+
Xylose	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	d	+	-	±	+	-	±
Sucrose	+	+	+	-	+	-	+	+	+	+

+, ≥90% strains positive; ±, ≥90% strains weakly positive; −, ≥90% strains negative; d, 11%-89% of strains positive; (d), delayed reaction; R, resistant; S, sensitive PYR-Pyrrolidonyl-α-naphthylamide

Table 2 : Species of *CoNS*(Coagulase negative *Staphylococci*)(n=86)

CoNS	Clinical specimens	Percentage
S.epidermidis	25	29.07%
S.haemolyticus	24	27.91%
S.hominis	20	23.25%
S.cohnii	5	5.81%
S. simulans	3	3.49%
S.warneri	3	3.49%
S.schieiferi	3	3.49%
S. intermedius	1	1.16%
S. hyicus	1	1.16%
S.saprophyticus	1	1.16%

Graph 1: Species of *CoNS* (Coagulase negative *Staphylococcus*)(n=86)

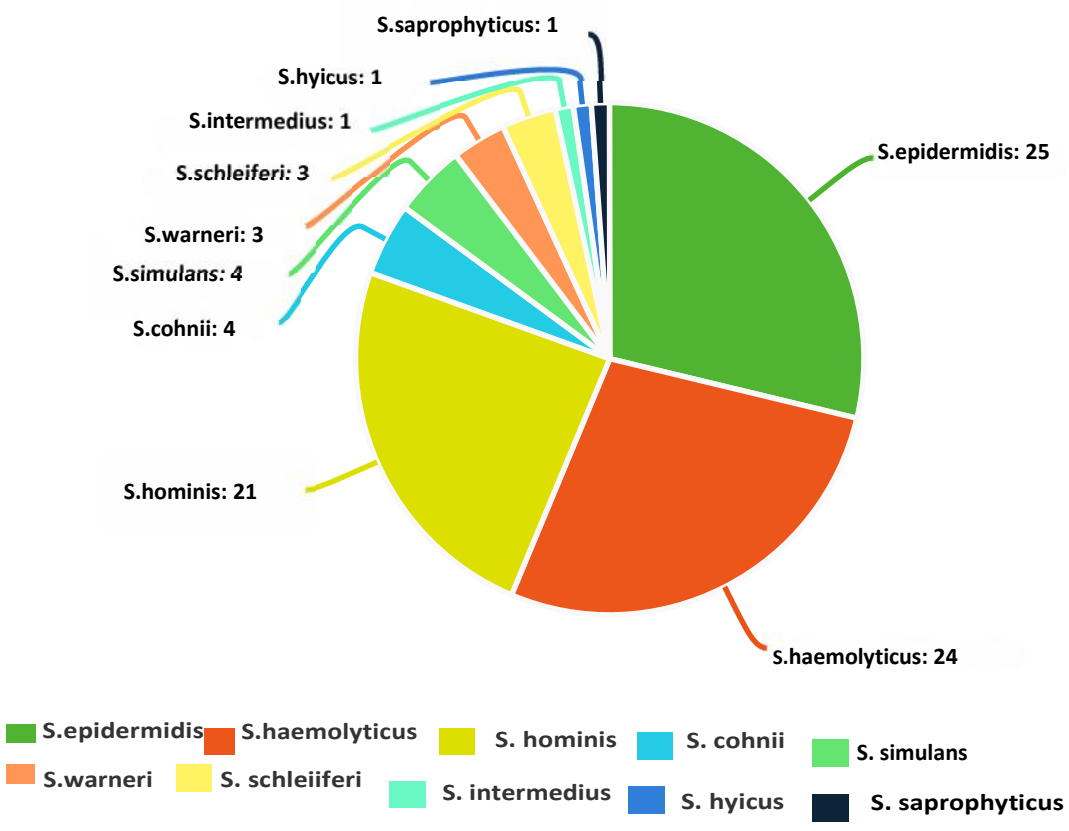


Table 3 : Distribution of species of *CoNS* (Coagulase negative *Staphylococci*) in clinical specimens. (n=86)

Species	Pus/wound swab	Blood	Endotracheal tube	Urine
S.epidermidis	**11**	**11**	**3**	**-**
S.haemolyticus	**3**	**21**	**-**	**-**
S.hominis	**1**	**18**	**1**	
S.cohnii	**4**	**-**	**-**	**-**
S. simulans	**2**	**2**	**-**	**-**
S.warneri	**-**	**3**	**-**	**-**
S.schleiferi	**-**	**3**	**-**	**-**
S. intermedius	**-**	**1**	**-**	**-**
S. hyicus	**-**	**1**	**-**	**-**
S.saprophyticus	**-**	**-**	**-**	**1**

Graph 2 : Distribution of species of *CoNS* in clinical specimens. (n=86)

