PHENOTYPIC AND GENOTYPIC METHODS FOR DETECTION OF BIOFILM PRODUCING COAGULASE NEGATIVE STAPHYLOCOCCI IN A TERTIARY CARE HOSPITAL

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BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled **"PHENOTYPIC AND GENOTYPIC METHODS FOR DETECTION OF BIOFILM PRODUCING COAGULASE NEGATIVE STAPHYLOCOCCI IN A TERTIARY CARE HOSPITAL''** submitted by **Dr.P.THILAKAVATHY** to the Tamil Nadu Dr.M.G.R. Medical University, Chennai in partial fulfillment of the requirement for the award of M.D degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision and guidance.

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DECLARATION

I, Dr.P.THILAKAVATHY declare that, I carried out this work on **"PHENOTYPIC AND GENOTYPIC METHODS FOR DETECTION OF BIOFILM PRODUCING COAGULASE NEGATIVE STAPHYLOCOCCI IN A TERTIARY CARE HOSPITAL''** at the Institute of Microbiology, Madurai Medical college. I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree or diploma to any other University, Board, either in India or abroad.

This is submitted to The Tamil Nadu Dr.M.G.R Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

Dr.P.THILAKAVATHY

Place: Madurai

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CONTENTS

INTRODUCTION

Gram positive bacteria are one of the most common isolates in the clinical microbiology laboratory. They are widespread in nature and can be recovered from the environment or as commensal inhabitants of the skin, mucous membranes and other body sites mostly in human and animals. The ubiquity of these gram positive bacteria in nature makes the interpretation of their recovery from patients specimens occasionally difficult unless clinical manifestations of an infectious disease process are apparent. Recovery of these organisms from specimens should always be correlated with the clinical condition of the patient before their role in an infectious process can be established.[13]

In 1880, Sir William Ogston, a Scottish surgeon first showed that a number of human pyogenic disease were associated with a cluster forming microorganism. He introduced the name "Staphylococcus" (Greek Staphyle - bunch of grapes ; kokkos – grain or berry) Staphylococci were resistant to dry conditions, high salt concentration and are well suited to their ecological niche, which is the skin. In 1884, Rosenbach used pigment production to classify Staphylococci as virulent Staphylococcus aureus which produces golden yellow pigmentation and avirulent Staphylococcus albus, producing white colour colonies.[135]

Major emphasis have been placed by early medical bacteriologist in distinguishing Staphylococcus aureus, the pathogenic species from Staphylococcus albus, the presumed commensal Staphylococci. Since S.aureus was a major cause of morbidity and mortality, this distinction was of considerable importance as the clinical specimens often carried both types of organisms. The practical value of the coagulating principle (coagulase) of S.aureus was first demonstrated by Von Daranyi(1925) and it is one of the most important tests used to identify this species. In the clinical microbiology laboratory, Staphylococci were typically categorized as those that have the ability to coagulate rabbit plasma (i.e., coagulase-positive staphylococci or S.aureus) and those that do not coagulate (i.e., coagulase-negative staphylococci). The medically important Staphylococci comprised of more than 46 described species and subspecies.[14]

Based on morphological, physiological and biochemical tests Baird-Parker (1963) subdivided the genera Staphylococcus and Micrococcus into six subgroups (I to VI). In subsequent schemes, the species S.aureus, S.epidermidis, S.saprophyticus were recognized by Baird Parker and the latter two species were divided into several biotypes. Medical and food diagnostic laboratories widely used his schemes for more than a decade. [14]

Coagulase negative Staphylococci (CoNS) are normal commensal of skin and mucous membrane and are indigenous to a variety of mammalian hosts. Depending on the anatomical site, healthy human skin or mucous membrane support from 10^1 to 10^6 colony forming units(CFU)/cm^2 of Coagulase negative Staphylococci. With more than 40 recognized species and subspecies, CoNS are the most abundant microbes inhabiting the normal skin and mucous membranes. CoNS infrequently causes primary invasive disease and most commonly encountered by clinicians as contaminants of microbiological cultures. With the advent of changes in the practice of medicine and changes in underlying host populations, CoNS became formidable pathogens.[3]

Over the past few decades, however these organisms had become recognized as important agents of human disease. Infection associated with CoNS are urinary tract infections, osteomyelitis, native-valve & prosthetic valve endocarditis, intravenous catheter infections, CSF shunt infections, peritoneal dialysis catheter-associated infections, vascular graft infections, septicemia, ocular & cutaneous infections.[13]

Currently, CoNS are predominant cause of nosocomial bacteremia and infect a wide variety of prosthetic medical devices. CoNS are frequently encountered in indwelling medical devices and have been implicated as a cause of primary bacteremia. The natural niche on human

skin which results in easy access to prosthetic medical device inserted or implanted across the skin and adheres to biomaterials forming **biofilm** are the two major features owing to pathogenicity of CoNS. CoNS causes bacteremia in patients with indwelling medical devices such as permanent pacemakers, orthopaedic prostheses, artificial heart valves, central venous catheters and other infections involving biofilm formation on implanted biomaterials, bacteremia. Indolent infections are often caused by CoNS and may be clinically difficult to diagnose by routine diagnostic methods. [3]

CoNS isolated from nosocomial environments are almost always resistant to multiple antimicrobial agents. [136] Phenotypic expression of methicillin (oxacillin) resistance in CoNS is much more heterotypic than that observed in Staphylococcus aureus and the percentage of the population that expresses high -level oxacillin resistance is smaller. [137] Regardless of the degree of heterotypy observed, all isolates containing mecA (the gene conferring oxacillin resistance) are clinically resistant to all β – lactam antibiotics. [138]

A particular onerous aspect of treatment of most CoNS infections is their ability to form biofilms on biomaterials. Treatment could be more difficult due to increasing rates of resistance to antibiotics in CoNS and which may due to effect produced by biofilms on the host defense

mechanism. The increasing use of indwelling medical devices will augment the biofilm producing strains of CoNS .[3]

Infections caused by CoNS:

Overall CoNS has low pathogenic potential and the infection arise in the severe immunocompromised state or associated with prosthetic material implantation. Infants who are preterm, low birth weight and individuals who are in immunocompromised states such as cancer and prolonged chemotherapy renders them neutropenic and susceptible to CoNS with the potential to form biofilm and acquire resistance to multiple broad spectrum antibiotics.[3]

In case of neonates, within days of their admission to the neonatal intensive care unit CoNS colonizes their skin and in their anterior nares, pharynx and umbilicus and these organism were not originating from the mother but are acquired from the hospital environment and health care workers. Low birth weight, the presence and duration of use of central venous catheters and umbilical catheters, mechanical ventilation and total parenteral nutrition especially with intravenous lipid emulsions are the risk factors for developing Coagulase negative Staphylococcal bacteremia. CoNS infections in the neonate is frequently associated with morbidity and requires prolonged intensive care in the hospital.[3]

Depending on the host factors, the site where affected and the presence or absence of prosthetic material the clinical manifestations of CoNS infection varies. When compared with that of infection with S.aureus, there is a relative lack of the host inflammatory response in CoNS infection involving prosthetic material. Device related CoNS infection in an immunocompetent individual would usually result in mild local signs and symptoms compared with immunocompromised individuals who may present with a systemic response, often resulting in bacteremia and end organ failure in which the diagnosis of CoNS infection will be more difficult.[3]

In contrast to Staphylococcus aureus, which produces an array of toxins and adherence factors, there are few defined virulence factors in CoNS. The ability of the organism to adhere and form biofilm on the surface of biomaterials is thought to be the most significant virulence factor. However, other factors such as the secretion of poly-gamma-DL-glutamic acid (PGA) and phenol soluble modulins (PSMs), appear to complement and increase virulence.[3]

Biofilms:

Biofilms are microbially derived sessile communities which are characterized by the cells that are attached to a substratum in an irreversible form.[91] They produce chemotactic particles or pheromones

and the bacteria communicate with each other within the biofilm, a phenomenon called quorum sensing.[93] The layers of cell clusters in biofilm are embedded in a matrix of extracellular polysaccharide called polysaccharide intercellular adhesin (PIA). The biofilm formation is mediated by Polysaccharide Intercellular Adhesion(PIA).[15] PIA is β-1,6-N-acetylglycosamine and is synthesized b N-acetylglucosaminyl transferase. PIA production is encoded by the **ica gene (intercellular adhesion)** which are organized in an operon structure. Some of the factors influencing biofilm formation are availability of key nutrients, surface chemotaxis, bacterial motility, surface adhesins and presence of surfactants.[93] According to a research done on microbial biofilms by National Institutes of Health, they have published that there is involvement of biofilm formation in more than 80% of all infections.[53] Many medical conditions including indwelling medical devices, dental plaque, peritonitis, urogenital infections and upper respiratory tract infections are associated with formation of biofilms.[134] Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas aeruginosa.[130]

Bacteria survives and multiplies in most environmental niches as surface attached biofilms but not as planktonic cells suspended in liquids. On abiotic and biotic (host mucosal tissues) surfaces there is development of complex communities of single or multiple species of microorganisms. The nature of the biofilm and physiological state of bacterial cells within the biofilm represents a barrier to eradication and confers high level of resistance to antimicrobial agents. So the microorganisms growing in a biofilm exihibits intrinsically more resistant to antimicrobial agents than planktonic cells. As antibiotic resistance can increase to 500 fold, high concentrations of antimicrobials are required to inactivate organisms growing in a biofilm. There are several properties of biofilms that could contribute to increased resistance to antibiotics. The exopolysaccharide matrix or slime that surrounds the cells may create an exclusive barrier to antimicrobials, or directly complex with these agents to inactivate them. Suboptimal concentrations of antibiotics may actually enhance antibiotic resistance.[14] Bacteria in biofilm grows slowly and slower growth may lead to decreased uptake of the drug and other physiologic changes that could affect drug resistance.[3] Prevention of CoNS infection has largely concentrated on prevention of indwelling catheter - associated infection. Catheters should be inserted with meticulous attention to aseptic practices. Staff should adhere to appropriate aseptic protocols in caring

out the indwelling catheterisation. Prophylactic antibiotics will help in slightly slow progression of biofilms in biomaterials.[3]

The critical role in patient management will be removal of the colonized devices to eliminate the colonization which will reduce implant failure. Treatment duration depends on the immune status of the host as well as persistent presence of implanted prosthetic material. The results of antibiotic susceptibility test would be guide in choosing the antibiotic. Decolonization of the biofilms along with prolonged antibiotics were required for cure and in such cases relapse were not unusual. The major attributing factor had been poor penetration of antibiotics into bacterial biofilms. A multidisciplinary team approach for the management of patients with these often complex conditions could make an important contribution towards high standards of care which will reduces the hospital stay, implant failure and morbidity in a patients who requires critical care in tertiary care hospitals.[14]

Currently, in medical areas various methods are used for the detection of biofilm production which includes use of different types of microscopes and visual assessment can be done by electron microscopy. Confocal laser scanning microscopy (CLSM) remains the most versatile and effective nondestructive approach for studying biofilms and markedly reduces the need for pretreatments such as disruption and fixation,

reducing or eliminating the evidence of microbial relationships, complex structures and biofilm organization, encountered with scanning electron microscopes without the limitations.[112] However, in the routine laboratories qualitative methods such as the tube method(TM), [25] Congo red agar (CRA) method [26] and quantitative methods such as the tissue culture plate (TCP)[24] are used. Molecular techniques such as the polymerase chain reaction (PCR), which amplifies the genes (ica) involved in biofilm production complement these methods.

The present study was undertaken to detect the prevalence of biofilm producing and nonproducing CoNS which were isolated from various clinical materials in our laboratory by three different phenotypic methods such as Congo red agar (CRA) method, Tube method (TM), Tissue culture plate (TCP) method, PCR for detection of the ica gene and to find out the reliable method from above which can be recommended for routine detection of biofilm production in CoNS.

AIMS & OBJECTIVES

1. To find out the prevalence of CoNS in various clinical samples collected from Govt. Rajaji Hospital (GRH), Madurai.
2. To isolate and characterise the CoNS among the clinical samples.
3. To identify the biofilm producing strains of CoNS in clinical isolates by various phenotypic methods.
4. The antimicrobial susceptibility pattern among the biofilm producing and non biofilm producing CoNS isolates.
5. To compare the various phenotypic methods in the identification of biofilm producing CoNS with PCR and to find out the most sensitive and economic method which is close to PCR that can be recommended for routine screening of Biofilm production in Microbiological laboratories.

REVIEW OF LITERATURE

Nosocomial infections are defined as infections acquired during or as a result of hospitalization. Generally, a patient who has been in the hospital for < 48 hrs and develops an infection is considered to have been incubating the infection before hospital admission. Most infections that become manifest after 48 hrs are considered to be nosocomial. A patient may develop a nosocomial infection after being discharged from the hospital if the organism apparently was acquired in the hospital. **(Odetola et al)**[1]

Harrison's Principles of Internal Medicine, (18th ed., p.no1112) [2] states that in hospitalized patients, nosocomial infections contributes to significant morbidity and mortality as well as to excess costs. About 5% of patients admitted to an acute care hospital acquire a new infection, with more than 2 million nosocomial infections occurring per year and an annual cost of more than $ 2 billion. Even though factors such as underlying disease and severity of illness play an important role in outcome, it is believed that patients who develop a nosocomial infection, the odds of death are doubled. Although immune suppressed hosts are especially vulnerable to infections acquired in a hospital, even immune competent hosts are also prone to develop

nosocomial infections. The potential impact of nosocomial infections is considerable when assessed in terms of incidence, morbidity, mortality and financial burden.

The most common nosocomial infections are urinary tract infections, pneumonia and surgical site infections. However, primary bloodstream infections have increased in frequency as have infections in medical and surgical Intensive Care Units(ICU's) especially those associated with intravascular devices. The combined effect of patient's own flora and invasive devices accounts for atleast 25-50% of nosocomial infections which highlights the importance of improvements in the use of such devices **(Kasper et al)**[101]. **Slade et al** [36] has estimated that about 15% to 25% of patients in general hospitals have a catheter inserted sometime during their stay and that the use of urinary catheters has increased over the last two decades.

Donlan et al [91], **Raad et al** [97], **Souli et al** [98] stated that the use of synthetic material for implantation is widely associated with "Implant associated infection" due to **biofilm** production. In the long run they may be very damaging because of immune complex disease. [13] It has been estimated that 80% of wide variety of microbial infections in the body, biofilms have been found to be involved.[53] Infectious processes in which biofilms have been implicated include common problems such as urinary

tract infections, catheter infections, middle-ear infections, formation of dental plaque,[54],[111] gingivitis,[54] coating contact lenses,[55] and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, infections of permanent indwelling devices such as joint prostheses and heart valves,[56] tracheal and ventilator tubing. [57] **(Roger et al** [54]**, Demmer et al** [111] **, Imamura et al** [55]**,Lewis et al** [56]**, Parsek et al** [57]**)**. More recently it has been noted that there is a reduction in topical antibacterial efficiency used for healing or treating infected skin wounds as there is impairment in cutaneous wound healing due to bacterial biofilm formation stated by **Davis et al**.[58] Biofilms can also be formed on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices documented by **Auler et al.**[59]

Most bacteria in natural environments are organized in biofilms **(Dalton et al** [108]**, Costerton et al** [109]**,Stickler et al** [110]**).** The first recorded observation concerning biofilm was probably given by **Henrici in 1933 ,** who observed that water bacteria are not free floating but grow upon submerged surfaces **(Toole et al).**[90] Biofilm extra cellular polysaccharide (EPS), which is also referred to as **slime**, is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. Biofilms may form on living or non-living surfaces and

can be prevalent in natural, industrial and hospital settings. **(Lear et al** [114]**, Hall-Stoodley et al** [113]**)**. **James et al** [86] in their study compared the bacterial composition of various chronic wound types and microscopically analysed the chronic wound tissue specimens using a high level electron microscope and revealed that in 60 percent of the specimens there is presence of dense colonies of bacteria often surrounded by an extracellular matrix.

There are five distinct stages of biofilm development namely **1)** reversible attachment **2)** irreversible attachment **3)** maturation 1 **4)** maturation 2 and **5)** dispersion **(Saucer et al)**[14].

MANDELL [3] explains that **Biofilm** formation is thought to occur in three stages-adherent, maturation and dispersal. Biomaterials placed within a human host were rapidly coated with serum matrix proteins, including fibrinogen and fibronectin, collagen, vitronectin and elastin.(**Zhang et al,** [66] **Nilsson et al,** [67] **Hartford et al,** [68] **Gill et al** [69]**, Bowden et al,** [70][71]**).** Autolysins had the ability to bind directly to plastic and contain matrix protein binding sites.(**Heilmann et al).**[72][73] Lipase, in addition to its enzymatic function binds to collagen.[70] Following adherence to the biomaterial, intercellular adherence of the bacteria was primarily mediated by polymeric molecules. Recently, it had been shown that eDNA (extracellular DNA) is a major component of

biofilm produced by Staphylococci and mutants defective in DNA release produce deficient biofilms(**Qin et al,**[74]**Rice et al)**[75]. The last stage of biofilm development is dispersal and subsequent spread to other potential sites[62]. The production of phenol soluble modulins by the organism mediates the detachment of upper layers of the biofilm. PSMs were regulated by the quorum- sensing global regulator, which acts as surfactant leading to loss of cellular clusters.