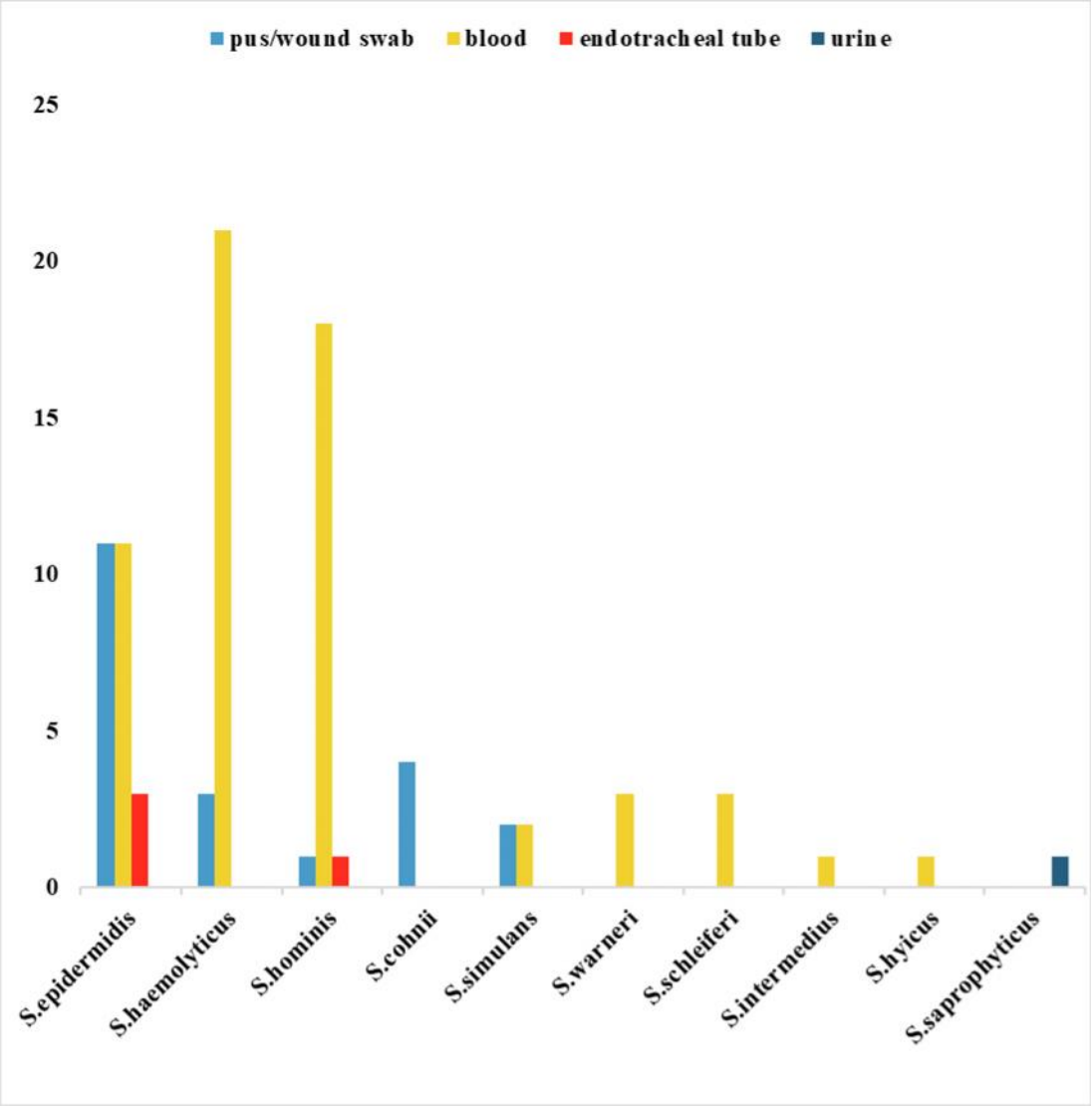


Table 4 : Distribution of *CoNS* (Coagulase negative *Staphylococci*) according to gender (n=86)

Gender	No.	Percentage
Male	55	63.9%
Female	31	36.1%

Graph 3 : Distribution of *CoNS* (Coagulase negative *Staphylococci*) according to gender (n=86)

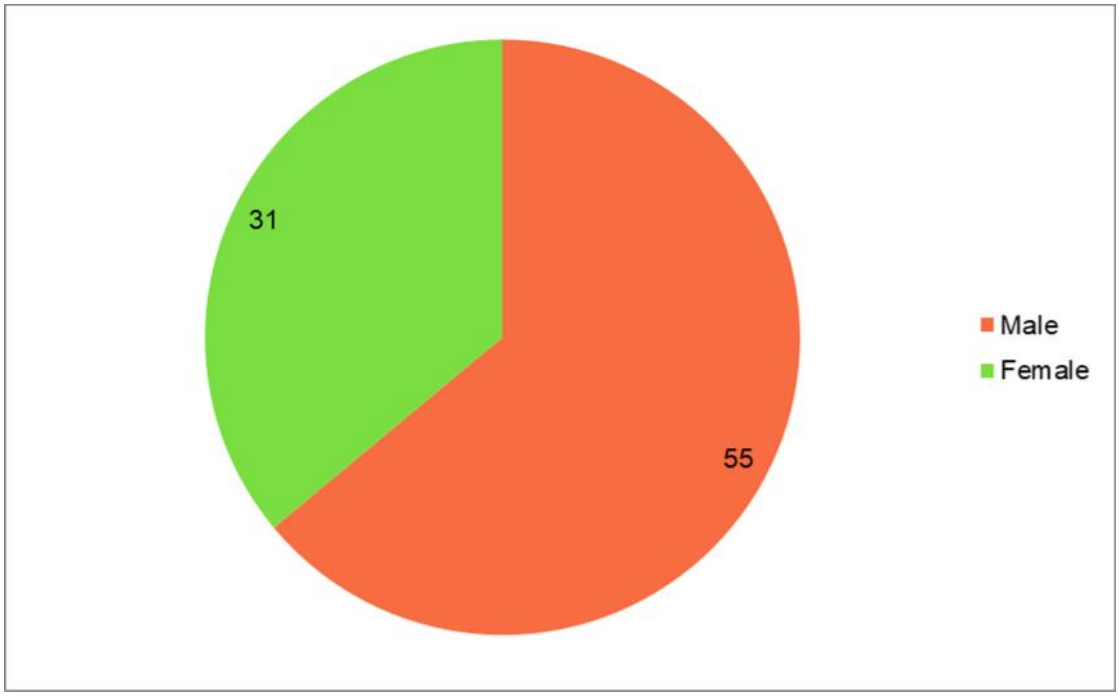


Table 5 : Distribution of *CoNS* among different Age groups(n=86)

Age in years	No.	Percentage
0-10	16	18.6%
11-20	2	2.32%
21-30	10	11.6%
31-40	6	6.9%
41-50	12	13.9%
51-60	7	8.0%
>60	32	37.2%

Graph 4 : Distribution of *CoNS* among different **Age groups(n=86)**

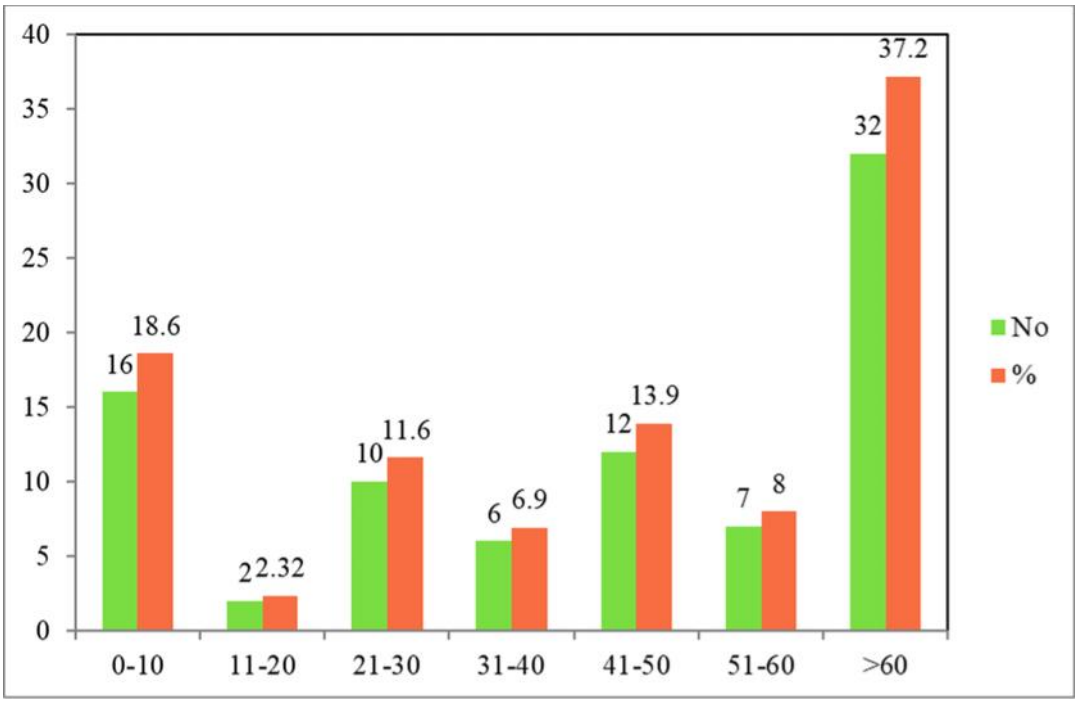


Table 6 : Biofilm production by *Staphylococcus epidermidis* by various methods(n=25)

Methods	Biofilm production
TCP method(phenotypic)	14(56%)
Modified CRA method(phenotypic)	10(40%)
PCR- *ica A* gene(genotypic)	17(68%)

Graph 5 : Biofilm production by *Staphylococcus epidermidis*- various methods(n=25)

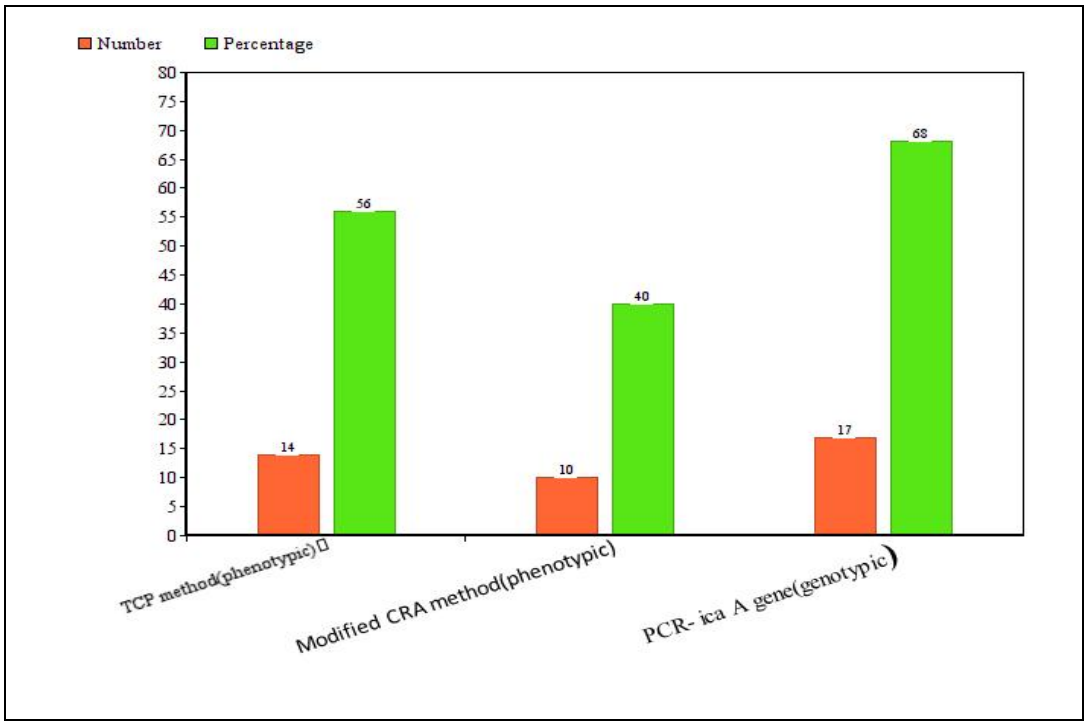


Table 7 : TCP Method for biofilm production by *Staphylococcus epidermidis* (n=25)

Biofilm producers	Number (%)
Strong producer	6(24%)
Moderate producer	8(32%)
Nonbiofilm producer	11(44%)

Graph 6 : TCP Method for biofilm production by *Staphylococcus epidermidis* (n=25)

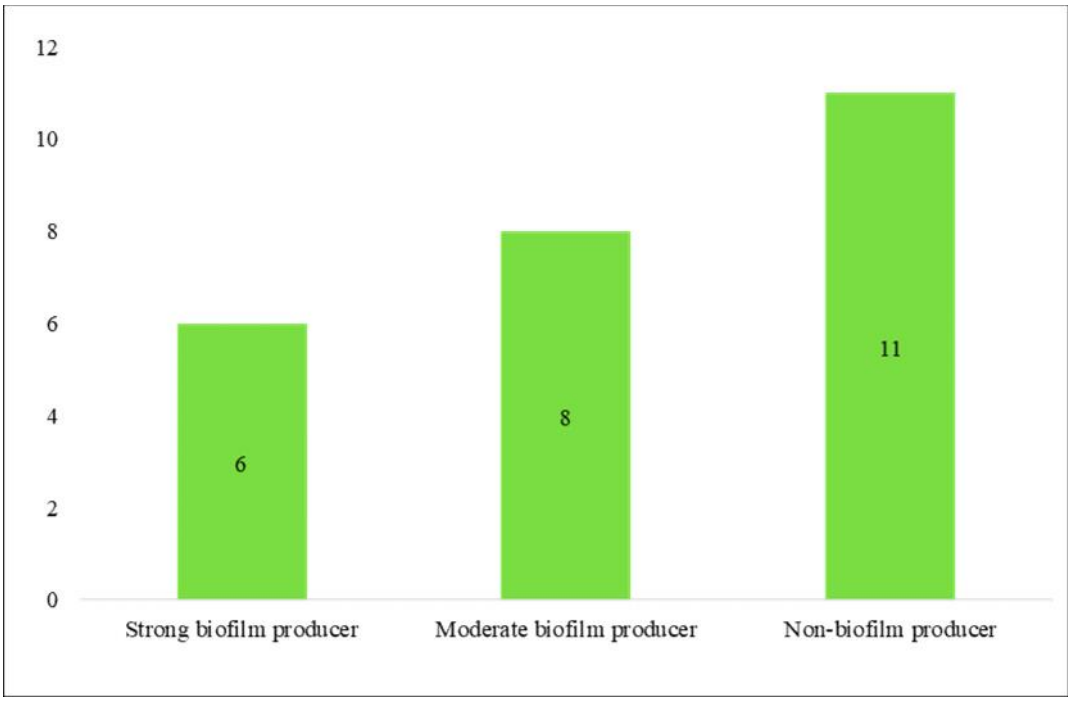


Table 8 : Classification of biofilm formation by *Staphylococcus epidermidis* isolates based on OD values of TCP

OD Values	Adherence	Biofilm formation
>0.24	Strong	High
0.12-.24	Moderate	Moderate
<0.12	None	None

Table 9 : OD Values of biofilms formed by *Staphylococcus epidermidis* strains from different clinical samples(n=25)

Clinical samples	OD > 0.24	VALUE 0.12-O.2	<0.12	Total
Pus/wound swab	1	3	7	11
Blood	4	5	2	11
Endotracheal tube	1	0	2	3

Table 10 : Number of isolates according to Modified Congo red agar method (n=25)

Isolates	Number	Percentage
Biofilm producers	10	40%
Non biofilm producers	15	60%

Graph 7 : Number of isolates according to Modified Congo red agar method (n=25)