In device - related bacteremias the most common pathogens isolated include CoNS, Staphylococcus aureus, Enterococci, nosocomial GNB and Candida [**Longo et al].**[2] **de Lalla et al** [4] stated that the predominant organism responsible for infective complications following surgical vascular grafts and implantation of prosthetic devices were Gram positive cocci and in particular Staphylococcus species. The Staphylococci are members of the family Micrococcaceae that also includes Micrococcus, Stomatococcus and Planococcus. These bacteria are catalase - positive, gram - positive cocci that divide in irregular clusters producing a "grapelike cluster" appearance **(Kloos et al)**[8]

O' Gara et al [7] has stated that CoNS, although considerably less virulent than Staphylococcus aureus, are among the most common cause of prosthetic device related infections. In most of the device related infections, in about 50-70% of catheter related infections CoNS are

responsible as causative organisms (**Huebner et al.**[6]) These infections are generally associated with the use of catheters and other medical devices. CoNS are the causative organism in 48-78% of infective complications following central nervous system shunt procedures (**Roos et al 1997),** [102]. CoNS are also responsible for a high proportion of prosthetic cardiac valve infection (40-50%) (**Ing et al)** [103], joint replacement infections (20-50%) (**Gentry et al)** [104] and majority of infections following neurosurgical procedures **(Roos et al)** [102]

Vuong C, Otto M et al .[5] stated that CoNS infections seems to be related to the health condition of the patient and also extracellular polysaccharide production. **Stoll BJ et al** [34] stated that among neonates the microorganisms most frequently encountered in nosocomial infections are the Coagulase-negative staphylococci (CoNS). In neonatal intensive care units (NICU) the most-frequent cause of late-onset sepsis among newborn infants are Coagulase-negative staphylococci (CoNS). Incidences of up to 66% of late-onset sepsis have been reported (**Vishal Hira et al**). Due to high rate of invasive procedures in immune compromised patients and also the bacterium's ability to form biofilms there is increased occurence of these infections. Biofilm producing Staphylococci frequently colonize catheters and medical devices and may

cause foreign body related infections. They easily get attached to polymer surfaces **(Thomas et al** [93]**, Murray et al**[94]**, Schwank et al** [95]**)**

Bannerman and Peacock et al [9] has stated that Staphylococcus epidermidis is the most frequently encountered CONS species associated with human infections and particularly associated with intravascular catheters. In indwelling medical device infections such as prosthetic-valve endocarditis, surgical wounds, central nervous system shunt infections, intravascular catheter-related infections, peritoneal dialysis-related infections, and infections of prosthetic joints, the predominant agent of nosocomial bacteremia is S. epidermidis. S. haemolyticus is the second most frequently encountered CONS species and has been implicated in native-valve endocarditis, septicemia, peritonitis, and wound, bone, and joint infections. Other CONS species are involved in a variety of infections. For example, in human urinary tract infections, especially in young, sexually active females the important pathogen is S.saprophyticus. S.lugdunensis has been implicated in arthritis, catheter infections and prosthetic joint infections. CoNS species were identified using the standard biochemical tests which includes catalase, DNase, coagulase production, growth and fermentation of mannitol on mannitol salt agar and susceptibility tests to bacitracin and novobiocin (**Kloos et al)** [8]

Archer et al [60] states that Staphylococcus epidermidis is the most frequently recovered organism accounting for 50% to over 80% of isolates. Staphylococcus epidermidis emerged as pathogen and has been synchronous with the widespread use of intravascular catheters in modern medicine. The inherent capacity of this organism to cause infection derives primarily from its ability to form biofilms on the inert substances of indwelling medical devices. **Rupp and Archer; Kloos and Bannerman et al** [10] has stated that in immunocompromised patients, S.epidermidis has emerged as a common cause of nosocomial infections. Septicaemia due to S. epidermidis is often associated with the use of catheters and other indwelling medical devices stated by **Kloos and Bannerman; Pfaller and Herwaldt; Peters et al** [11],[12]**.** The prevalence of methicillin - resistant Staphylococcus epidermidis (MRSE) strains (**Giacometti et al** [105]**, Jarlov et al** [106]**,Tammelin et al** [107]) and the emergence of vancomycin resistant in this species further complicate treatment of biomaterial infections.

Rupp et al [40][41] stated that the major virulence factor of S.epidermidis is the ability to adhere on the surface of biomaterials and form biofilms. other virulence factors such as Poly-Gamma-DL-Glutamic Acid(PGA), Lantibiotics documented by **Kocianova et al** [76] **Vuong et al**[77] S.epidermidis produces PGA. PGA is a cell surface –

associated, antiphagocytic polymer first described as a virulence factor in Bacillus anthracis.[78] PGA has bifunctional role and functions to inhibit innate host defense as well as facilitate colonization of human skin. S.epidermidis produces several lantibiotics (eg., epidermin, Pep5, epilancin, epicidin) which are bacteriocins. These thio-ether aminoacid – containing antimicrobial peptides are active against a variety of bacteria and may play a role in bacterial interference and successful colonization and persistence on human skin.(**Kupke et al**).[79] The major pathogenic factor in biofilm formation is mediated by a Polysaccharide Intercellular Adhesion(PIA) documented by **Eftekhar et al.** [15]

Koneman's textbook of diagnostic microbiology[13] explains the Biofilm formation by Staphylococcus epidermidis : Staphylococcus epidermidis strains from infections of indwelling medical devices have shown that these bacteria produce cell-surface and extracellular macromolecules that initiate and subsequently enhance bacterial adhesion to the plastic surfaces of foreign bodies to form a biofilm. Initial specific adherence to be largely mediated by a capsular polysacch aride-adhesin called PS/A(**Muller et al)**[79] PS/A is a high molecular weight, variably N-succinylated,β-1,6-linked polyglucosamine molecule that is encoded by the ica locus of the Staphylococcus epidermidis genome **(McKenney et al)**.[80] Purified PS/A can block

adherence of PS/A - producing S.epidermidis to plastic catheters in vitro, antibodies directed against PS/A also appear to block adherence to biomaterials. PS/A is also able to protect the organisms from complement mediated phagocytic killing (**Muller et al** [79]**, Shiro et al).**[81][82] Following initial adherence to biomaterials, pathogenesis of S.epidermidis infection apparently involves adhesion between cells that are adherent to the plastic surface, forming the rest of the bacterial cell/polysaccharide matrix biofilm.(**Aricola et al**).[51] Intercellular adhesion is mediated by a polysaccharide called PIA (Polysaccharide Intercellular Adhesion) along with some other cell -associated proteins (**Mack et al**).[83] PIA in the initiation and synthesis of biofilms on biomaterials is supported by studies with animal models documented by **Rupp et al**.[17],[84]

Mack D et al [16] stated that the layers of cell clusters in biofilm are embedded in a matrix of extracellular polysaccharide called polysaccharide intercellular adhesin (PIA), which consists of β-1,6-N-acetylglycosamine and is synthesized by N-acetylglucosaminyl transferase. **Nilsdotter - Augustiinsson et al** [18] documented that the products of the chromosomal ica gene(intercellular adhesion) mediates the synthesis of PIA , which are organized in an operon structure. This operon contains the icaADBC genes, in addition to the icaR gene which

exerts a regulatory function and is transcribed in the opposite direction. Four proteins are transcribed after activation of this operon namely IcaA, IcaD, IcaB and IcaC , which are necessary for the synthesis of PIA.

Recent studies have shown that accumulation-associated protein (Aap), DNA and RNA independently or in cooperation with the ica operon are the additional components, have also been suggested to be involved in CoNS biofilms stated by **Chokr et al.**[19] **Tomo et al** [20] documented that in the initial attachment, intercellular adhesion and biofilm formation of Staphylococcus aureus there has been association of Bap [biofilm-associated protein]. Interestingly, an alternative mechanism of biofilm formation that does not depend on PIA has been induced by Bap homologue protein (Bhp) and is found in human S. epidermidis strains.

Mack et al [30]**; Cramton et al** [32]**; Rachid et al** [33]**; Cerca et al** [31]**; Eftekhar and Speert**[15] documented that the amounts of biofilm produced in vitro by S.epidermidis are highly variable and greatly influenced by glucose and other environmental and growth conditions. **Kim L. Riddle et al**[35] has documented that many recalcitrant infections produced by biofilm producing bacteria are notoriously difficult to eradicate. Resistances to antibiotics were exhibited by various mechanisms like restricted penetration of antibiotic

into biofilms, decreased growth rate and resistant gene expression. **Klingenberg C, Aarag E, Ronnestad A et al**[38] stated that the biofilm protects CoNS against the action of antibiotics administered for the treatment of these infections and also against the patient's immune system and for cure, it is often necessary to remove the foreign body. **Afreenish Hassan et al**[37] documented in their study that biofilm producing bacteria had higher antibiotic resistance than non-biofilm producers.

Several genetic regulation mechanisms have been implicated in biofilm regulation such as quorum sensing and the novel secondary messenger cyclic-di-GMP unveils the rapid progress in biofilm research. Understanding the molecular mechanisms of biofilm formation has facilitated the exploration of novel strategies to control bacterial biofilms stated by **An S et al.**[85]

Zufferey et al[29]**,Freeman et al**[26]**,Christensen et al**[24],[25]**,Donlan et al**[27]**, Aparna et al**[28] has documented various methods of detection of biofilm formation which includes Tissue Culture Plate (**TCP**)[24], Tube method (**TM**)[25], Congo Red Agar method (**CRA**)[26], bioluminescent assay, piezoelectric sensors, and examination by fluorescent microscopy.[29] **Afreenish Hassan et al**[37] compared various methods to detect biofilm formation (TM , CRA & TCP

methods), and concluded that the for detection of biofilm producing bacteria, TCP can be recommended as a general screening method. **Mathur et al**[39] has documented that out of 152 Staphylococcal isolates tested, by TCP 53.9 % were biofilm producers and 46% were non-biofilm producers, performed by addition of 1% glucose in trypticase soy broth.

Adilson Oliveira et al[42] has investigated production of biofilm by the tube adherence test, among the 82 of the 100 isolates of CoNS were found to be biofilm producers, which includes 44 strains isolated from catheter tips, 23 isolated by blood culture, and 15 isolated from nares specimen. **Bose et al**[41]has stated that the TCP is the better screening test for biofilm production than CRA and TM. The test is easy to perform and assess both qualitatively and quantitatively. In his study, positivity rate of CRA method was higher than observed by other workers. **Ruzicka et al**[43] noted that among 147 isolates of S. epidermidis, biofilm formation detected by TM is 79 (53.7%) and 64 (43.5%) isolates by CRA and documented that TM is better for biofilm detection than CRA. **Baqai et al**[44] studied the biofilm formation among uropathogens by TM. According to their results, biofilm formation was detected by TM in 75% of the isolates CRA detected biofilm production in only 11isolates and 99 as non-biofilm producers. The sensitivity (11%), specificity (92%)

and accuracy (41%) of CRA method were very low when compared with other methods.

Knobloch et al[45] recommended the TCP method for detection of biofilm. Out of 128 isolates of S. aureus, 3.8% were detected as biofilm producers by CRA as compared to TCP which detected 57.1% biofilm producers. **Morales et al**[89]**, Cunha et al**[88] documented that TCP assay provides reliable result for biofilm detection in CNS and is adequate for routine use. CoNS growing within biofilm consists of atleast four metabolic states : aerobic growth, anaerobic growth, dormant cells and dead cells.[64] **Lewis et al** [65] hypothesized that these unique physiologic states found within a biofilm allow for tolerance to antibiotics and development of persister and dormant cells. **Crampton et al** [96] showed that like S epidermidis, S aureus also has ica locus encoding the function of intracellular adhesion and biofilm formation.

Gad et al[99], **Cafiso et al**[100] demonstrated the molecular technique(PCR) is more efficient in detecting the genes of the ica operon. In addition, these genes are important virulence markers of clinical CNS isolate since their expression is associated with the production of PIA, the most clearly characterized component of staphylococcal biofilms.

Aricola et al[46]; **Ziebuhr et al** [47]**; Galdbart et al** [48] suggested that a good predictor of biofilm formation for distinguishing blood and catheter related infecting organisms from contaminating bacteria is the detection of the ica locus in S.epidermidis**. de Silva et al**[49] evaluated the hypothesis that there is association of ica operon and biofilm production and concluded that the quantity of biofilm produced may be associated with the ability to cause CoNS infection. This conclusion suggests that the regulation of biofilm expression may play a central role in the disease process. **Gerke et al**[21]**, Freeman et al**[22] stated that the ica operon consisting of icaA, icaD, icaB and icaC genes mediates the synthesis of PIA production in S.epidermidis and is regulated by the product of icaR. Nacetylglucoseaminyl transferase which synthesizes the PIA polymers is encoded by IcaA and D and icaC is responsible for formation of long polymer chains and icaB deacetylates the poly-N-acetylglucoseamine molecule.

Seung – Hak - Cho et al[23] has analyzed 41 S.epidermidis isolates obtained from catheter-related urinary tract infections for the presence of the icaADBC operon and biofilm formation and found that ica-specific DNA was present in 18 out of 41 isolates (44%), but only 11 isolates (27%) produced biofilms spontaneously under normal growth conditions. Upon induction by external stress or antibiotics, biofilm

formation could be stimulated in five of seven ica-positive, biofilm-negative isolates, indicating that the icaADBC expression was down-regulated in these strains.

Galdbart, J. O.,et al[50] studied the association of S.epidermidis isolated from prosthetic material related joint infection and demonstrated that ica was positive in 44 out of 54 isolates, compared with 2 of 23 isolates from eight healthy individuals. **Arciola, C. R**[51] documented that out of 68 S.epidermidis isolates associated with intravenous-catheter-related infection ica was positive in 33 isolates compared with none of 10 isolates from the skin or mucosa of healthy volunteers. **Frebourg, N. B**[52] showed that ica was more than twice as frequent in isolates associated with infection when he compared the ica gene in S.epidermidis associated with either bacteremia, blood culture contamination, or colonized intravenous devices and S. epidermidis from normal flora of healthy volunteers who were not hospitalized.

MATERIALS AND METHODS

The present study was conducted in Government Rajaji Hospital, Madurai, attached to Madurai Medical College. The study period was from June 2011 to May 2012. Ethical committee clearance from the institution was obtained before starting the study and informed written consent was received from the patients before collecting the specimens. A total of 456 clinical samples were collected from the patients admitted in various wards at Government Rajaji Hospital, Madurai.

SOURCE AND SAMPLE SIZE:

Pus, wound swab samples (from infected bone & joint prosthetic implants, surgical site infections), indwelling catheter samples, blood samples, urine samples were collected from 456 patients admitted at Govt. Rajaji Hospital during the study period.

INCLUSION CRITERIA:

1. All age groups and both sexes were included
2. Patients admitted to various wards (ICU, CCU, IRCU, Orthopeadic, plastic surgery, medicine) with signs and symptom suggestive of impending infections such as infected implants, surgical site infection, urinary tract infection, septicemia, pyrexia of unknown origin were included in this study.

COLLECTION OF SPECIMEN:

COLLECTION OF BLOOD SAMPLE:

Blood samples were collected by sterile aseptic procedures . The hands were kept clean and dry, sterile gloves were worn. The skin over the venepuncture site was disinfected by applying 70% alcohol followed by 1% iodine or 1-2% chlorhexidine for atleast 1 min and allowed to dry. With precautions to avoid touching and recontaminating the venepuncture site, 5 ml of blood was withdrawn. The withdrawn volume of blood was inoculated into the appropriate volume of brain heart infusion broth.

COLLECTION OF URINE SAMPLES :

Urine sample was collected from catheterized patients. Urinary catheterization will allow collection of bladder urine with less urethral contamination. Specimen collection from such patients was done with scrupulous aseptic techniques. A pair of gloves were worn while handling urinary catheters. The catheter tubing was clamped off above the port to allow collection of freshly voided urine. With 70% alcohol, clean vigorously the catheter port or wall of the tubing and by using sterile syringe with needle aspirate the urine, introduction of organisms into the bladder was prevented by maintaining the integrity of closed drainage system. The collected urine transported in a sterile, wide

mouthed, screw capped container. Clean catch midstream urine sample was collected from other patients with proper instructions.

COLLECTION OF WOUND SWAB SAMPLE:

Two sterile cotton swabs were used to collect the samples from patients, which can be used for direct smear examination and culture respectively. The swabs were transported in sterile test tubes to the laboratory.

COLLECTION OF PUS SAMPLE:

The site from which the culture was to be obtained should first be decontaminated with 70% ethyl or isopropyl alcohol. After which the wound was washed well with sterile saline and dried. Using sterile syringe and needle, pus was aspirated and then specimen transported in a puncture proof container.

PROCESSING OF SAMPLES:

The collected samples were properly labeled with Name. Age, Sex and IP / OP no of the patient, date and time of collection of the sample and brought to the laboratory and processed immediately.

WOUND SWAB AND PUS SAMPLES:

Direct Gram stain was done on samples followed by inoculation on to the following solid media

a) Nutrient Agar

b) Mac Conkey Agar

c) Blood Agar

BLOOD SAMPLES:

Blood culture broth were incubated at 37^0C for 18-24 hrs after which the broth were examined for turbidity and subcultured in to above solid media for isolation of organisms. The broths which were clear kept for further incubation.

URINE SAMPLES:

Urine samples were inoculated on to the NA, Mac Conkey, Blood agar. Before inoculation, urine was mixed thoroughly and the top of the container was then removed. The calibrated loop was inserted vertically into the urine in a cup. The centre of the plate was touched with the loop and the inoculum was spread in a line across the diameter of the plate. Without flaming or re-entering urine, the loop was drawn across the entire plate, the first inoculum was crossed numerous times to produce isolated colonies. A colony count of $>10^5$ CFU/ml ml was taken as indicative of a positive culture .

CULTURE IDENTIFICATION:

After 24 hr of incubation, plates were examined for the presence of growth and the organisms were identified by Gram stain, colony morphology on solid media and biochemical reactions and other identification tests.

IDENTIFICATION OF COAGULASE NEGATIVE STAPHYLOCOCI (CoNS):

1. Gram staining : Gram positive cocci arranged in clusters
2. Catalase test : Positive
3. Nutrient agar : Small, circular, low convex, opaque colonies
4. Mannitol : Not fermented
5. Tube Coagulase test : Negative
6. DNase test : Negative

SPECIES IDENTIFICATION OF CoNS:

Based on biochemical assays such as production of Phospahatase, Susceptibility to Novobiocin, Ornithine decarboxylase test and Beta hemolysis on Blood agar, species of CoNS were identified. Staphylococcus epidermidis was the most frequently encountered CONS species followed by S.saprophyticus, S.lugdunensis & S.haemolyticus.

TEST	S.epidermidis	S.haemolyticus	S.lugdunensis	S.saprophyticus
NOVOBIOCIN	S	S	S	R
PHOSPHATASE	+	–	–	–
ORNITHINE DECARBOXYLASE	–	–	+	–
β -HEMOLYSIS	–	+	–	–

IDENTIFICATION TESTS:

GRAM STAINING:

Smear was prepared from the test organism taken from the agar plate, air dried and heat fixed. The smear was flooded with 0.5% methyl violet and washed with water after 1 minute. Then Gram's iodine is added to the smear and washed with water after 1 minute. This was decolorized with few drops of acetone and immediately washed with water. The counter stain, 1:10 dilute carbol fuchsin was added to the smear and washed with water after 1 minute, the smear was dried with blotting paper and viewed under oil immersion objective. Violet colored spherical cocci arranged in clusters were identified as Gram positive cocci.

CATALASE TEST: (Tube method)

Procedure:

Two to three ml of 3% Hydrogen peroxide was taken in a clean test tube. Few colonies of the test organism were taken from the nutrient agar plate with a sterile wooden stick or glass rod and immersed in the Hydrogen peroxide solution.

Interpretation:

Brisk effervescence produced immediately is considered as Catalase positive. The organisms producing Catalase will split Hydrogen peroxide into water and oxygen, and the effervescence was due to release of nascent oxygen. The genus Staphylococci were catalase positive.

COAGULASE TEST:

This test was done to identify Coagulase negative Staphylococci from Staphylococcus aureus. The enzyme coagulase causes clotting of plasma by converting fibrinogen to fibrin. There are two types of coagulase :

a) Free Coagulase: it activates coagulase reacting factor present in plasma and converts fibrinogen to fibrin resulting in clot formation.

b) Bound Coagulase (clumping factor) which converts fibrinogen to fibrin not depending on reacting factor. It can be detected by clumping of bacterial cells in the rapid slide test.

Procedure :

Slide Test Method:

1. Place a drop of saline on each end of a clean slide.
2. Make two thick suspensions by emulsifying the colony of test organism with each of the drops.
3. To one of the suspension add a loopful of rabbit plasma and mix it gently, plasma was not added to 2nd suspension which was used as a control.

Interpretation:

Positive slide Coagulase test : Clumping within 10 seconds.

Negative slide Coagulase test : No clumping.

Tube Test Method:

Procedure:

1. Three small clean test tubes were taken. One tube was used for testing the isolate from primary culture plate. Emulsify a small amount of the colony growth of the organism in a tube containing 0.5ml of undiluted rabbit plasma.
2. The other tubes were used as positive and negative controls. A known Staphylococcus aureus strain and a Staphylococcus epidermidis strain serve as positive and negative controls.

3. The tubes were incubated at 35^0C for 4 hours and observe for clot formation by gently tilting the tube. If no clot was observed at that time, the tubes were reincubated at room temperature and read after 18 hours

Interpretation:

Positive tube Coagulase test: Clotting of tube contents or fibrin clot in tube.

Negative tube Coagulase test: No clotting.

DNase TEST:

This test was done to identify CoNS which incapable of producing deoxyribonuclease (DNase) enzyme from S.aureus which produces DNase enzyme, which is capable of hydrolyzing DNA.

Procedure :

1. The DNase agar plate was divided into sections by drawing lines on its bottom and the sections were numbered to denote the strain to be applied to them.
2. A colony was picked from the primary culture plate spot inoculated on to a small area of the medium.
3. Known DNase positive and DNase negative cultures were spot inoculated on other sections as control. The plate was kept for overnight incubation.

4. Then the plate was flooded with a few ml of 1 mol/L, 3.6% hydrochloric acid to precipitate unhydrolysed DNA.

5. The plate was examined within 5 minutes.

Interpretation:

DNase positive strain : Clearing around the colonies.

DNase negative strain : No clearing around the colonies

TEST FOR SPECIES IDENTIFICATION OF CoNS:

PHOSPHATASE TEST:

Culture growth was inoculated on to Phenolphthalein diphosphate agar medium and kept for overnight incubation. After overnight incubation a few drops of ammonia solution added.

Interpretation :

Positive colonies turned pink within a few minutes. S.epidermidis produces phosphatase enzyme.

NOVOBIOCIN SUSCEPTIBILTY TEST:

The novobiocin test is performed as a disk susceptibility test using a novobiocin disk (5µg). The test done by including a 5µg novoobiocin disc in the disc diffusion test for antibiotic sensitivity.

Interpretation:

Strains resistant to novobiocin will show zones measuring 6 mm (no zone) to 12 mm; susceptible strains will have zones of 16 mm or larger. S.saprophyticus is novobiocin resistant.

ORNITHINE DECARBOXYLASE TEST:

For determining the decarboxylase capability Moeller decarboxylase medium was used as the base. Before inoculating the test organism ornithine was added to the base. The base without the amino acid is kept in a tube as control. Inoculate both the two tubes with the test organism. Both the tubes were overlaid with sterile mineral oil to cover about 1 cm of the surface and then kept for incubation. S.lugdunensis produces ornithine decarboxylase enzyme.

Interpretation:

Violet colouration of the medium denotes production of the decarboxylase, and a yellow colouration as a negative reaction.

ANTIBIOTIC SUSCEPTIBILITY TEST:

PREPARATION OF INOCULUM:

Morphologically similar 4 to 5 isolated colonies of CoNS were taken from 24 hr culture plate with the help of a sterile loop and transferred to a test tube containing sterile peptone water and incubated

for 4 hrs at 35^0C . Then the turbidity was adjusted to 0.5 McFarland turbidity standards by using Vickeum chart. This inoculums was used for antibiotic susceptibility testing .

According to CLSI guidelines (CLSI document M02-A10), the antibiotic susceptibility test was performed by using the Kirby-Bauer disc diffusion technique. Mueller Hinton agar was used for antibiotic susceptibility test. Zone size was interpretated using control strain Staphylococcus aureus (ATCC 25923) and following antibiotic discs were used.

Antimicrobial Drug	**S**	**I**	**R**
Ampicillin (AMP 10µg)	≥29	-	≤ 28
Gentamicin (GM 10µg)	≥ 15	13 – 14	≤ 12
Ciprofloxacin (CIP 5µg)	≥ 21	16 – 20	≤ 15
Cefotaxime (CTX 30µg)	≥ 23	15 – 22	≤ 14
Chloramphenicol (C30 µg)	≥ 18	13 – 17	≤ 12
Cotrimoxazole (COT 25 µg)	≥ 16	11 – 15	≤ 10
Erythromycin (ERM 15µg)	≥ 23	14 – 22	≤ 13
Doxycycline(DOX 30µg)	≥ 16	13 – 15	≤ 12

Zone size(mm): **S-** sensitive, **I-** intermediate, **R-** resistant

DETECTION OF BIOFILM PRODUCTION :

Detection of biofilm production in CoNS isolates was done by the following methods :

1..Congo Red Agar method (CRA)

2.Tube method (TM)

3.Tissue Culture Plate (TCP)

4.Detection of ica genes by PCR.

CONGO RED AGAR METHOD:

A qualitative method for detection of biofilm production and medium used was Congo Red Agar (CRA) medium

Procedure

The test organisms were inoculated in CRA and kept for incubation at 37^{o}C for 24 h aerobically.

Interpretation:

Biofilm producer:	Colony morphology
High	colonies with black colour and a dry crystalline consistency
Moderate	darkening of the colonies without dry crystalline consistency
Weak/Non-biofilm producers	pink colored colonies.

TUBE METHOD:

A qualitative method for detection of biofilm production.

Procedure:

1. The test organisms were inoculated in 10 mL of trypticase soy broth taken in the sterile test tubes.The tubes were kept for incubation.
2. Then the tubes were decanted and by using phosphate buffer saline pH 7.3) the tubes were washed and then allowed to dry.
3. By using safranin (0.1%) the tubes were stained and deionized water was used to remove excess stain.
4. Tubes were kept in inverted position and allowed to dry. The control strains were included in the test and according to the results the scoring was done

Interpretation:

Biofilm production:

- The wall and the bottom of the tube were lined by a visible film.
- The amount of biofilm formed was scored as 1- weak/none, 2-moderate, 3-strong.

TISSUE CULTURE PLATE METHOD:

This is a quantitative method for biofilm detection.

Procedure:

1. The test organisms were inoculated in10 mL of trypticase soy broth broths and kept for overnight incubation.
2. A dilutions of :100 was done for the cultures by using fresh medium. Add 200 μL of the diluted cultures into individual wells of sterile 96 well flatbottom polystyrene tissue culture and then incubated.
3. Then the organisms used for control (positive and negative) were diluted and added to the microtitre plate and kept for incubation [Staphylococcus epidermidis ATCC 35984 (biofilm producer) and ATCC 12228 (biofilm nonproducer)]
4. Then gentle tapping was done to remove the contents of the well
5. Washing of the wells was done with 0.2 mL of phosphate buffer saline (pH 7.2) and then wells were washed four times to remove the free floating bacteria.
6. After washing 2% sodium acetate was used to fix adherent bacteria in the wells and by using crystal violet (0.1%) the wells were stained and deionized water was used to remove excess and then allowed to dry.

7 Reading was taken at wavelength 570 nm by micro ELISA autoreader. As the bacteria forms biofilm and adheres to the wells, these OD values were taken as an index of bacterial adherence to the wells.

Interpretation:

Mean OD values	Biofilm production
<0.120	Non/weak
0.120-0240	Moderate
>0.240	High

OD cut off value = average of negative control

+3x standard deviation (SD) of negative control.

DETECTION OF icaADBC GENE:

POLYMERASE CHAIN REACTION (PCR)

PCR was done by the following protocol to detect ica gene in CoNS isolates.

DNA extraction from CoNS isolates:

1 1.5 ml of overnight CoNS culture was transferred to 1.5 ml centrifuge tube.

2 Centrifuged at 10000 rpm for 5 mins; supernatant discarded.

3 Bacterial Pellet is suspended in 200µl of PBS

4 50µl of Lysozyme [50mg/ml] is added and Incubated at 37ºC for 15min.

5 Added 400µl of Lysis Buffer and 40µl of Proteinase K [Reconstituted]

6 Mix immediately by inverting and incubate at 70ºC for 10min.

7 Added 100µl of Isopropanol and mixed well.

8 Pipetted entire sample into the PureFast® spin column.After centrifuging for 1 min discard the flow through and column was placed back into the same collection tube.

9 Added 500µl Wash Buffer-I to the PureFast® spin column. After centrifuging for 30-60 seconds discard the flow-through and the column was placed back into the same collection tube.

10 Added 750µl Wash Buffer-II to the PureFast® spin column. After centrifuging for 30-60 seconds discard the flow-through and the column was placed back into the same collection tube.

11 Repeat Step 8 once.

12 After discarding the flow-through an additional centrifugation for 1 min was done to avoid residual ethanol.

13 PureFast® spin column is transferred into a fresh 1.5 ml micro centrifuge tube.

14 Added 100µl of the pre-warmed Elution Buffer to the PureFast® spin column.

15 Then incubated for two minutes at room temperature and centrifuged for two minutes.

16 Discard the column and stored the purified DNA at -20°C. For gel analysis,

loaded 10 - 20µl of elute.

PCR Procedure:

Reactions set up as follows;

Components :(total volume - 50µl)

1 Mastermix - 25µl

2 ica primer (10pmoles/µl)

F - **iCA-F-TCCAGAAACATTGGGAGGTC**

ica primer (10pmoles/µl)

R - **iCA-R-TGGGTATTCCCTCTGTCTGG**

3 Nucleus free water 22 µl

4 Test samples (Genomic DNA) 1µl Mixed gently and spin down briefly.

5 Place into PCR machine and program it as follows;

Program: (total cycles run – 30)

Initial Denaturation: 94ºC for 3 min

Denaturation	:	94ºC for 1 min	30 cycles
Annealing	:	58ºC for 1min	
Extension	:	72ºC for 1min	

Agarose gel electrophoresis:

1. Prepared 2% agarose.
2. When the temperature of agarose gel was around 60ºC, 5µl of Ethidium bromide was added.
3. Warm agarose solution was poured slowly into the gel platform.
4. The gel was kept undisturbed till the agarose sets and get solidified.
5. To the submarine gel tank ,1XTAE was poured.
6. The gel platform is carefully placed into tank and a buffer level 0.5cm was maintained above than the gel.
7. After mixing with the gel loading dye and 10µl 100bp DNA Ladder the PCR Samples were loaded.
8. Run electrophoresis at 50V till three fourth distance had been reached in the gel.
9. UV Transilluminator was used to view the gel and the bands pattern were observed.

Interpretation:

The presence of ica gene was indicated by the amplification of 100bp PCR product from the clinical isolates.

STATISTICAL EVALUATION:

Considering the PCR as the standard test, statistics was applied for calculating Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) for each method .

RESULTS

Samples of pus, wound swab, blood and urine collected from 456 cases admitted at Govt.Rajaji Hospital, Madurai were included in this study. This study included both sexes of all age group. Out of 456 samples, 165 from wound swab, 103 from pus, 96 from blood and 92 from urine. Among the 456 samples, 424 showed growth and 32 samples showed no growth. Among the 424 isolates, 252 Gram positive cocci (GPC) in groups were Staphylococci species and 172 were Gram negative bacilli (GNB).

Table -1

SPECIMEN WISE ISOLATION OF ORGANISMS

n=456

Specimen	**Staphylococci**	**GNB**	**No growth**	**Total**
Wound swab	108(23.68%)	51(11.18%)	6(1.32%)	165
Urine	55(12.06%)	34(7.45%)	3(0.65%)	92
Blood	54(11.84%)	30(6.58%)	12(2.63%)	96
Pus	35 (7.68%)	57(12.50%)	11(2.41%)	103
Total	252(55.26%)	172(37.71%)	32(7.01%)	456

Among the 252 Staphylococci species, 108 were isolated from wound swab, 55 from urine specimen, 54 from blood and 35 from pus

samples. From the above observation it is inferred that **Staphylococcal species were the common isolates in various clinical specimens and wound swab specimen was the common specimen in which more Staphylococci were isolated.**

Out of 252 Staphylococci species, 96 were found to be Coagulase test negative and 156 were Coagulase test positive.

Table – 2

IDENTIFICATION OF COAGULASE NEGATIVE STAPHYLOCOCCI (CoNS)

n=25

Tube Coagulase test	**Staphylococci (no of isolates)**
Positive	156(61.9%)
Negative	96(38.1%)

From the above table it is inferred that nearly **1/3rd of Staphylococcal species were Coagulase negative Staphylococci.**

Among the 96 CoNS, 31 were isolated from wound swab, 28 from blood, 19 from urine specimen and 18 from pus specimen.