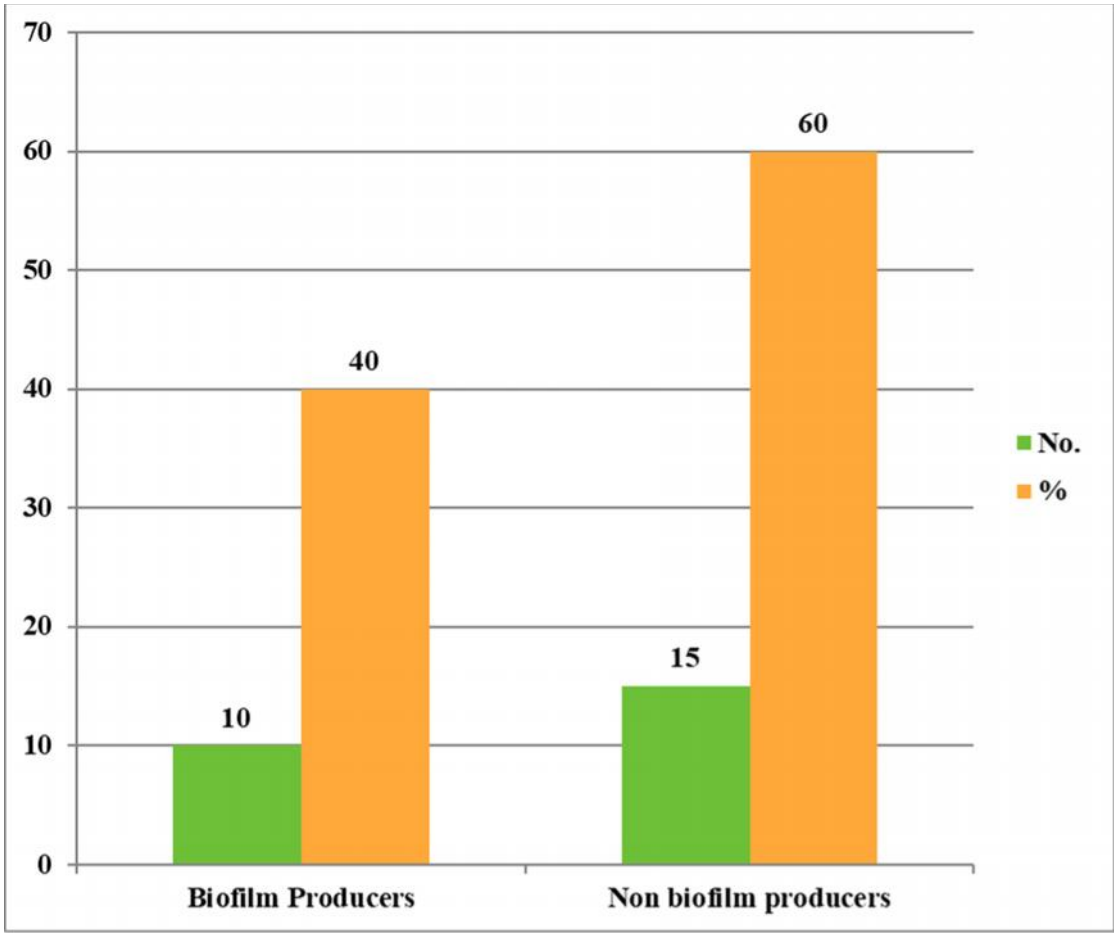


Table 11 : Distribution of samples according to biofilm production by Modified Congo red agar method (n=25)

Isolates	Pus/woundswab	Blood	Endotracheal tube
Biofilm producers	2	7	1
Nonbiofilm producers	9	4	2
Total	11	11	3

Graph 8 : Distribution of samples according to biofilm production by Modified Congo red agar method (n=25)

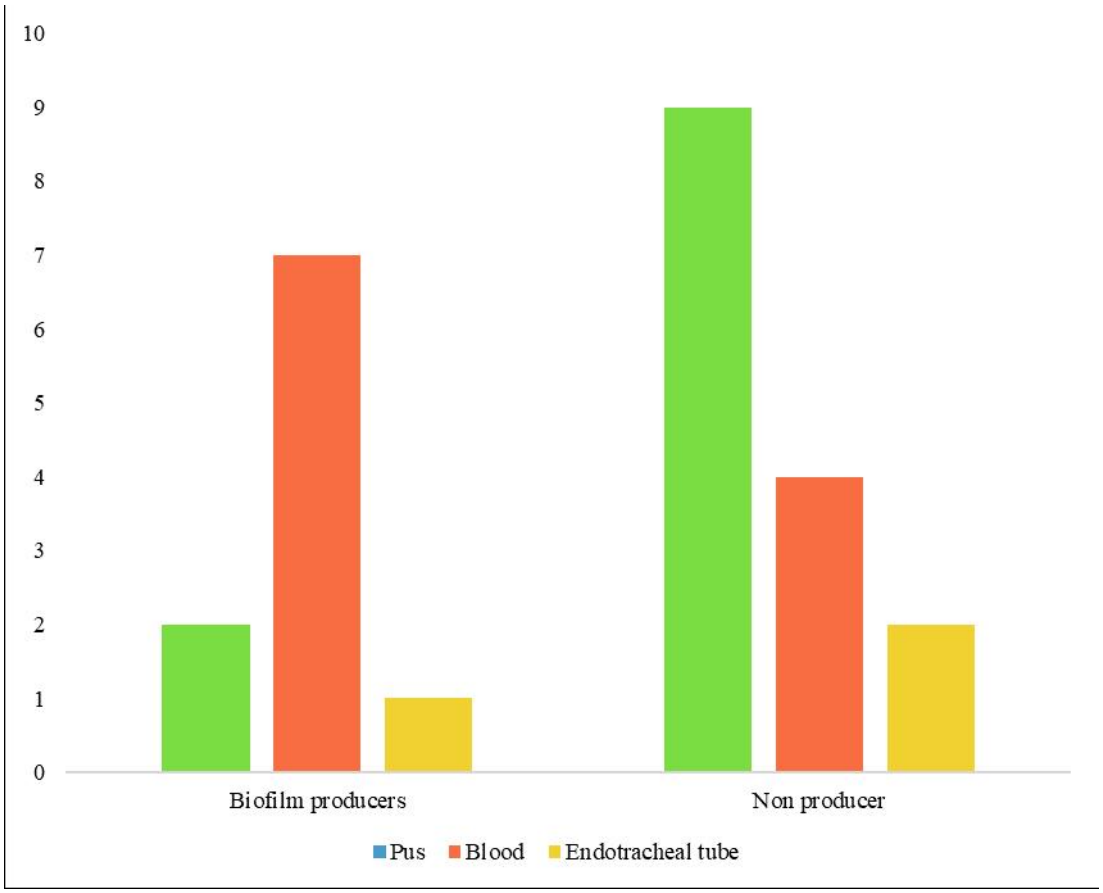


Table 12 : Distribution of *ica A* gene in *S.epidermidis* from different clinical samples(n=25)