Table - 3

SPECIMEN WISE ISOLATION OF CoNS

n=96

Specimen	No.of cons isolates	Percentage
Wound swab	31	32.29%
Blood	28	29.17%
Urine	19	19.79%
Pus	18	18.75%

From the above observation it was found **that CoNS was isolated more from wound swab samples.**

Out of 96 CoNS isolates, 76 isolates were Staphylococcus epidermidis, 5 isolates were Staphylococcus haemolyticus, 3 isolates were Staphylococcus lugdunensis, 3 were Staphylococcus saprophyticus and 9 were other species of CoNS.

Table -4

SAMPLE WISE IDENTIFICATION OF CoNS SPECIES

n=96

Specimen	Staphylococcus Epidermidis	Staphylococcus Haemolyticus	Staphylococcus Lugdunensis	Staphylococcus saprophyticus	Other species	Total
Wound swab	26 (27.08%)	2 (2.08%)	1 (1.04%)	-	2 (2.08%)	31
Blood	22 (22.92%)	2 (2.08%)	1 (1.04%)	-	3 (3.13%)	28
Urine	16 (16.67%)	-	-	3 (3.13%)	-	19
Pus	12 (12.50%)	1 (1.04%)	1 (1.04%)	-	4 (4.17%)	18
Total	76 (79.17%)	5 (5.21%)	3 (3.13%)	3 (3.13%)	9 (9.38%)	96

Thus it is inferred that **Staphylococcus epidermidis was the most common CoNS species isolated from specimens more commonly in wound swabs followed by blood, urine and pus specimens.**

All the 96 CoNS isolates were subjected to Biofilm detection by various phenotypic methods such as Congo red agar, Tube method, Tissue culture plate method. By Congo red agar method only 7 isolates showed black colonies with crystalline appearance, 10 isolates showed black colonies but no dry crystalline morphology and 79 isolates displayed pink colored colonies. By Tube method, the number of strong biofilm producers were 12, moderate were 17 and weak or non-biofilm producers were 67. In Tissue culture plate method, strong biofilm producers were 17, 21 were moderate biofilm producers and 58 considered as weak or none biofilm producers.

Table – 5

SCREENING OF THE CoNS ISOLATES FOR BIOFILM PRODUCTION BY PHENOTYPIC METHODS

n=96

No. of CoNS isolates (96)	Biofilm production	Tissue Culture plate (TCP)	Tube method (TM)	Congo red agar(CRA)
No. of biofilm producers	High	17(17.70%)	12(12.5%)	7(7.29%)
	Moderate	21(21.87%)	17(17.71%)	10(10.41%)
	Total	38(39.58%)	29(30.21%)	17(17.71%)
No. of non biofilm producers	Weak/None	58(60.42%)	67(69.79%)	79(82.29%)

From the above table, it is observed that **the number of biofilm producer detected by tissue culture plate method was high 38 (39.58%)**, followed by Tube method and Congo red agar method.

Among the 38 biofilm producing CoNS isolates, sample wise analysis of Biofilm production showed that 16 out of 31 CoNS isolates were detected as Biofilm producer from wound swab specimens, 11 out of 28 from blood, 9 out of 19 from urine, 2 out of 18 CoNS isolates from pus specimen were identified as Biofilm producer

Table - 6

SAMPLE WISE ISOLATION OF BIOFILM PRODUCING CoNS

n=96

Specimen	No. of Cons isolates	No. of Biofilm producers	Percentage
Wound swab	31	16	16.67%
Blood	28	11	11.46%
Urine	19	9	9.37%
Pus	18	2	2.08%
Total	96	38	39.58%

From the above table it is inferred that the **percentage of biofilm producing CoNS isolated from wound swab sample was high** followed by blood, urine and pus specimens.

Out of 38 biofilm producing CoNS isolates, 37 were Staphylococcus epidermidis, 1 was Staphylococcus saprophyticus .

Table-7

SPECIES AND SPECIMEN WISE DISTRIBUTION OF BIOFILM PRODUCING CoNS

n=96

Specimen	Staphylococcus Epidermidis	Staphylococcus Saprophyticus	Total
Wound swab	16 (16.67%)	-	16
Blood	11 (11.46%)	-	11
Urine	8 (8.33%)	1 (1.04%)	9
Pus	2 (2.08%)	-	2
Total	37 (38.54%)	1 (1.04%)	38

From the above table it is inferred that **Staphylococcus epidermidis was the most common biofilm producing CoNS species and more number of Biofilm producers were isolated from wound swab specimen** followed by blood, urine and pus specimens.

Analysis of agewise distribution of biofilm producing CoNS showed that among the biofilm producing CoNS isolates 7(18.42%) were from the age group 0 -14yrs, 9(23.68%) from the age group of 15-45 yrs, 10 (26.32%) from the age group of 46-60 yrs and 12 (31.58%) from the age group of >50yrs.

Table – 8

AGE WISE DISTRIBUTION OF BIOFILM PRODUCING CoNS

n=38

Age group	No. of Biofilm producer	Percentage
0-14	7	18.42%
15-45	9	23.68%
46-60	10	26.32%
>60	12	31.58%

From the above table it is inferred that the percentage of Biofilm producing CoNS isolated from the age group of >60yrs was the highest followed by46-60 yrs of age.

Among the 38 Biofilm producing CoNS isolates, 29(76.32%) were associated with risk factors. Analysis of Biofilm production with risk factors showed that 15 patients had orthopaedic implants which were infected, 8 patients had urinary catheterization, 6 patients had history of prosthetic valve implantation.

Table-9

PERCENTAGE OF BIOFILM PRODUCING CoNS ASSOCIATED WITH RISK FACTORS

n=29

Risk factors	No.of Biofilm producer	Percentage
Infected Orthopaedic implants	15	39.47%
Urinary catheterization	8	21.05%
Prosthetic valve	6	15.79%

From the above table it is found that **Biofilm production had a strong association with medical device related infections such as infected orthopaedic implants, urinary catheterization and prosthetic valve.**

All the 96 CoNS isolates (Biofilm and non biofilm producers) were subjected to antibiotic susceptibility testing by Kirby Bauer disc diffusion method.

Table-10

ANTIBIOGRAM OF BIOFILM PRODUCING AND NON BIOFILM PRODUCING CoNS ISOLATES

Antibiotics	Biofilm producers (38)		Non biofilm producers (58)	
	Sensitive	**Resistant**	**Sensitive**	**Resistant**
Ampicillin	3(7.89%)	35(92.11%)	8(13.79%)	50(86.21%)
Gentamicin	20(52.63%`)	18(47.37%)	36(62.06%)	22(37.93%)
Ciprofloxacin	12(31.58%)	26(68.42%)	39(67.24%)	19(32.75%)
Cefotaxime	23(60.53%)	15(39.47%)	45(77.59%)	13(22.41%)
Chloramphenicol	15(39.47%)	23(60.52%)	38(65.51%)	20(34.48%)
Cotrimoxazole	14(36.84%)	24(63.15%)	41(70.69%)	17(29.31%)
Erythromycin	22(57.89%)	16(42.11%)	43(74.13%)	15(25.86%)
Doxycycline	21(55.26%)	17(44.73%)	42(72.41%)	16(27.59%)

From the above table it is observed that **Biofilm producers showed higher antibiotic resistance than non biofilm producers**. Among the **Biofilm producer highest antibiotic resistance were noted against Ampicillin followed by Ciprofloxacin, Cotrimoxazole, Chloramphenicol, Gentamicin, Doxycycline**, **Erythromycin and Cefotaxime.**

PCR was done to identify **ica gene** in CoNS isolates which were found to be biofilm producers by phenotypic methods. Among the 35 PCR positive Biofilm producing CoNS isolates, TM identified 6 isolates, CRA 18 isolates as false negative and none of the isolate was identified as false negative by TCP. Among the PCR negative isolates, TCP method identified 3 isolates as falsely positive .The false positive identified by TM and CRA were 5 and 7 respectively.

Table -11

COMPARISON OF PCR WITH OTHER

PHENOTYPIC METHODS

Methods	**True positives**	**False negatives**	**False positives**
PCR	35	-	-
TCP	35	-	3
TM	24	6	5
CRA	10	18	7

From the above table it was found that **Tisssue culture plate method showed only three false positives and thus very close to PCR in identifying True positives**.

Table-12

SENSITIVITY AND SPECIFICITY OF VARIOUS PHENOTYPIC METHODS

Methods	Sensitivity	Specificity	PPV	NPV
TCP	100%	95.08%	92.1%	100%
TM	80%	92.42%	82.75%	97.04%
CRA	35.71%	89.71%	58.82%	77.22%

From the above observation it was found that **Tissue culture plate method had the highest sensitivity and specificity, the PPV and NPV were 92.1% and 100% respectively.** TM showed 80% sensitivity and 92.42% specificity with 82.75% PPV and 97.04% NPV. CRA method had the least sensitivity (35.71%) and specificity (89.71%) with PPV and NPV of 58.82% and 77.22%.

DISCUSSION

CoNS have been major cause of nosocomial infections in tertiary health care settings.[132] Since 1950, these organisms have been reported with increasing frequency. Taking into consideration, the increased frequency of isolation of CoNS from clinical specimens, they must be individually evaluated as potentially true pathogens.[119] The major virulence factor determining the pathogenicity of CoNS has now well defined and found to be **Biofilm production**. Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. In this study we evaluated 96 CoNS isolates by Phenotypic and Genotypic methods for their ability to form biofilms.

In the present study, out of 456 samples processed, 252 (55.26%) were Staphylococcal species. Among the 252 Staphylococcal species, 96(38.1%) were Coagulase negative Staphylococci (CoNS). The results were correlated with the study conducted by **Mahajan VM et al** [124] who documented that out of 145 Gram positive, catalase positive isolates, 88(60.6%) isolates were Coagulase negative Staphylococci. However, **Fule RP et al**[119] reported that only 39(23.6%) isolates of CoNS and 165 of Coagulase positive Staphylococci from various clinical specimens. Nearly 1/3rd of CoNS were isolated among the Staphylococcal species from various clinical specimen in the above studies. CoNS were generally

considered as saprophytes with little pathogenic potential. Over the past four decades, however these organisms have become recognized as important agents of human disease.[13] .CoNS are pathogenic when alterations in the integument allow these normal skin inhabitants to gain entry into the body.[128]

The present study showed that CoNS was isolated more 31(32.29%) from wound swab specimen collected from infected joint prostheses and surgical site infections. The same were reported by **Sewell CM et al**,[128] in that majority of CoNS isolates (43%) were from exudates, **Gaikwad SS and Deodhar LP et al**,[126] documented that drainage from wound was the frequent source of CoNS 40(76.92%). However **Narayani TV et al** [127] had reported majority of CoNS isolates from urine specimen(52%) will counter this current study. CoNS has been isolated and documented as a pathogen in infections of various prosthetic devices, surgical wound infections, urinary tract infections, septicaemia etc. CoNS are opportunistic pathogens that cause infection in debilitated patients often by colonizing biomedical devices such as prostheses, implants and intravascular lines.[125] CoNS produce cell-surface and extracellular macromolecules that initiate and subsequently enhance bacterial adhesion to the plastic surfaces of foreign bodies to form biofilms. [13] Biofilm drastically affects the human cellular immune

response by its affect on the lymphoproliferative response to mononuclear cells to polyclonal stimulators. This inhibition of cellular response may contribute to infection of implanted prostheses.[139]

In this study, Staphylococcus epidermidis was the most commonly isolated species 76(79.17%) and isolated in more from wound swab 26(27.08%) specimens. The second most common isolate was S.hemolyticus followed by S.lugdunensis and S.saprophyticus. The species predominance was also reported by **Mohan et al** [116] who documented in their study that S. epidermidis was the most commonly isolated species 82.3% (158/192) and 94% isolated from pus sample and also **Marsik et al** [117] reported that 72.41% of bacteremia's caused by S. epidermidis and most commonly isolated from bone, joint and wound infections, S.hemolyticus (7.47%) was the second most common isolate from wound infections and S.saprophyticus (6.41%) was most commonly isolated from urinary tract infection. **Gaikwad SS and Deodhar LP et al,**[126] **Seetha et al.** [125] also documented that S. epidermidis was the predominant isolates among CoNS species. The formation of multilayered biofilm appears to be essential for the pathogenesis of device related S.epidermidis infections. The other virulence factors such as phosphatase, gelatinase, hemolysins, lipases, proteases also contributes to its pathogenicity. S.saprophyticus is a well documented urinary

pathogen and uroepithelial tissue tropism and production of urease contributes to its pathogenicity.[13]

The present study showed that 38(39.58%) of CoNS isolates were biofilm producers. This finding correlated with studies conducted by **Seetha et al,**[125] **Mohan et al,**[116] . **Makhija SK et al** [121] who showed that 43(42.5%) CoNS isolates were slime producers. However **Pal N et al**[129] reported 100% slime production by CoNS. The factor determining the pathogenicity of CoNS was found to be extracellular slime.[121] Biofilm appears to act as a barrier protecting bacteria from host defense mechanisms while providing a suitable environment for bacteria survival.[101] A significant association between the ability of an isolate to produce biofilm and its propensity to cause disease has been found in other studies also.[129]

Afreenish et al [37] and **Donlan et al**[130]in their study found that the majority of biofilm producing organisms were from urinary catheter tips (26.3%). But in the present study, majority of Biofilm producers were isolated from wound swab sample (16.67%) collected from infected joint prostheses. The inherent capacity of this organism to cause infection in device related infections is the ability to form mucoid biofilms on the inert synthetic surfaces of indwelling medical devices.[7]

In this study, Staphylococcus epidermidis 37 (38.54%) was the most common Biofilm producing CoNS species followed by Staphylococcus saprophyticus 1(1.04%).The same findings were also noted in the study conducted by **Mohan et al,** [116] who documented that 77 (48.7%)isolates of S.epidermidis and 8(26.7%) isolates of S.saprophyticus were Biofilm producers. **Sujata et al** [120] reported that 47.5% of Staphylococcus epidermidis were slime producers. **Aricola et al** [51] noted that 48.5% of their clinical isolates of Staphylococcus epidermidis were slime positive. The emergence of Staphylococcus epidermidis as a pathogen has been increased with the widespread use of indwelling medical devices and intravascular catheters in modern medicine. The ability of the organism to adhere and form biofilm on the surface of biomaterials is thought to be the most significant virulence factor.

In the present study, the percentage of Biofilm producing CoNS isolated from the age group of >60yrs was the highest followed by 46-60 yr of age. More incidence in higher age group may be due to the increasing use of indwelling medical devices which will augment the biofilm producing strains of CoNS. Due to high rate of invasive procedures in immunocompromised patients and also the bacterium's ability to form biofilms there is increased occurence of these infections **.**

In the current study, Biofilm production had a strong association with medical device related infections (76.32%) such as infected implants, urinary catheterization and prosthetic valve. This in correlation with study by **Sujata et al** [120] Who had documented that out of 55 S.epidermidis isolates from various device related infections, 26 (65.2%) were biofilm producers and S. epidermidis was mainly from patients with indwelling catheters as documented by **Narmata Kuma[ri et al** .[123] **Donlan et al** [91], **Raad et al.**[97]The use of synthetic material for implantation is widely associated with "Implant associated infection" due to **biofilm** production.[98] Biofilm producing Staphylococci easily gets attached to polymer surfaces and frequently colonize catheters and medical devices causing foreign body related infections. [93],[94],[95]

In the present study, it was observed that there was higher antibiotic resistance in Biofilm producing CoNS isolates than non-Biofilm producers. This in correlation with **Afreenish Hassan et al** [37] **de Silva et al**[131] who observed higher antibiotic resistance in biofilm producing bacteria than non-biofilm producers. **Sujata et al** [120] reported in their study that among the 55 implant associated S. epidermidis isolates, 23(41.8%) were multidrug resistance strains and 26(65.2%) were slime producers. **Kim L. Riddle et al** [35] had documented that biofilm producing organism exhibit resistance to antibiotics by various methods

like restricted penetration of antibiotic into biofilms, decreased growth rate and expression of resistance genes.