Samples	Positive	Negative
Pus	7	4
Blood	7	4
Endotracheal tube	3	0

Graph 9 : Distribution of *ica A* gene in *S.epidermidis* from different clinical samples(n=25)

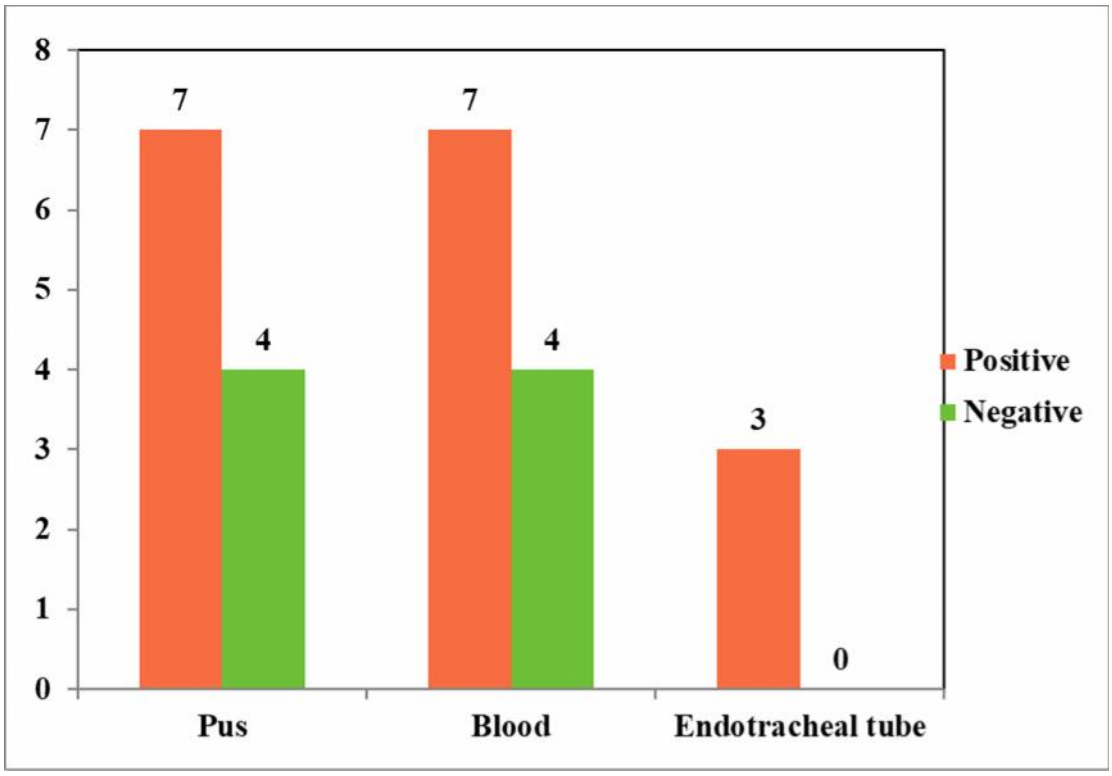


TABLE 13 : Statistical evaluation of the phenotypic analysis compared to the detection of ica A genes in S. epidermidis isolated (n=25)

Phenotypic Analysis		icaA Detection						X^2	P	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		Negative		Positive		Total							
		No.	%	No.	%	No.	%						
MCRA	Negative	7	87.5	8	47.1	15	60	3.7071	0.054181	52.9	87.5	90	46.6
	Positive	1	12.5	9	52.9	10	40						
	Total	8	100	17	100	25	100						
TCP	Negative	6	75	5	29.4	11	44	4.5884	0.032189	70.5	75	85.7	54.5
	Positive	2	25	12	70.6	14	56						
	Total	8	100	17	100	25	100						

X^2 : chi square

P: Probabilty

PPV: Positive Predictive Value

NPV: Negative Predictive value

TABLE 14 : Comparison of TCP and PCR detection of ica A genes in *S.epidermidis* isolates

TCP	ica A detection					
	Negative		Positive		Total	
	No.	%	No.	%	No.	%
Negative	6	75	5	29.41	11	44
Moderate positive	2	25	6	35.29	8	32
Strong positive	0	0	6	35.29	6	24
Total	8	100	17	100	25	100

TABLE 15 : Qualitative (MCRA) method versus Quantitative (TCP) method for detection of biofilm forming *S.epidermidis*(n=25)

MCRA	TCP		
	Positive (14)		Negative (11)
	Strong Positive	Moderate Positive	
Positive	6	4	0
Negative	0	4	11
Total	6	8	11

DISCUSSION

Coagulase Negative *Staphylococci* is a part of normal flora. Moreover, if it happens to be isolated along with another organism, its pathogenic potential is totally neglected. Hence it becomes mandatory to speciate *CoNS* and understand the pathogenic potential of individual *CoNS* isolates. Coagulase-negative *Staphylococci* species formerly considered as a contaminant bacteria, are now important possible pathogens with the increase in number of foreign body-related infections and infections in newborns who are preterm.. In this study, *Staphylococcus epidermidis* was the most commonly isolated species of *CoNS.* The major virulence factor of *Staphylococcus epidermidis* is its ability to attach on the surface of biomaterials to make biofilms. In this study the presence of *icaA* gene in comparison with different phenotypic methods to detect biofilms was assessed.

In this study among the 86 isolates of *CoNS*, most of them were from blood, followed by pus/wound swab, endotracheal tube and urine. (69.7%, 24.4%, 4%, 1.1%, respectively). Majority of the patients were males >60years of age. Studies by Boynukara B et al[62], Choudhari, Arora and Sharma[63] also demonstrated that *CoNS* were isolated from blood, pus, urine, indwelling catheters, and seldom from body fluids. In this study, the species which was most common in blood samples were *S. epidermdis, S. hemolyticus* and *S. hominis,* which is similar to the study by Weinstein et al.[64]The study compred Microscan rapid and Dried overnight Gram positive panels versus a conventional reference method to identify *CoNS*. The rate of isolation from the blood specimen was high in this study as compared with other studies. The reason could be due to a higher number of blood samples tested for *CoNS* and testing of more

patients who had predisposing factors (in situ medical devices or immunocompromised status, cancers etc).

The present study recognised *Staphylococcus epidermidis* as the predominant species (29.07% of the total isolates) . Goyal R et al[66], Sheik et al[67], have recognised findings with 41% and 46% of isolation of *Staphylococcus epidermidis* in their studies respectively . Senger et al[65] recognised a large proportion of *Staphylococcus epidermidis* (68% of the total *CoNS* isolates) in their study .

The second most common isolate in the present study was *S. haemolyticus* accounting for 27.9% of the total isolates. The third most common organism isolated was *S. hominis* 23.25%.

Asangi et al.[68] documented *S. epidermidis* as the most frequent isolate (43, 44.8%), followed by *S. saprophyticus* (26, 27.1%), *S. haemolyticus* (19, 19.7%), *S. lugdunensis* (2, 2.1 %), *S. warneri* (2, 2.1%), *S. cohnii* (1, 1%).