In the present study, the percentage of biofilm production detected by Tissue culture plate method (39.58%) was high followed by Tube method (30.21%) and Congo red agar method (17.71%). This finding correlated with **Mathur et al** [122] showed that the number of biofilm producers identified by TCP method was high (53.9 %) and followed by Tube method(11.8%) and CRA (5.17%). In another study**,** conducted by **Ruzicka et al** [43] 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 reported that TM is better for biofilm detection than CRA. **Afreenish et al** [37] documented in their study that the number of isolates showing biofilm formation by TCP was 70 (64.7%), and non or weak biofilm producers were 40 (36.3%) and Tube method detected 49% isolates as biofilm producers. However, **Baqai et al** [44] reported that 75% of the isolates exhibited biofilm formation by TM. The tube test correlates well with the TCP test for strongly biofilm producing isolates but it was difficult to discriminated between weak and biofilm negative isolates due to the variability in observed results by different observers. Consequently, high variability was observed and classification in biofilm positive and negative was difficult by tube method. In congo red agar

method out of 17 positive isolates only 7 showed black colonies with dry crystalline consistency and remaining isolates showed no correlation with TCP and TM.In agreement with the previous reports and based on our results TM and CRA cannot be recommended as general screening test to identify biofilm producing isolate.[122]

In this study, out of 38 Biofilm producing CoNS isolates detected by phenotypic method, **ica gene** was identified by PCR in 35 (36.45%) isolates. The results were concordance with the study by **Sujata et al** [120] who had reported in their study that ica gene was present in 23(41.8%) among 26(47.2%) of Biofilm producers. The results were in discordance with the study by **Seung- Hak- Cho et al** [23] who had reported that 18 S. epidermidis isolates obtained from catheter-related urinary tract infections showed the ica speficic DNA and only 11isolates biofilms spontaneously under normal growth conditions and **Galdbart, J. O.,et al** [50] in their study showed that 44 out of 54 S. epidermidis isolates from prosthetic-material related joint infection showed ica positivity. **Gad et al** [99] , **Cafiso et al** [100] demonstrated the detection of ica operon by molecular technique (PCR) with high efficiency. In addition, these genes are important virulence markers of clinical CoNS isolate since their expression is associated with the production of PIA, the most clearly characterized component of Staphylococcal biofilms.

The present study showed that ica gene was present in 35 (36.45%) of CoNS isolates which were detected as Biofilm producers by Tissue culture plate and sensitivity and specificity of Biofilm detection by this method was high in comparison with Tube method and Congo red agar. TCP can be recommended as a general screening test for biofilm production than CRA and TM .Though PCR detects ica genes, the virulence marker of staphylococcal infection and Biofilm non-producers are negative for icaA and icaD and lack the entire ica ADBC operon. But in a developing country like ours, a low cost method for detection of biofilm is needed which require inexpensive equipment and less technical expertise.

To compare PCR with TCP, the test share the specific identification rates. Although the genotypic methods will be absolute detection methods, it was not done in all centres. Considering the cost and specialized man power and sophisticated infrastructure, TCP can be performed to detect the Biofilm producing strains of CoNS with same sensitivity and specificity coinciding with genotypic methods.

SUMMARY

- A total of 456 samples were collected to study Biofilm production in Coagulase negative Staphylococci.

- Among the 456 clinical samples, 252 were Staphylococcal species. Out of 252 Staphylococcal species, 108 had been grossly isolated from wound swab specimen.

- Out of 252 Staphylococci isolates, 96 were Coagulase negative Staphylococci, which were identified by Coagulase test.

- Wound swab had the highest percentage (32.29%) in respect with Coagulase negative Staphylococci isolation among various clinical samples. Coagulase negative Staphylococci had been isolated from blood, pus, urine samples in greatest way when compared with routine isolation of Staphylococci.

- The most common Coagulase negative Staphylococci species were Staphylococcus epidermidis followed by Staphylococcus haemolyticus, Staphylococcus lugdunensis, and Staphylococcus saprophyticus. Staphylococcus epidermidis had the highest isolation in wound swab specimen.

- Phenotypic detection of Biofilm production among CoNS isolates were high in tissue culture plate, when compared with other phenotypic methods such as Tube method and Congo red agar method.

- The Biofilm producing CoNS isolates showed higher antibiotic resistance than non biofilm producers. Among the Biofilm producer highest antibiotic resistance were noted against Ampicillin followed by Ciprofloxacin, Cotrimoxazole, Chloramphenicol, Gentamicin, Doxycycline, Erythromycin and Cefotaxime.

- Biofilm production had a strong association with medical device related to orthopaedic implants followed by urinary catheterization and prosthetic valve.

- Among the phenotypic method, Tissue culture plate had the highest sensitivity and specificity as compared with genotypic method.

- Though genotypic method had the absolute value in detection of Biofilm production in an infectious agent, the Tissue culture plate can be recommended for the identification of Biofilm producing organism due to cost effectiveness, short turn around time and capability of being used in routine diagnostics.

CONCLUSION

- Coagulase negative Staphylococci are responsible for nosocomial infections at GRH, Madurai and Staphylococcus epidermidis is the most commonly isolated CoNS species.

- Resistance among CoNS are increasing especially among the patients with the indwelling medical devices due to Biofilm production.

- As infection caused by Biofilm producing bacteria are difficult to treat, early diagnosis and management is necessary to reduce morbidity and mortality.

- This study was focused on finding out of a simple, economic and more accessible method with high sensitivity and specificity to identify Biofilm production.

- Various phenotypic methods were compared with PCR and found that Tissue culture plate method showed high sensitivity and specificity and it is closer to PCR.

- Tissue culture plate method was proved to be simple, economical method can be recommended for early and prompt diagnosis of Biofilm production.

- Prophylactic antibiotic therapy to cover surgical insertion of most biomaterials and this will help in slightly slow progression of biofilms in biomaterials. Catheters should be inserted with meticulous attention to aseptic practices. Staff should adhere to appropriate aseptic protocols in caring out the indwelling catheterization.

The clinical significance of CoNS is increasing day by day in device related infections, urinary tract infections, endocarditis. Very soon CoNS may emerge as one of the leading nosocomial pathogen. Staphylococcus epidermidis can cause a number of human infections and should no longer be considered as a harmless commensal. The virulence of CoNS is directly related to its capability to establish multilayered, highly structured biofilms on artificial surfaces. There is association between biofilm production with persistent infection and antibiotic failure. Hence, in small microbiological laboratories where PCR cannot be done, Tissue culture plate method can be recommended which is simple and cost effective.

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ANNEXURE -1

PREPARATION OF GRAM STAIN

GRAM STAIN REAGENTS

1. Methyl violet – Primary stain
   Methyl violet 10g
   95% Ethyl alcohol 100ml
   Distilled water 1 L

2. Gram's Iodine – Mordant
   Iodine 10g
   Potassium Iodide 20 g
   Distilled water 1 L

3. Acetone – Decolouriser

4. Dilute carbol fuchsin – Counter stain
   Basic fuchsin 0.3g
   95% Ethyl alcohol 10ml
   Phenol crystals,melted 5ml
   Distilled water 95ml

Dissolve fuchsin in alcohol. Add the 5% phenol solution. Allow it to stand overnight. Filter through coarse filter paper.

ANNEXURE – 2

PREPARATION OF MEDIA

CONGO RED AGAR PLATE : To prepare 1L of CRA medium

1.Brain heart infusion broth 37 g/L

2. Sucrose 50 g/L

3. Agar No.1 10 g/L and

4. Congo Red indicator 8 g/L.

First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121^{o}C for 15 minutes) separately from the other medium constituents. Then it, was added to the autoclaved brain heart infusion agar with sucrose at 55^{o}C.

ANNEXURE – 3

PROFORMA

1. CASE NO. :

2. NAME :

3. AGE :

4. SEX :

5. ADDRESS :

6. IP/OP NO :

7. UNIT/WARD :

8. SPECIMEN :

9. DATE OF SAMPLE COLLECTION :

10. BRIEF CLINICAL HISTORY :

11. CLINICAL DIAGNOSIS :

12. TREATMENT HISTORY :

13. GENERAL PHYSICAL EXAMINATION:

MICROBIOLOGICAL INVESTIGATIONS:

1. DIRECT SMEAR STUDY : PUS CELLS:

(GRAM'S STAIN) EPITHELIAL CELLS:

BACTERIAL MORPHOLOGY:

2. GROWTH ON CULTURE MEDIA:

a. NUTRIENT AGAR PLATE:

b. MAC CONKEY AGAR PLATE:

c. BLOOD AGAR PLATE:

3. CULTURE SMEAR:

(GRAM'S STAIN)

4. CATALASE TEST:

5. OXIDASE TEST:

6. COAGULASE TEST:

SPECIATION

7. PHOSPHATASE TEST

8. ORNITHINE DECARBOAXYLASE TEST

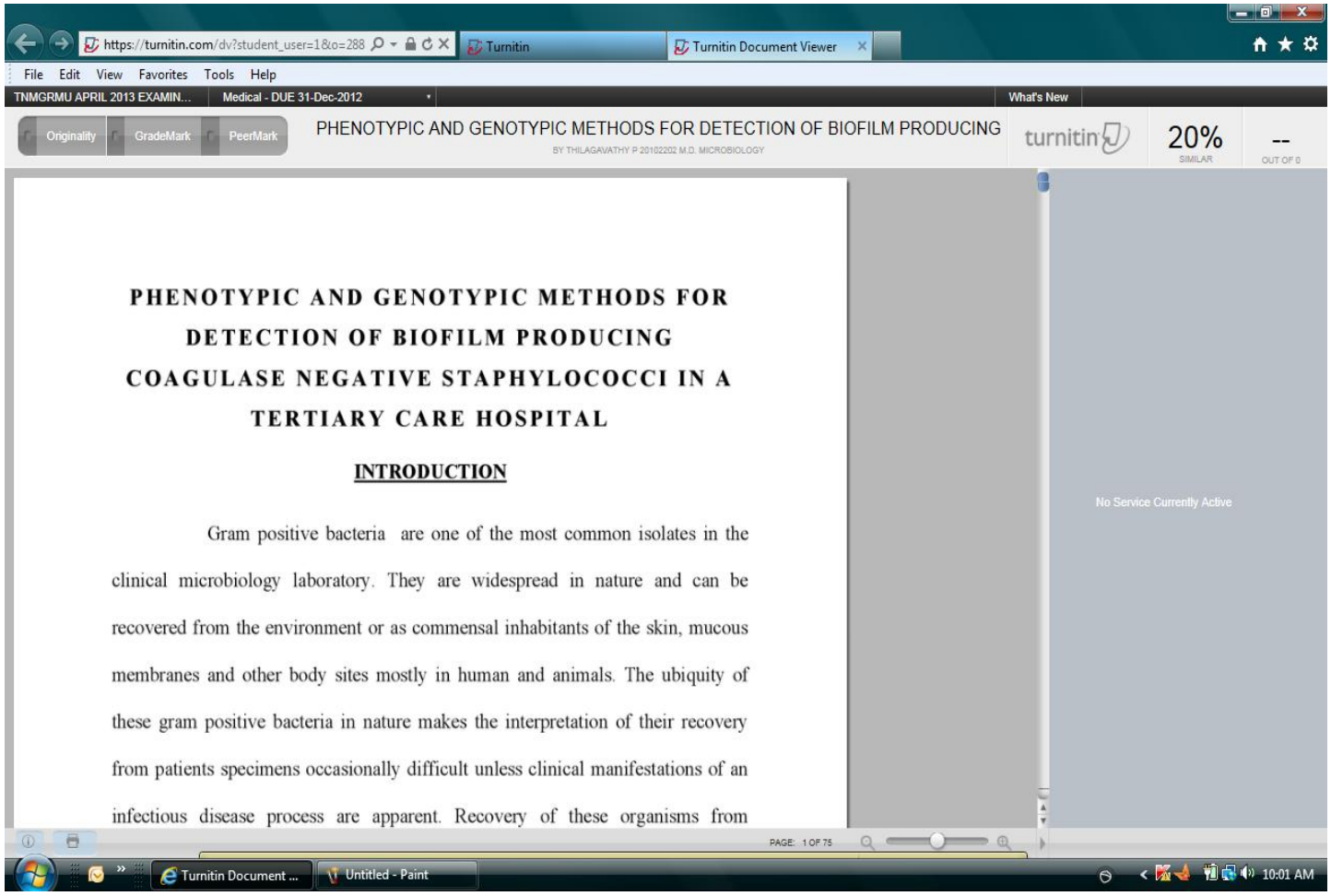
9. NOVOBIOCIN SENSITIVITY TEST:

10. ANTIBIOTIC SENSITIVITY

AMP		**C**		**CIP**		**CTX**		**GM**		**COT**		**ERM**		**DOX**	
S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**

BIOFILM DETECTION:

BIOFILM PRODUCTION	**TISSUE CULTURE PLATE**	**TUBE METHOD**	**CONGO RED AGAR**	**PCR ica gene**
High				
Moderate				
Weak/None				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PHENOTYPIC AND GENOTYPIC METHODS FOR DETECTION OF BIOFILM PRODUCING COAGULASE NEGATIVE STAPHYLOCOCCI IN A TERTIARY CARE HOSPITAL
INTRODUCTION
Gram positive bacteria are one of the most common isolates in the clinical microbiology laboratory. They are widespread in nature and can be recovered from the environment or as commensal inhabitants of the skin, mucous membranes and other body sites mostly in human and animals. The ubiquity of these gram positive bacteria in nature makes the interpretation of their recovery from patients specimens occasionally difficult unless clinical manifestations of an infectious disease process are apparent. Recovery of these organisms from
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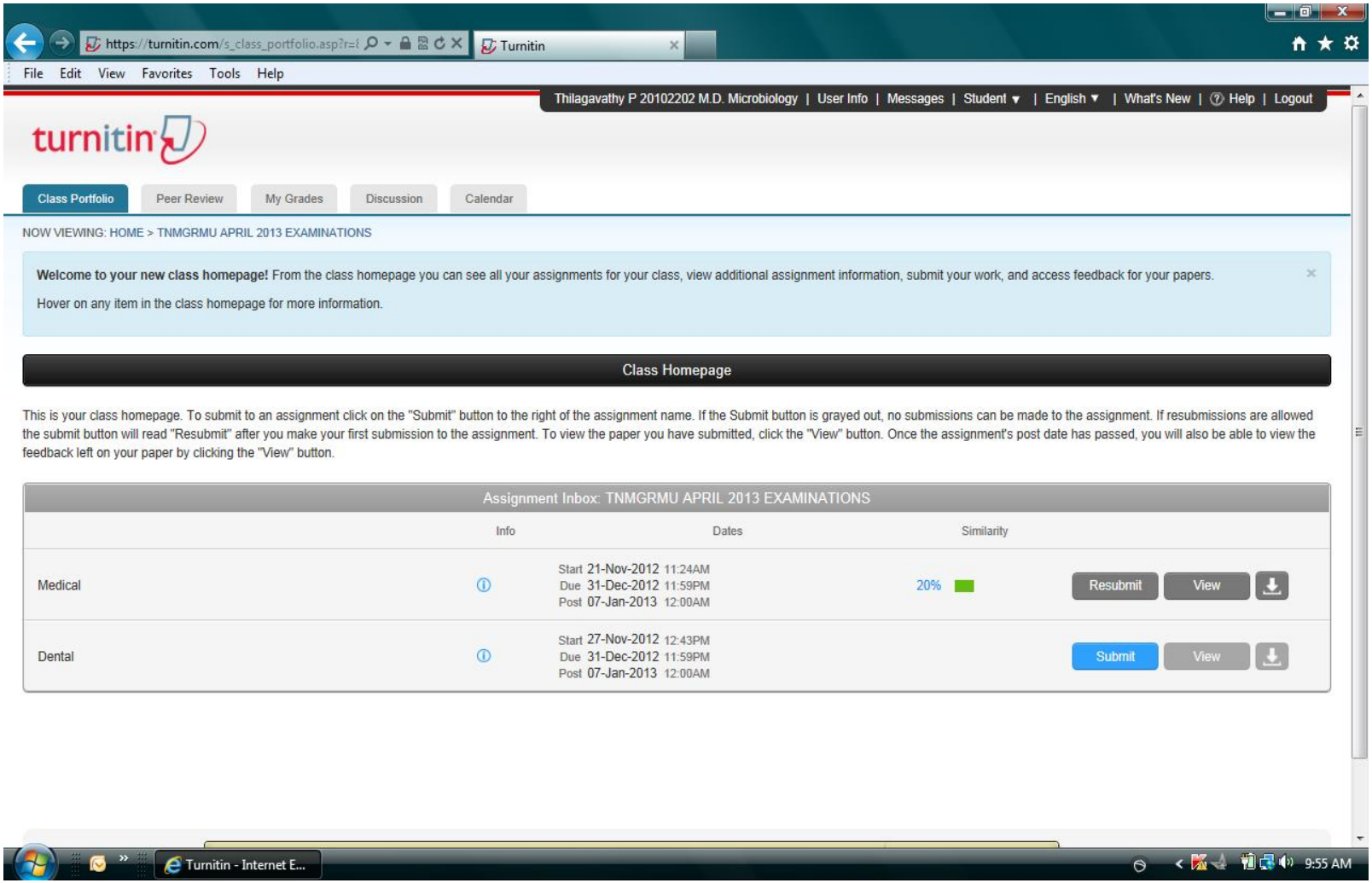
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MASTER CHART