A study done by Shubhra Singh and Gopa Banerjee et al.[69], identified *S. epidermidis* (40%) as the most commonly recognised clinical isolate in their hospital followed by *S. saprophyticus* (14%), *S. haemolyticus* (12%).

The present study is in concordance with the study conducted by M. G. Usha et al[70]. Out of the 100 isolates studied, *S.epidermidis* was the most frequent species isolated, seen in (32%), followed by *S.hemolyticus* (18%), *S. cohni* and *S. warneri* (3% each).

The differences in the incidence of individual species could be due to different clinical samples from different sections of hospital like NICU,orthopaedic wards,cardiac ICU, out patient department(OPD) etc.

In this study out of 86 *CoNS* ,32 patients were more than 60 years of age. The isolations were more in males (55, 63.9%) than females (31, 36.1%). A study by Larry M. Baddour, David L.et al[71],concluded that 19 (54.2%) of 35 patients were males and 30 (85%) patients were above the age of 40 years.

In this present study prevalence of *Staphylococci epidermidis* with biofilm formation ability, isolated from various clinical samples has been studied by three methods, the growth on MCRA, biofilm formation ability by TCP method and PCR-based detection of *icaA* operon genes associated with biofilm formation.

TCP, CRA and/or PCR methods have been used by many scientists to determine the important virulence factors of *CoNS*, i.e. the ability of biofilm formation (Christensen et al[72], Freeman et al[73], Arciola et al[74 75], Bozkurt et al.[76], El-Mahallawy et al[38]). Some reports (Frebourg et al.[78], Galdbart et al[79].,Vandecasteele et al[80].,Chokr et al.[81],Satorres & Alcar´az,[82] Mateo et al.,[83] Jain & Agarwal[84]) indicate that these methods, can be helpful to distinguish between colonizing or commensal, staphylococcal strains. This can lead to early detection and management of potentially pathogenic isolates responsible for device-associated nosocomial infections.

In the present study, the percentage of biofilm production detected by phenotypic method that is tissue culture plate (TCP) method (56%) was high compared to modified Congo red agar method (40%).In a study by Mathur et al.[85],who demonstrated that the number of biofilm producers identified by TCP method was high(53.9%) and was followed by Tube method (TM)(11.8%) and CRA (5.17%).

In their study on 110 isolates Hassan et al.[86], concluded that the TCP method detected biofilm in 70 isolates (63.6%), TM (tube method) in 54 (49%) and CRA in 11 (10%) isolates

In another study, done by Ruzicka et al.[87], noted that out of 147 isolates of *S. epidermidis,* TM detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates and concluded that TM is a better method for biofilm detection than CRA.Same findings were also reported by Oliveira et al[88], of the 100 *CoNS* isolates 82% were biofilm positive by PCR and the tube test, 81% by the TCP assay, and 73% by the CRA method. The low effectiveness of the CRA method to evaluate biofilm production, was also recognised by de Silva et al.[89]

In this study the phenotypic ability to biofilm formation was depicted better by TCP method than MCRA method.Most of the studies have used CRA method to detect slime production. In a study by Panda PS et al[19] comparison of four different methods to detect slime production were done. Out of the 300 isolates, the TCP method detected biofilm in 137 isolates (45.6%), TM detected biofilm in 118 isolates (39.3%), CRA and MCRA detected biofilm in 33 isolates (11%). The TCP was found to be most sensitive which was followed by the TM, CRA and the MCRA method. There was no difference in the frequency of biofilm detection between CRA and MCRA in other isolates, but MCRA was found to be more effective to CRA to detect staphylococcal biofilm formation.

In this study, out of 14 Biofilm producing isolates detected by phenotypic method, *icaA* gene was identified by PCR in 12 (70.5%) isolates. The results were in concordance with the study by Sujata et al.[90], who reported in their study that *icaA* gene was present in 23 among 26 of biofilm producers. The results were different

when compared with the study by Seung- Hak- Cho et al.,[91] who had concluded that 18 *S. epidermidis* isolates obtained from catheter-related urinary tract infections showed the *icaA* speficic DNA. Only 11 isolates produced biofilms spontaneously under normal growth conditions. In a study conducted by Agarwal et al[92] showed that the use of glucose or NaCl or combination of both increased biofilm producing capability of staphylococcal isolates regardless of presence or absence of *icaA* operon. Galdbart Jo et al.[92], in their study reported that 44 out of 54 *S. epidermidis* isolates from prosthetic material related joint infection identified *ica A* positivity. Gad et al.[93], Cafiso et al.[94],demonstrated the detection of *ica* operon by molecular technique (PCR) with high efficiency. In addition, these genes are important virulence markers of clinical *S.epidermidis* isolate as their expression is associated with the production of PIA, the most important component of staphylococcal biofilms.

The data in this study also demonstrated that five *S.epidermidis* isolates were positive for *icaA* gene but did not phenotopically produce biofilm by TCP or MCRA method.This is in cordance with a study conducted by Wojtyczka R D et al.[95] The study demonstrated only nine (7.4%) out of 122 tested strains were considered as biofilm-formers by CRA method and not a single *S. epidermidis* strain was among them. Among 32 analyzed *S. epidermidis* strains, 12 (37.5%) produced biofilms by TCP method.PCR of 32 *S. epidermidis* strains showed the presence of *icaADBC* operon genes in 15 (46.9%) isolates.The *icaA* and *icaD* were present in 34.4% and 28.1% of strains respectively and *icaC* gene in 37.5% of strains. Only 21.9% showed *icaB* gene . The presence of all *icaADBC* operon genes was found in five (15.6%) *S. epidermidis* isolates, while in four (12.5%) isolates both of *icaA and icaC* were found. The frequencies of *icaB/icaD, icaA/icaD/icaB, and icaA/icaB/icaC* genotypes were on the same level (3.1%) suggesting that the use of TCP method together with the PCR-

based techniques should be used as a gold standard to evaluate biofilm formation ability by *CoNS* strains isolated from hospital strains. All *S. epidermidis* strains with *ica* genes gave negative results for biofilm production done by the CRA method while only12 strains were biofilm positive by the TCP method.

There is enough evidence that the presence of *icaABCD* operon genes in the staphylococcal genome is associated with biofilm formation ability. Oliveira[88] and Cucha's [96]study on the prevalence of *icaA*, *icaC* and *icaD* genes in *CoNS* strains depicted that the *icaA* and *icaD* genes could be demonstrated in 40% of isolates, and *icaA*, *icaC* and *icaD* in 42% of isolates. In the above study only 18% of analyzed isolates did not have all *icaABCD* genes. In studies carried out by Arciola et al[74]., 101 *S. aureus* and *S. epidermidis* clinical isolates were analysed for the presence of *icaA* and *icaD* genes. Sixty strains of *S. epidermidis* and 23 strains of *S. aureus* were isolated from infections associated with the implementation of vascular catheter and 10 strains of *S. epidermidis* were isolated from the skin and mucous membranes of fit subjects. The presence of both genes (*icaA* and *icaD)* were demonstraed in 48.5% (33) of *S. epidermidis* clinical isolates. The remaining 35 strains of *S. epidermidis* and 10 strains isolated from the skin and mucous membranes, did not have *ica* operon genes.