S. NO	NAME	AGE	SEX	IP/OP NO	WARD /UNIT	SPECIMEN	DIAGNOSIS	ORGANISM ISOLATED	ANTIBIOGRAM								BIOFILM DETECTION			
									AMP	C	CIP	CTX	GM	COT	ERM	DOX	TCP	TM	CRA	PCR
1.	Pandiyammal	64	F	36253	O – IV	Wound swab	Infected implant	S.epidermidis	R	S	R	R	S	S	S	S	+	+	+	+
2.	Radha	46	F	37267	S -1	Pus	Abscess	S.epidermidis	R	S	R	S	R	R	S	R	+	+	+	+
3.	B/o Janaki	1/365	M	37289	I PU	Blood	Neonatal septicaemia	S.epidermidis	R	S	S	S	S	R	R	R	-	+	-	-
4.	Lakshmi	51	F	28882	S -3	Wound swab	Ulcer	S.epidermidis	S	S	S	R	S	R	S	R	-	-	+	-
5.	Muthulaxmi	47	F	41117	S -5	Wound swab	Surgical site infection	S.hemolyticus	R	R	S	S	S	S	S	S	-	-	-	-
6.	Mahalingam	35	M	38804	M -6	Blood	IE	S.epidermidis	R	R	R	S	R	R	R	R	+	+	+	+
7.	Venkatadharn	46	M	38307	S -4	Pus	Abscess	S.epidermidis	R	S	S	R	S	R	R	R	-	+	-	-
8.	Poomari	63	F	33415	M-3	Urine	UTI	S.saprophyticus	R	R	S	S	S	S	R	R	+	-	-	-
9	Kathiresan	13	M	38916	M -1	Blood	PUO	S.epidermidis	S	R	R	S	R	R	R	R	+	-	-	+
10.	Vanishree	8	F	24869	O-III	Wound swab	Infected implant	S.epidermidis	R	S	S	S	S	R	R	R	+	+	-	+
11.	Vallimuthu	21	M	34129	M-7	Blood	PUO	S.epidermidis	S	S	S	R	R	S	R	R	-	-	+	-
12.	Santhi	57	F	31589	S-3	Pus	Abscess	S.epidermidis	R	R	S	S	S	S	S	S	-	-	-	-
13	Indira	11	F	36952	S-7	Wound swab	Surgical site infection	S.epidermidis	R	S	R	S	S	R	S	S	+	+	-	+
14	Vijayajaeyanthi	38	F	34652	M-3	Urine	UTI	S.epidermidis	R	R	S	S	R	S	S	S	-	-	-	-
15	Rani	62	F	40785	O- II	Wound swab	Infected implant	S.epidermidis	R	R	S	S	S	S	S	R	+	-	-	+
16	Subramani	61	M	42468	M-1	Urine	UTI	S.epidermidis	S	R	R	S	S	R	R	S	+	+	+	+
17	Kalimuthu	63	M	42249	O-I	Wound swab	Osteomyelitis	S.hemolyticus	R	S	S	S	S	R	S	S	-	-	-	-
18	Mookammal	32	F	24750	S-5	Pus	Stricture abscess	S.epidermidis	S	R	S	S	R	S	S	S	-	-	-	-
19	Rupa	13	F	42991	M-4	Blood	PUO	S.epidermidis	R	S	S	R	S	R	R	R	-	-	-	-
20	Muthukumar	31	M	37812	S-1	Wound swab	Ulcer	S.epidermidis	R	S	S	S	S	R	S	S	-	-	-	-
21	Vimala	48	F	42821	M-2	Urine	UTI	S.epidermidis	R	R	S	S	R	R	R	S	+	-	-	+
22	Sankarammal	64	F	42916	S-5	Pus	Abscess	S.epidermidis	R	S	S	S	S	R	S	S	-	-	-	-
23	Madhavi	25	F	252205	M-3	Blood	PUO	S.epidermidis	S	S	S	R	R	S	R	R	-	-	-	-
24	Pitchaimuthu	65	M	40926	O- I	Wound swab	Infected implant	S.epidermidis	R	R	S	S	R	R	S	R	+	+	-	+
25	Swetha	14	F	36455	O-IV	Wound swab	Infected implant	S.epidermidis	R	R	S	S	R	S	R	S	+	+	-	+
26	Murugesan	47	M	34729	M-5	Blood	PUO	S.epidermidis	R	R	S	S	S	R	S	S	-	-	+	-
27	Sundarammal	32	F	73512	S-4	Wound swab	Surgical site infection	S.epidermidis	R	R	S	S	R	S	R	R	-	-	-	-
28	Periakaruppu	48	M	17711	S-6	Pus	Abscess	S.epidermidis	R	R	S	S	S	R	S	S	-	-	-	-
29	Veluthayi	44	F	41410	M-1	Blood	IE	S.epidermidis	R	S	R	R	S	R	R	S	+	+	+	+
30	Angaleeswari	29	F	39722	M-5	Blood	PUO	S.hemolyticus	R	S	S	S	S	R	S	S	-	-	-	-

31	Ponnaiyan	47	M	44348	S-3	Wound swab	Ulcer	S.lugdunensis	R	R	S	S	S	S	S	S	-	-	-	-
32	Chinnakalai	51	M	53223	S-6	Wound swab	Surgical site infection	S.epidermidis	S	R	S	S	S	S	R	R	-	-	-	-
33	Esaikiraja	60	M	42171	PSW	Wound swab	Wound infection	S.epidermidis	R	S	S	S	S	S	S	S	-	-	-	-
34	Perumal	65	M	41679	NSW	Pus	Cerebral abscess	S.epidermidis	S	R	S	S	S	R	S	S	+	+	-	+
35	Nathiya	11	F	34592	IV PU	Blood	Septicemia	S.epidermidis	R	R	R	R	R	R	R	R	-	-	+	
36	Kothaiammal	36	F	20659	S-1	Pus	Abscess	S.epidermidis	R	S	S	S	S	R	S	S	-	-	-	-
37	Kannan	64	M	44950	M-6	Urine	UTI	S.epidermidis	R	R	S	S	S	R	S	R	+	-	-	-
38	Udhayan	38	M	41191	S-1	Pus	Abscess	S.epidermidis	S	R	S	R	R	R	R	R	-	-	-	-
39	Poongodi	33	F	41538	M-2	Blood	PUO	S.epidermidis	R	R	R	S	S	S	S	S	-	-	-	-
40	Thayammal	55	F	45175	M-5	Blood	IE	S.epidermidis	R	S	S	R	S	R	S	S	+	+	-	+
41	Mangayarkarasi	28	F	40157	M-1	Blood	PUO	S.epidermidis	R	R	R	S	S	S	S	S	-	-	-	-
42	Pandi	66	M	35922	O-III	Wound swab	Infected implant	S.epidermidis	R	R	R	S	R	R	S	S	+	+	+	+
43	Mariyammal	45	F	51912	M-4	Urine	UTI	S.epidermidis	S	R	S	R	S	S	R	R	-	+	-	-
44	Ponnusamy	50	M	53851	S-6	Pus	Abscess	S.epidermidis	R	R	S	S	S	S	S	S	-	-	-	-
45	Santhosh	9	M	51938	II PU	Blood	Septicemia	S.epidermidis	R	S	R	S	S	S	S	S	-	-	-	-
46	Selvi	18	F	55934	S-5	Pus	Abscess	S.epidermidis	R	R	S	R	S	S	S	S	-	-	-	-
47	Mogana	23	F	55987	M-1	Blood	PUO	S.epidermidis	R	R	R	S	S	S	S	S	-	-	-	-
48	Palanisamy	52	M	55956	M-5	Urine	UTI	S.epidermidis	R	R	S	S	R	R	S	R	+	-	-	-
49	Dhanam	31	F	56025	O-I	Wound swab	Infected implant	S.epidermidis	R	R	S	S	S	R	R	S	+	-	-	+
50	Krishnan	61	M	43210	O-1I	Wound swab	Infected implant	S.epidermidis	R	S	R	S	S	R	R	R	+	+	+	+
51	Geetha	27	F	53486	S-5	Pus	Abscess	S.hemolyticus	R	R	S	S	R	S	S	S	-	-	-	-
52	Revathi	35	F	54923	M-6	Blood	PUO	S.epidermidis	R	S	R	S	S	S	S	S	-	-	+	-
53	Mallika	13	F	57212	O-1	Wound swab	Osteomyelitis	S.epidermidis	R	S	S	S	S	R	S	S	-	-	-	-
54	Muthu	45	M	57277	M-4	Blood	PUO	S.epidermidis	R	S	R	S	R	S	S	S	-	-	+	-
55	Nagammal	54	F	52817	S-4	Wound swab	Surgical site infection	S.epidermidis	R	S	R	S	S	R	S	S	-	-	-	-
56	Ramesh	46	M	56915	O-1V	Wound swab	Infected implant	S.epidermidis	R	R	R	R	R	R	S	S	+	+	+	+
57	Rosammal	41	F	58406	M-5	Blood	IE	S.epidermidis	R	R	R	S	S	S	R	R	+	+	-	+
58	Siva	16	M	55683	M-2	Blood	PUO	S.epidermidis	R	S	S	S	R	S	S	S	-	-	-	-
59	Dhanalakshmi	36	F	59201	S-4	Pus	Abscess	S.epidermidis	R	S	S	S	R	S	S	S	-	+	-	-
60	Bhuvaneswari	21	F	56710	M-6	Blood	PUO	S.epidermidis	R	S	S	S	R	S	S	S	-		-	-
61	Kamali	12	F	41296	I PU	Blood	PUO	S.epidermidis	R	R	R	R	S	S	R	R	+	+	+	+
62	Subbaiah	52	M	55910	O-I	Wound swab	Infected implant	S.epidermidis	R	S	R	R	S	S	S	R	+	-	-	+
63	Kanimozhi	20	F	58168	M-4	Urine	UTI	S.saprophyticus	R	S	S	S	R	S	S	S	-	-	-	-
64	Rajalaxmi	48	F	65514	M-5	Blood	IE	S.epidermidis	R	S	R	R	R	R	S	S	+	+	-	+
65	Alagarsamy	67	M	72427	M-1	Urine	UTI	S.epidermidis	R	R	R	R	S	S	S	R	+	+	-	+
66	Chinnapriya	13	F	57432	S-6	Wound swab	Cellulitis	S.epidermidis	R	S	S	S	S	S	S	S	-	-	-	-
67	Rajammal	39	F	46877	M-3	Blood	IE	S.epidermidis	R	S	R	S	R	R	S	R	+	-	-	+

68	Sakthivel	21	M	83815	S-5	Pus	Abscess	S.epidermidis	R	S	S	S	R	S	S	S	-	-	-	-
69	Shanmugavalli	47	F	60345	M-6	Urine	UTI	S.epidermidis	R	R	R	S	R	S	S	S	+	-	-	+
70	Ponnusamy	52	M	60558	O-II	Wound swab	Infected implant	S.epidermidis	R	R	R	R	S	R	R	S	+	+	-	+
71	Annarani	31	F	48076	M-6	Urine	UTI	S.epidermidis	R	S	S	S	R	S	S	S	-	-	-	-
72	Urkavalan	47	M	59289	S-6	Pus	Abscess	S.epidermidis	R	S	R	S	S	S	S	S	-	-	-	-
73	Kanaga	14	F	54949	O-1V	Wound swab	Infected implant	S.epidermidis	R	S	R	R	R	S	S	R	+	+	-	+
74	Sathish	10	M	53616	II PU	Blood	PUO	S.epidermidis	R	R	R	S	S	R	S	S	+	-	-	+
75	Mohameddusain	9	M	49895	III PU	Blood	Septicemia	S.epidermidis	R	S	R	S	R	S	S	S	-	-	-	-
76	Tamilselvi	19	F	51298	O-II	Wound swab	Infected implant	S.epidermidis	R	R	R	R	S	S	S	S	+	+	-	+
77	Rajamani	23	F	56070	M-5	Urine	UTI	S.saprophyticus	R	S	S	S	S	R	R	R	-	-	-	-
78	Kotaimuthu	49	M	63797	M-1	Blood	PUO	S.hemolyticus	R	R	R	S	S	S	S	S	-	-	-	-
79	Tamilrani	49	F	63717	O-II	Wound swab	Osteomyelitis	S.epidermidis	R	S	R	S	R	S	S	S	-	+	-	-
80	Balu	50	M	57212	M-4	Urine	UTI	S.epidermidis	R	R	R	R	R	S	S	R	+	+	+	+
81	Rajeswari	34	F	44504	M-6	Urine	UTI	S.epidermidis	R	S	R	S	S	S	S	S	-			-
82	Abinaya	8	F	61944	O-II	Wound swab	Infected implant	S.epidermidis	R	R	S	S	R	R	S	S	+	+	-	+
83	Narayanan	7	M	64974	IV PU	Blood	PUO	S.lugdunensis	R	S	S	R	R	S	S	R	-	-		-
84	Periyakaruppan	72	M	58283	M-2	Urine	UTI	S.epidermidis	R	R	R	R	S	S	R	R	-	-	+	-
85	Mahalakshmi	47	F	61384	O-I	Wound swab	Infected implant	S.epidermidis	R	S	R	S	S	S	S	S	-	-	-	-
86	Kunjaram	31	F	59583	M-6	Urine	UTI	S.epidermidis	R	S	S	S	R	S	S	S	-	-		-
87	Mariammal	41	F	64778	M-2	Urine	UTI	S.epidermidis	R	S	R	S	R	S	S	S	-	-	-	-
88	Kaliraja	14	M	68841	S-6	Pus	Abscess	S.epidermidis	R	S	R	S	S	S	S	R	-	-	-	-
89	Velammal	33	F	57212	M-1	Blood	PUO	S.epidermidis	R	S	R	R	R	S	S	S	+	-	-	+
90	Sowmini	12	F	58834	S-1	Pus	Abscesss	S.epidermidis	R	S	S	S	R	S	S	S	-	-	-	-
91	Amaravathy	45	F	69270	0-IV	Wound swab	Infected implant	S.epidermidis	R	S	R	S	R	R	R	S	+	-	-	+
92	Manikandan	9	M	70722	O-I	Wound swab	Infected implant	S.epidermidis	R	S	S	R	R	R	R	S	-	-	-	-
93	Priya	10	F	61753	M-I	Blood	PUO	S.epidermidis	R	S	R	S	R	S	R	R	-	-	-	-
94	Kaliammal	62	F	64684	M-1	Urine	UTI	S.epidermidis	R	S	R	R	R	S	R	S	+	+	-	+
95	Selvam	7	M	63747	S-4	Pus	Abscess	S.lugdunensis	R	S	R	R	S	S	R	S	-	-	-	-
96	Balamani	60	F	68195	O-II	Wound swab	Infected implant	S.epidermidis	R	S	R	R	S	R	R	S	+	-	-	+