Phenotypic methods for detection of biofilm formation are among the easy and cheap methods which are available for routine laboratory use, but are liable to cause some difficulties in result interpretation as they are liable to be influenced by variations in temperature, pH, medium composition and cultivation conditions and are also prone to subjective errors.

The studies have shown that the *aap* and *bhp* genes can be responsible in an alternative PIA-independent mechanism of biofilm formation. This indicates that if *icaABCD* operon is not present, it does not rule out biofilm formation ability of the isolate[55]. In another study by Eftekhar and Mirmohamadi[97], the ability of biofilm production by two groups of *S. epidermidis* strains isolated from nosocomial infections and skin of healthy subjects was analyzed. It depicted that the prevalence of biofilm-forming strains in the TCP method was at the 52% and 56% level for the groups which were assessed respectively. The genetic analysis using single pair of primers for the *icaA*, *icaB* and *icaD* yielded positive results in 30% *S. epidermidis* isolates from hospital borne infections and in 8% of the isolates from the skin of healthy volunteers.

CONCLUSION

Coagulase negative *Staphylococcus* have emerged as important cause of opportunistic and health care associated infections. Among 86 isolates of *CoNS*, isolates *S.epidermidis* was the most common isolate 25 (29.07%). Majority of the isolates were obtained from blood culture followed by pus samples. Maximum number of isolates were obtained from patients of more than 60 years. Males were more affected than females. The species identification is needed to monitor the reservoir and distribution of *CoNS* in hospital borne infections.

The present study depicted that *icaA* gene was present in 17 (68%) of *S.epidermidis* isolates . 14 (56%)of the isolates were detected as biofilm producers by Tissue culture plate method. The sensitivity and specificity of biofilm detection by this method was high when compared with modified Congo red agar method. Although the genotypic methods are the absolute detection methods, it cannot be done in all centres, moreover it is expensive too. TCP being cost effective can be recommended as a general screening test for biofilm production than MCRA method. The results in this study have confirmed the previous data presented by other authors that the molecular presence of *ica* operon genes in the bacterial genome is associated with the ability to form biofilms, but if these genes are absent, it does not preclude this phenomenon phenotypically.

Therefore, it seems appropriate to use both genotypic and phenotypic methods to improve the results of identification of biofilm formation by *S. epidermidis* strains isolated from various clinical samples. Biofilm-forming strains are difficult to treat so biofilm formation ability is crucial for health care providers to implement hospital infection prevention and control plans. Physicians also need to know about them in building up adequate and emperical antibacterial therapies.

SUMMARY

The present study was conducted in the Department of Microbiology, J.N.M.C. , KLE University, Belagavi from January 2016 – December2016

A total of 86 isolates of Coagulase negative Staphylococci isolated from different clinical samples were identified to the species level by using conventional biochemical tests.

Out of the total 86 *CoNS* isolates *S.epidemidis* was the most frequent isolate 25(29.07%), followed by *S. hemolyticus* 24 (27.9%), *S. hominis* 20 (23.25%), *S. cohnii* 5 (5.81%), *S. simulans* 3 (3.49%), *S. warneri* 3 (3.49 %), *S. schleiferi* 3 (3.49 %), *S. intermedius* 1 (1.16 %), *S. hyicus* 1(1.16 %)and *S. saprophyticus* 1 (1.16 %).

Majority of the isolates were isolated from blood (60) followed by pus/wound swab (21), endotracheal tube (4) and urine (1). Maximum number of isolates were obtained from male patients more than 60 years of age who had a history of indwelling catheters and intravascular devices. Biofilm formation by *Staphylococcus epidermidis* (25 isolates) was detected by phenotypic and genotypic methods. By MCRA method 10 (40%) Staph epidermidis isolates were defined as biofilm producers through their black colonies. By TCP method 14 isolates (56%) were found to be biofilm producers with different grades: 6 (24%) strong producers and 8(32%) moderate producers.The i*caA* gene was detected concomitantly in 17(68%) isolates. In comparison with the data of *icaA* gene detection MCRA had the sensitivity of 52.9% and specificity of 87.5% while TCP method represented 70.4% sensitivity and 75% sepecificity. It was also noticed that all strong biofilm producers by TCP method were positive for i*caA* genes.

For maximum evaluation of biofilm production by *Staphylococcus epidermidis* both genotypic and phenotypic analysis is needed.

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ANNEXURE – I – ETHICAL CLEARANCE LETTER

K.L.E.UNIVERSITY'S
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI-590010 (KARNATAKA-INDIA)
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Ref: MDC/DOME/

Date: 18/11/2015

To,

REG NO.BI0115002

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"COMPARISON OF PHENOTYPIC AND GENOTYPIC METHODS FOR BIOFILM FORMING STAPHYLOCOCCUS EPIDERMIDIS FROM VARIOUS CLINICAL SAMPLES A CROSS SECTIONAL STUDY",** is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr. Arathi Darshan)
Member Secretary
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

(Dr. Ganga Pilli)
Chairman,
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

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ANNEXURE-II

PROFORMA

CASE NO.-

NAME OF PATIENT-

AGE-

SEX-

ADDRESS-

IP/OPD NO.-

UNIT/WARD-

DATE OF SAMPLE COLLECTION-

TYPE OF SAMPLE-

CHIEF COMPLAINTS-

HISTORY OF PRESENTING ILLNESS-

TREATMENT HISTORY-

CLINICAL DIAGNOSIS-

OTHER INVESTIGATIONS-

Complete haemogram

Liver function tests

Kidney function tests

Blood Sugar

Fasting

Post Prandial

MICROBIOLOGICAL INVESTIGATIONS

LAB NO.-

SOURCE OF DATA-

All Staphylococcus epidermidis isolated from different clinical samples received in Microbiology Department.

RECONFIRMATION-

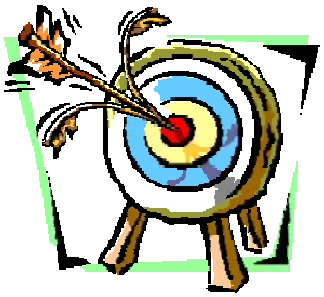
1. GRAM STAINING-
2. BLOOD AGAR-
3. NUTRIENT AGAR-
4. CATALASE-
5. SLIDE COAGULASE-
6. TUBE COAGULASE-
7. UREASE-
8. MANNITOL FERMENTATION-
9. OTHER BIOCHEMICAL TESTS-
10. DETECTION OF BIOFILM FORMATION BY-
    - TISSUE CULTURE PLATE METHOD
    - MODIFIED CONGO RED AGAR METHOD
    - PCR FOR DETECTION OF *ica A*

ANNEXURE-III- MASTER CHART

S.NO.	age	sex	hosp no.	lab no.	specimen	species
1	D3	Male	742385	3090	Blood	*S.hominis*
2	70yr	Male	742602	3114	Blood	S.haemolyticus
3	64yr	Female	740649	2916	Blood	S.hominis
4	52yr	Male	740719	2926	Blood	S.hominis
5	D4	Male	740694	2927	blood	S.haemolyticus
6	64yr	Male	741333	2975	Blood	S.hominis
7	D4	Male	741366	2980	Blood	S.hominis
8	45yr	Female	741400	2986	Blood	S.haemolyticus
9	D6	Male	741524	2992	Blood	S.haemolyticus
10	55yr	Male	741528	2994	Blood	S.haemolyticus
11	D8	Female	741777	3018	Blood	S.haemolyticus
12	39yr	Male	741827	3028	Blood	S.haemolyticus
13	1mnth	Female	742336	3081	Blood	S.epidermidis
14	63yr	Male	743326	6212	Pus	S.simulans
15	64yr	Male	744509	3436	Blood	S.schleiferi
16	55yr	Male	743503	3224	Blood	S.hominis
17	48yr	female	743765	3244	Blood	S.hominis
18	17yr	Male	743768	3246	Pus	S.cohnii
19	2.5mnth	Male	743826	3250	Blood	S.hominis
20	30yr	Female	743951	3263	Pus	S.cohnii
21	21yr	Female	744137	3374	Blood	S.hominis
22	36yr	Male	744122	3375	Blood	S.haemolyticus
23	45yr	Male	744123	3376	Blood	S.epidermidis
24	43yr	Female	744183	3381	Blood	S.epidermidis
25	D20	Female	742610	3117	Blood	S.hominis
26	68yr	Female	743021	3167	Blood	S.haemolyticus
27	23yr	Male	743031	3168	Blood	S.hominis
28	52yr	Male	743271	3190	Blood	S.hominis
29	63yr	Male	743486	3213	Blood	S.schleiferi
30	82yr	Female	742383	3087	Blood	S.haemolyticus
31	67yr	Male	745120	3488	Blood	S.hominis
32	D3	Female	744552	3493	Pus	S.haemolyticus
33	45yr	Male	745311	3501	Blood	S.cohnii
34	68yr	Female	744895	3454	Blood	S.hominis
35	74yr	Male	744933	3458	Blood	S.warneri
36	30yr	Male	743499	3222	Blood	S.hyicus
37	45yr	Male	744945	3460	Blood	S.haemolyticus
38	81yr	Male	744991	3464	Blood	S.epidermidis
39	40yr	Female	744823	7448	Blood	S.epidermidis
40	75yr	Male	744799	3446	Blood	S.epidermidis
41	58yr	Male	744759	3439	Blood	S.hominis
42	55yr	Female	742216	3450	Blood	S.warneri
43	35yr	Male	744269	3393	Blood	S.hominis

44	22yr	Male	744504	3409	Blood	S.hominis
45	46yr	Male	743662	6219	Pus	S.simulans
46	82yr	Male	744976	6846	ET	S.epidermidis
47	24yr	Female	743927	6674	urine	S.saprophyticus
48	43yr	Male	740934	6666	PUS	S.epidermidis
49	69yr	Male	745257	7014	Pus	S.epidermidis
50	33yrs	Male	745341	3505	Blood	S. simulans
51	45yr	Female	3274540	7197	Pus	S.epidermidis
52	58yr	Male	746532	7340	ET	S.epidermidis
53	60yr	Male	748770	7657	Pus	S.epidermidis
54	77yr	Male	748972	7696	Pus	S.epidermidis
55	80yr	Female		7895	Pus	S.hominis
56	81yr	Male	750515	7937	Pus	S.haemolyticus
57	26yr	Female	3938685	7999	Pus	S.haemolyticus
58	60yr	Female	745282	17936/37	Pus	S.epidermidis
59	22yr	Female	3946298	3136	Pus	S.epidermidis
60	19yr	Male	740422	6199	Pus	S.epidermidis
61	3 mnths	Female	742604	3110	Blood	S. intermedius
62	60yr	Female	741745	5855	Pus	S.haemolyticus
63	D6	Male	742344	6075	Blood	S.epidermidis
64	25yr	Male	3458397	6087	Pus	S.epidermidis
65	62yr	Male	750171	4105	Blood	S.epidermidis
66	40yr	Male	750579	4158	Blood	S.haemolyticus
67	5yr	Male	750630	4168	Blood	S.simulans
68	61yr	Male	751050	4209	Blood	S.haemolyticus
69	68yr	Female	740760	5725	Pus	S.epidermidis
70	81yr	Male	748886	3978	Blood	S.haemolyticus
71	58yr	Male	749010	3996	Blood	S.epidermidis
72	66yr	Male	749254	4026	ET	S.epidermidis
73	5yr	Female	749911	4080	Blood	S.haemolyticus
74	79yr	Male	748884	3977	Blood	S.epidermidis
75	72yr	Male	747378	3719	Blood	S.epidermidis
76	8yr	Male	748439	3927	Pus	S.cohnii
77	49yr	Male	748509	3941	Blood	S.haemolyticus
78	68yr	Male	748680	3954	Blood	S.warneri
79	74yr	Female	745591	3520	Blood	S.haemolyticus
80	65yr	Male	745794	3539	Blood	S.schleiferi
81	67yr	Male	746708	3651	Blood	S.epidermidis
82	46yr	Male	746975	3676	Blood	S.haemolyticus
83	75yr	Male	744648	3467	Blood	S.haemolyticus
84	25yr	Female	745070	3484	Blood	S.hominis
85	65yrs	Male	741645	3008	ET	S. hominis
86	D17	Male	741081	2962	Blood	S.haemolyticus

Introduction



Objectives

Review of Literature

Methodology

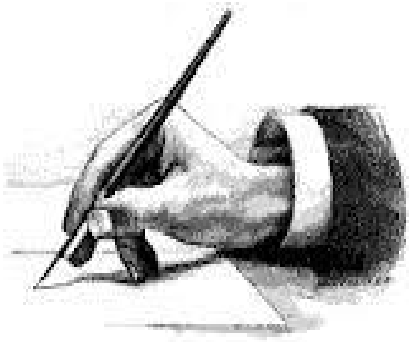
Results

Discussion

Conclusion

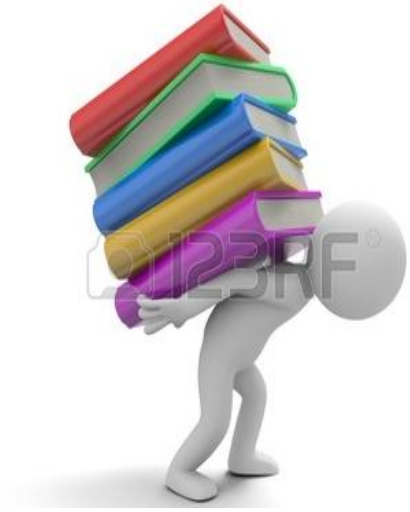
Summary

Bibliography



Annexure-I

Annexure-II



Annexure-III