FIGURE 1 : SAMPLE WISE ISOLATION OF ORGANISMS

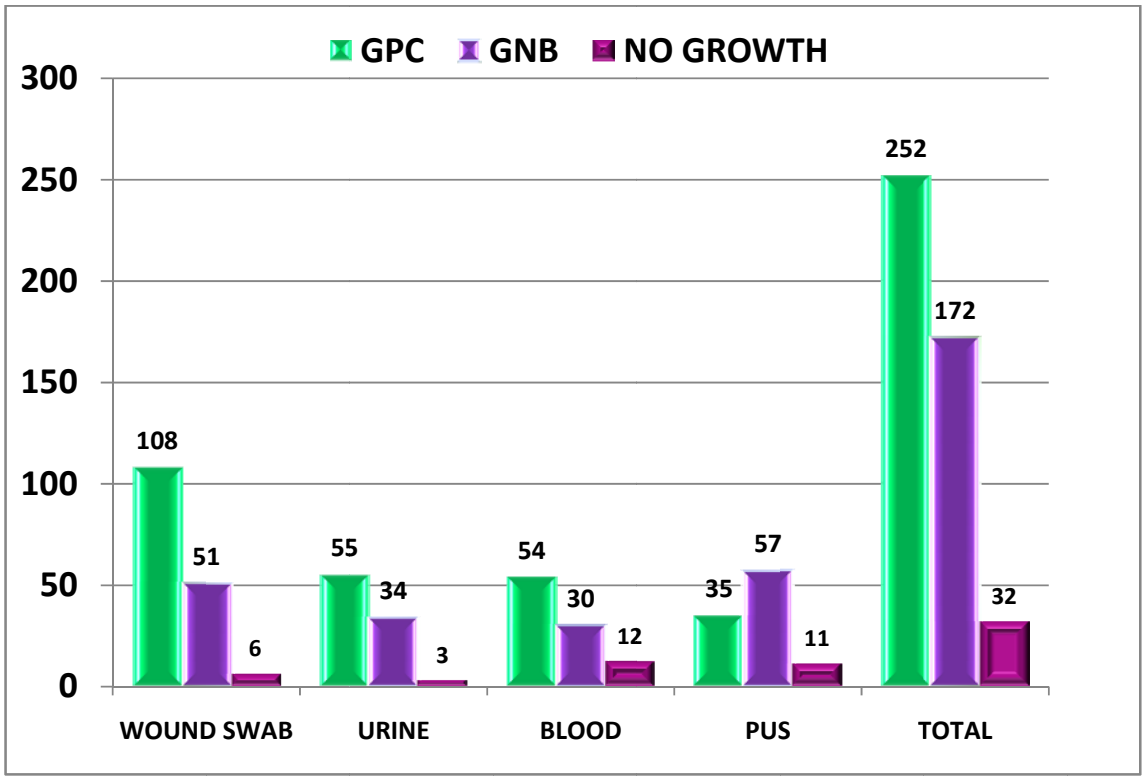


FIGURE 2 : IDENTIFICATION OF COAGULASE NEGATIVE STAPHYLOCOCCI

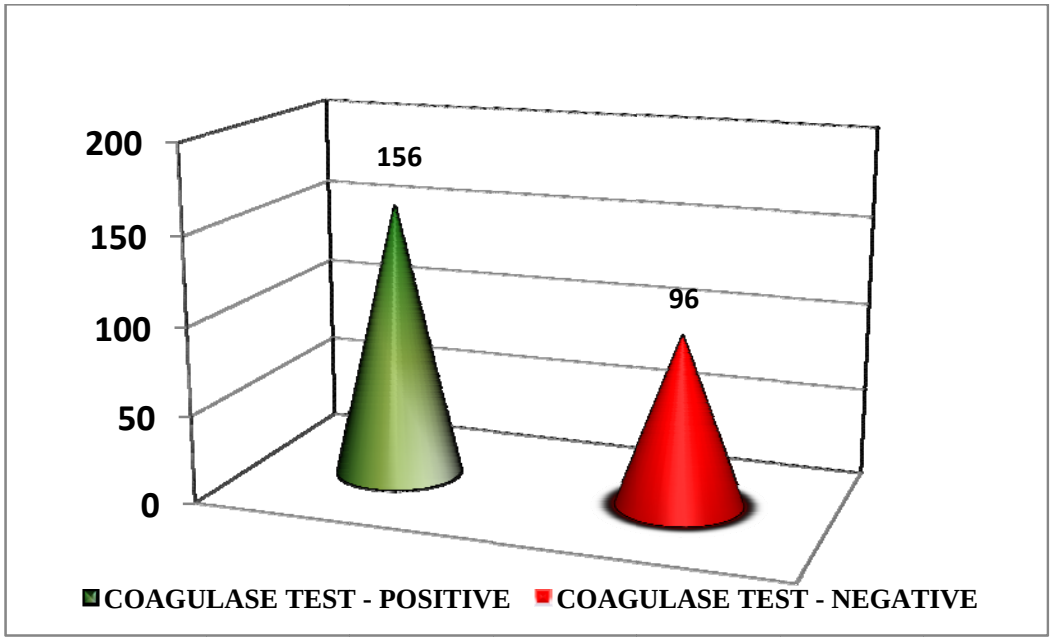


FIGURE 3 : SPECIMEN WISE ISOLATION OF COAGULASE NEGATIVE STAPHYLOCOCCI

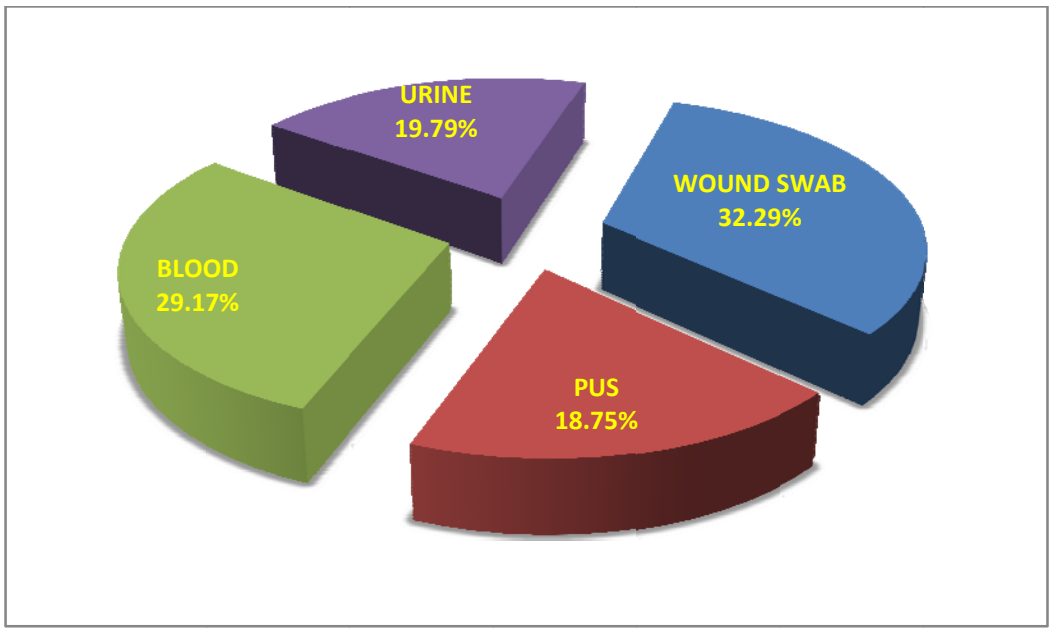


FIGURE 4 : SAMPLE WISE IDENTIFICATION OF CoNS SPECIES

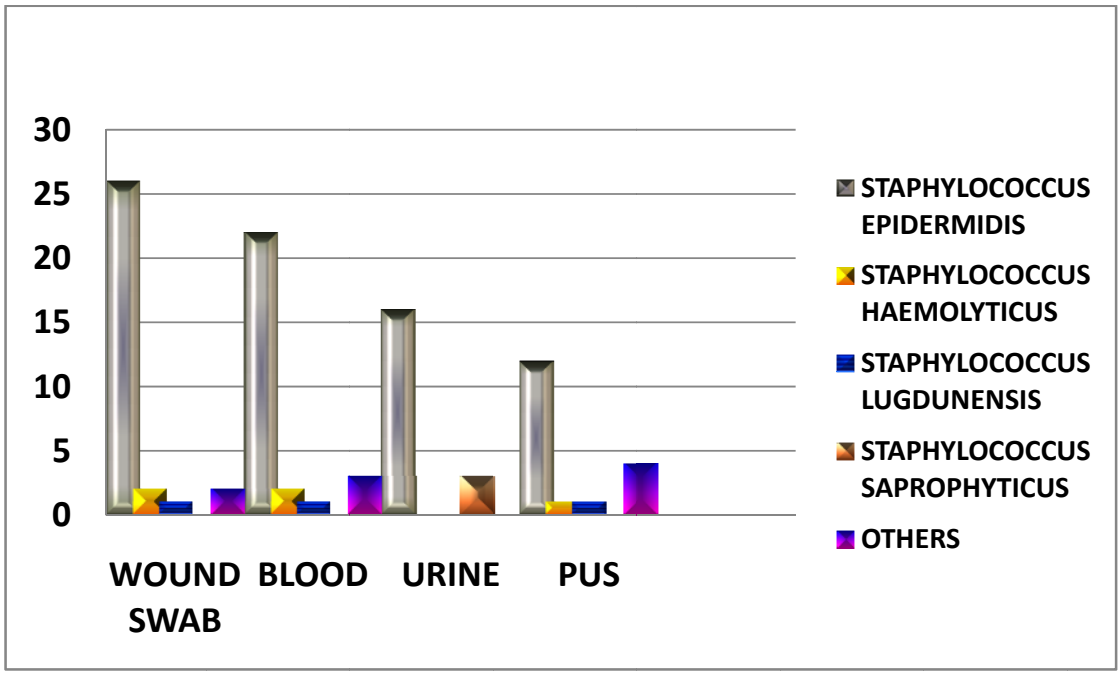


FIGURE 5: SCREENING OF THE CoNS ISOLATES FOR BIOFILM PRODUCTION BY PHENOTYPIC METHODS

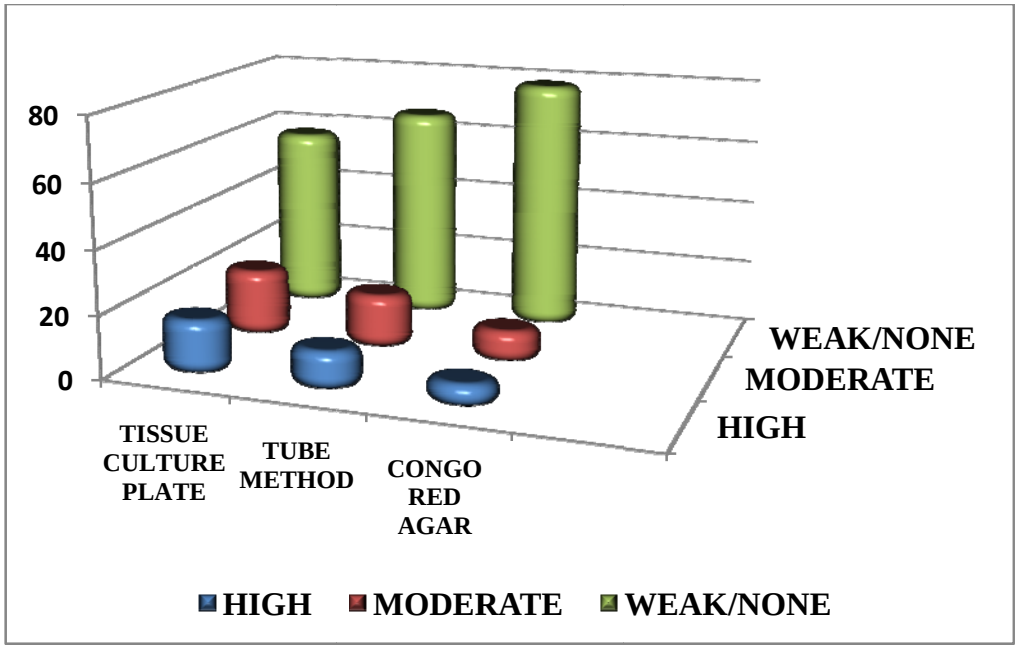


FIGURE 6 : SAMPLE WISE ISOLATION OF BIOFILM PRODUCING CoNS

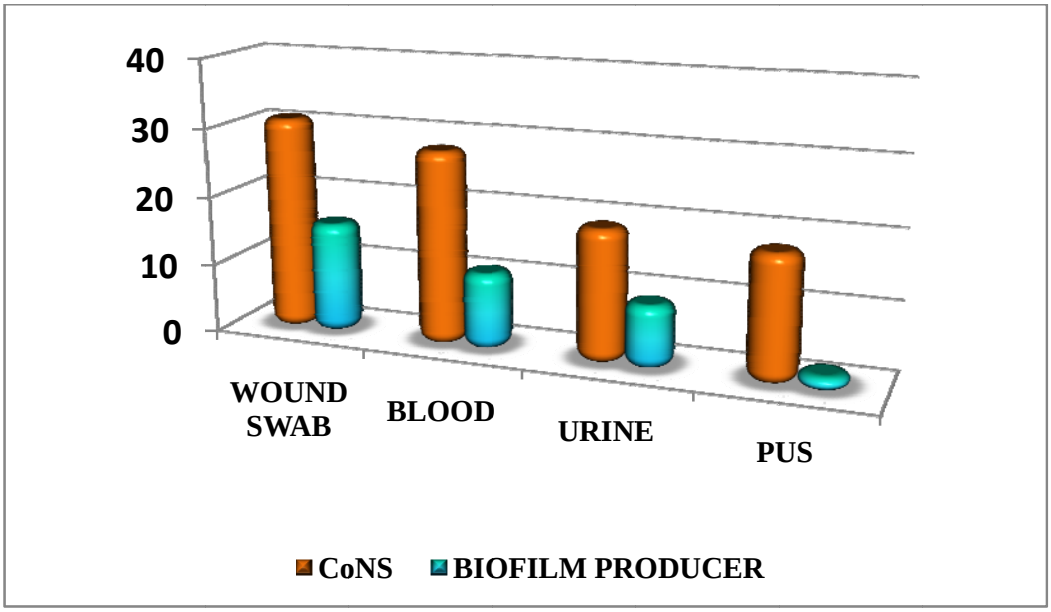


FIGURE 7: SPECIES AND SPECIMEN WISE DISTRIBUTION OF BIOFILM PRODUCING CoNS

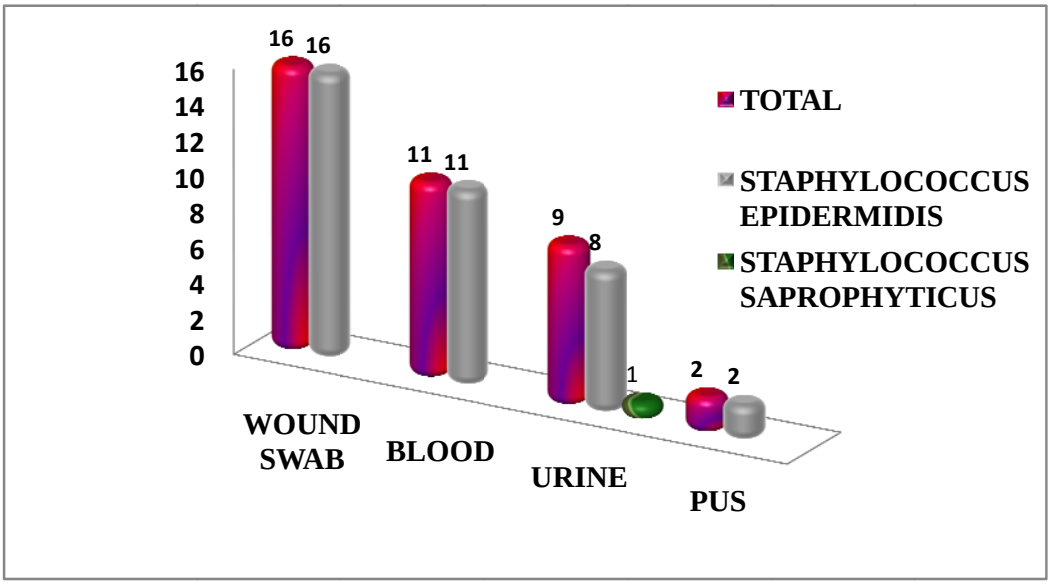


FIGURE 8 : AGE WISE DISTRIBUTION OF BIOFILM PRODUCING CoNS

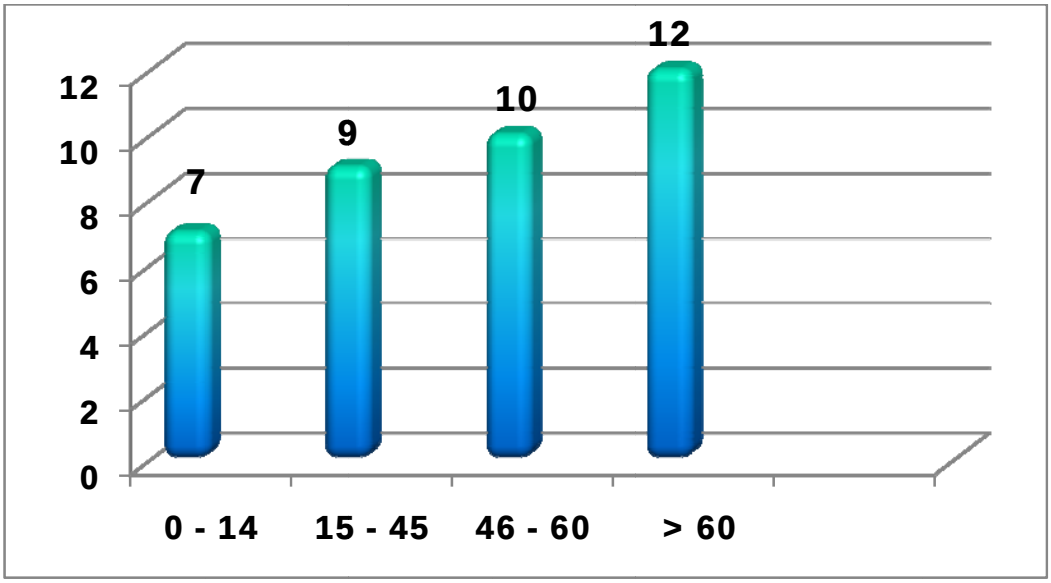


FIGURE 9 :PERCENTAGE OF BIOFILM PRODUCING CoNS ASSOCIATED WITH RISK FACTORS

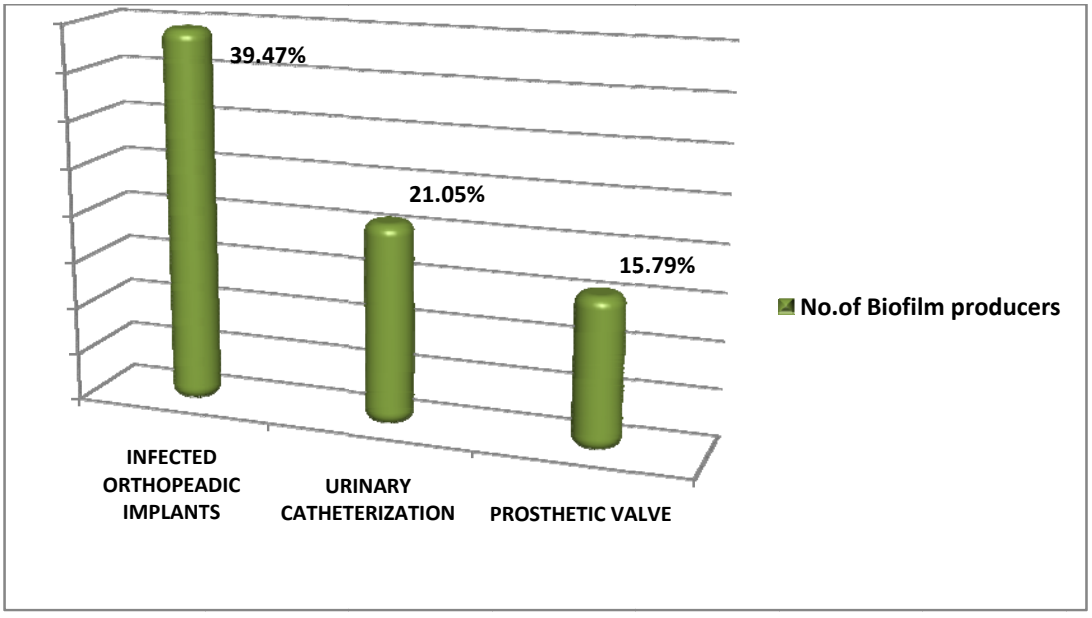


FIGURE 10A: ANTIBIOGRAM OF BIOFILM PRODUCING CoNS ISOLATES

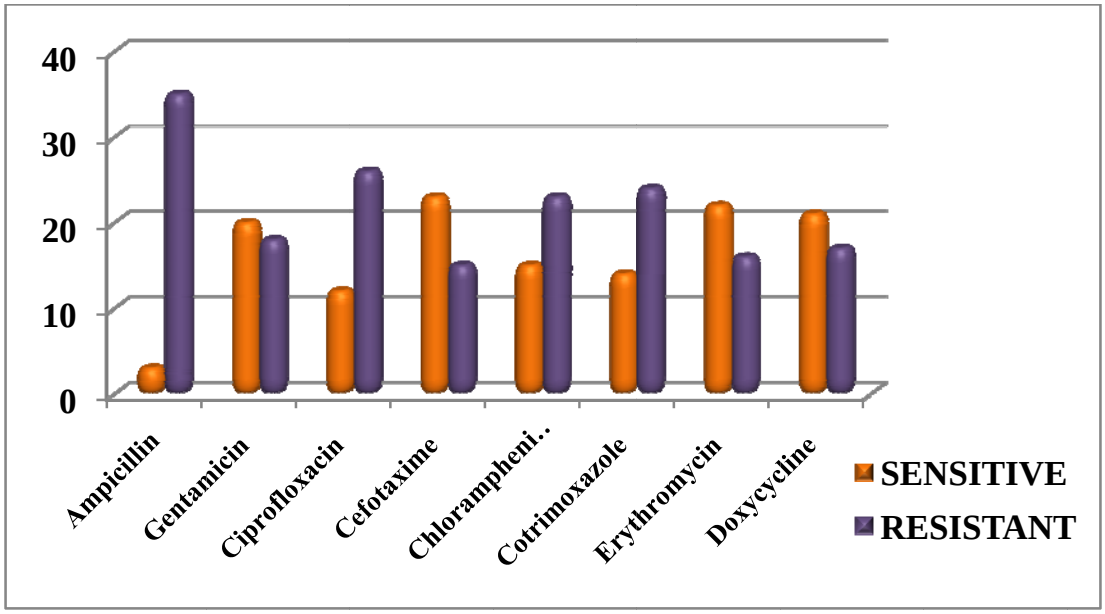


FIGURE 10B: ANTIBIOGRAM OF NON BIOFILM PRODUCING CoNS ISOLATES

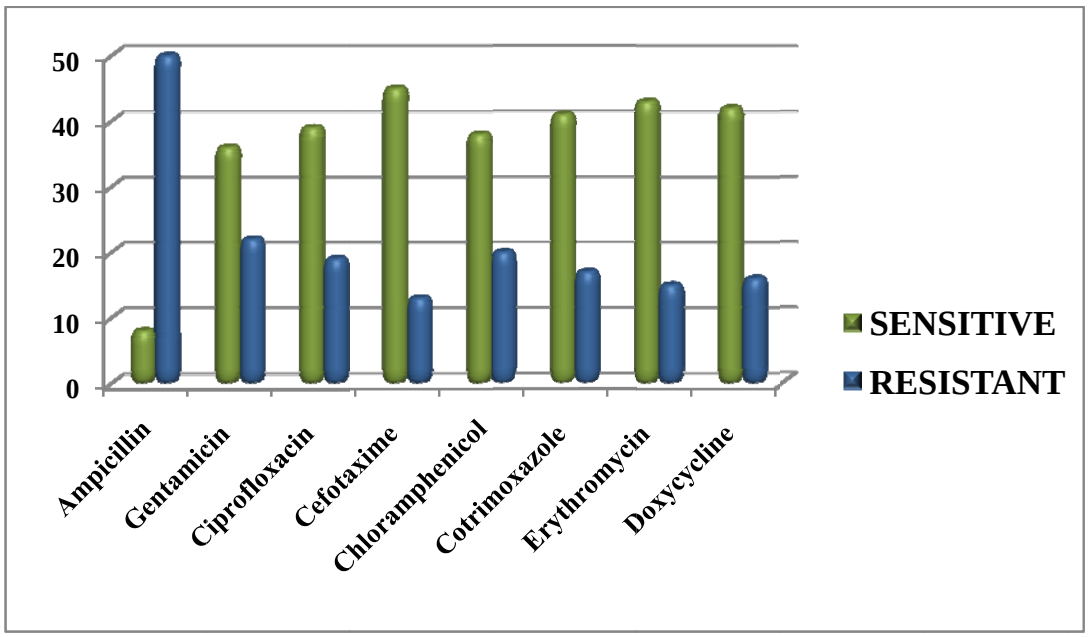


FIGURE 11 : NO. OF BIOFILM PRODUCING CoNS ISOLATES BY VARIOUS METHODS

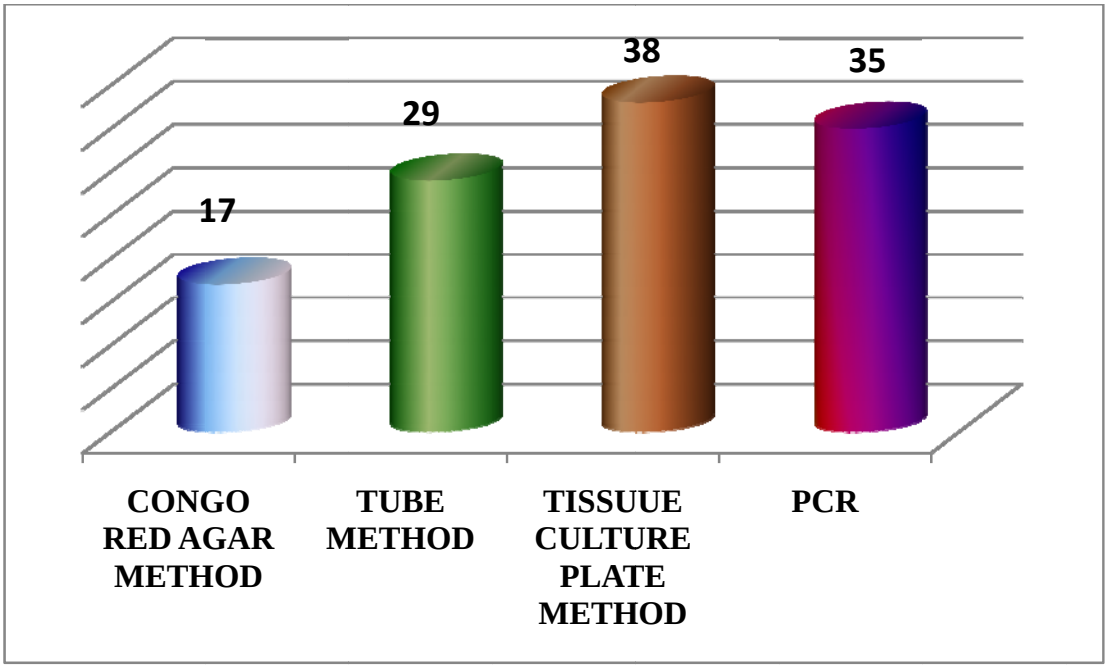


FIGURE12: SENSITIVITY AND SPECIFICITY OF VARIOUS PHENOTYPIC METHODS

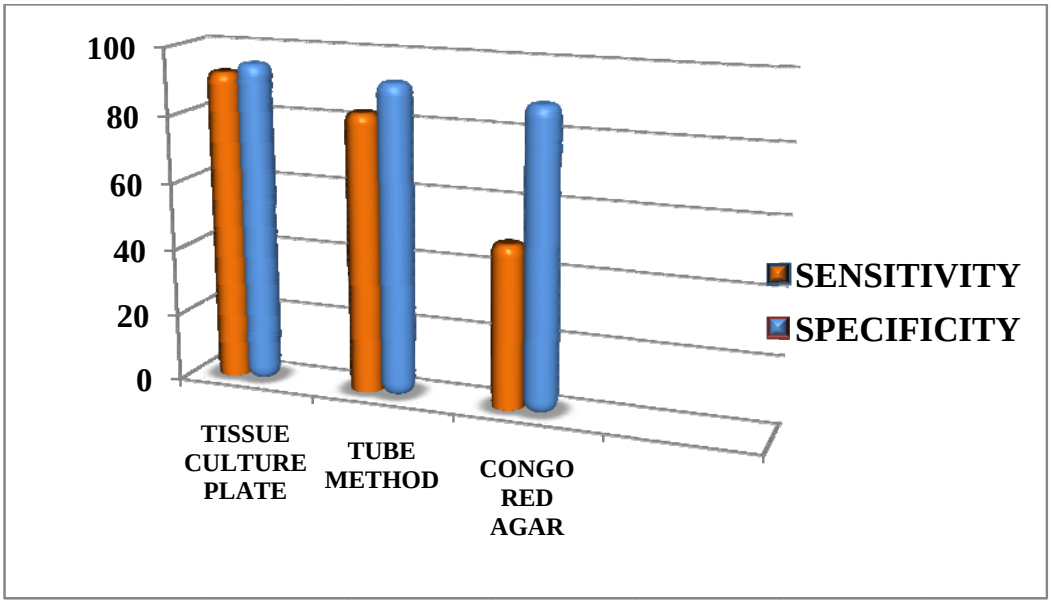


FIGURE 1

GRAM STAIN SHOWING

GRAM POSITIVE COCCI IN CLUSTERS

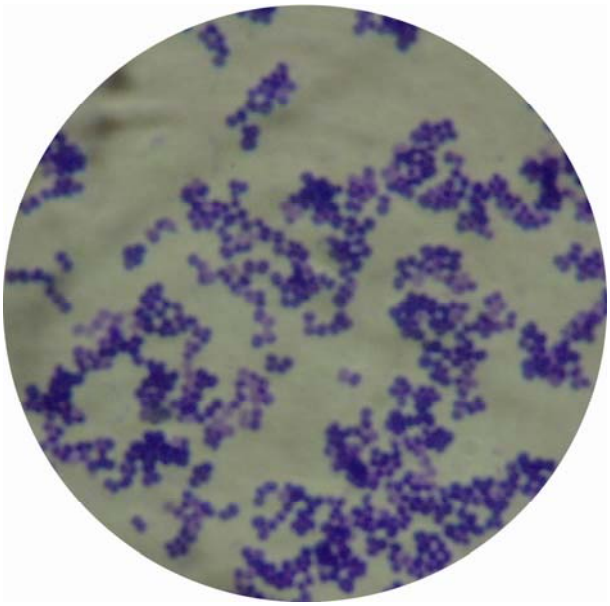


FIGURE 2

NUTRIENT AGAR PLATE SHOWING WHITE COLOUR COLONIES

FIGURE 3

MANNITOL AGAR PLATE SHOWING WHITE COLOUR COLONIES

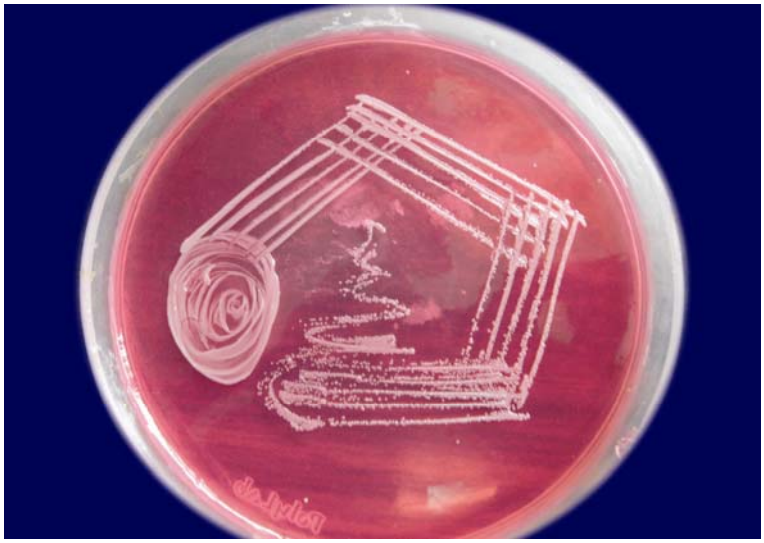


FIGURE 4

CATALASE TEST

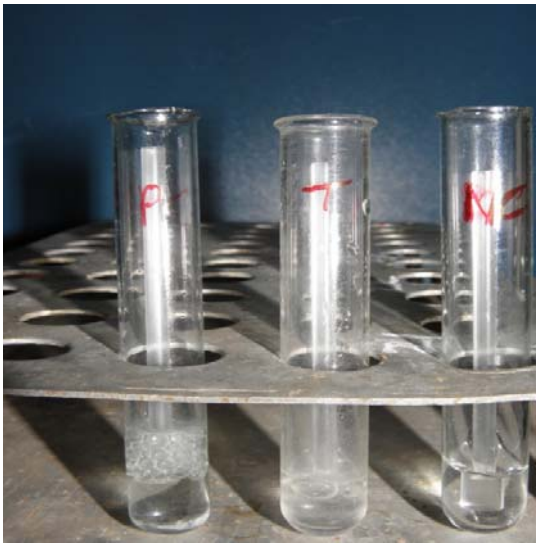


FIGURE 5

TUBE COAGULASE TEST

FIGURE 6

PHOSPHATASE TEST

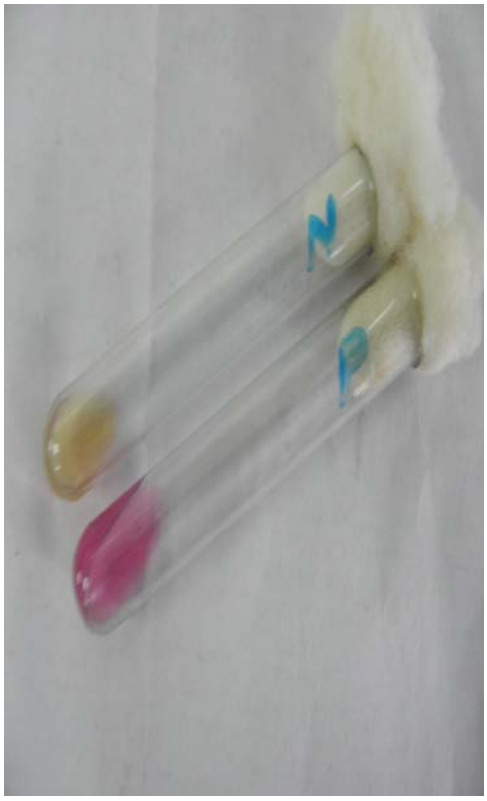


FIGURE 7

ORNITHINE DECARBOXYLASE TEST

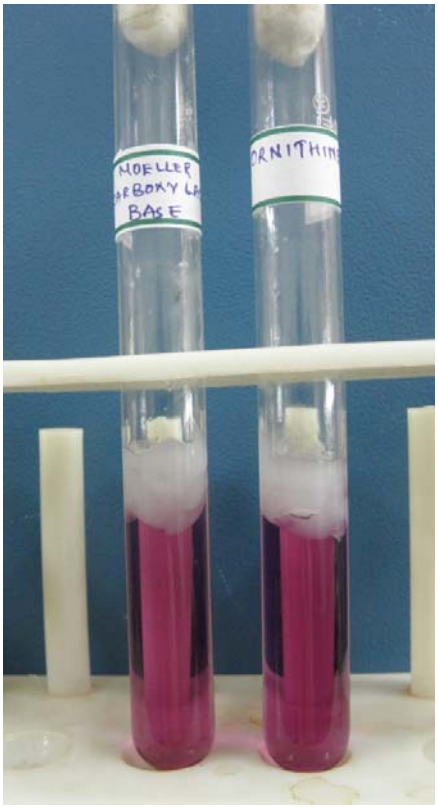


FIGURE 8

DISC DIFFUSION TEST – NOVOBIOCIN

SENSITIVE **RESISTANT**

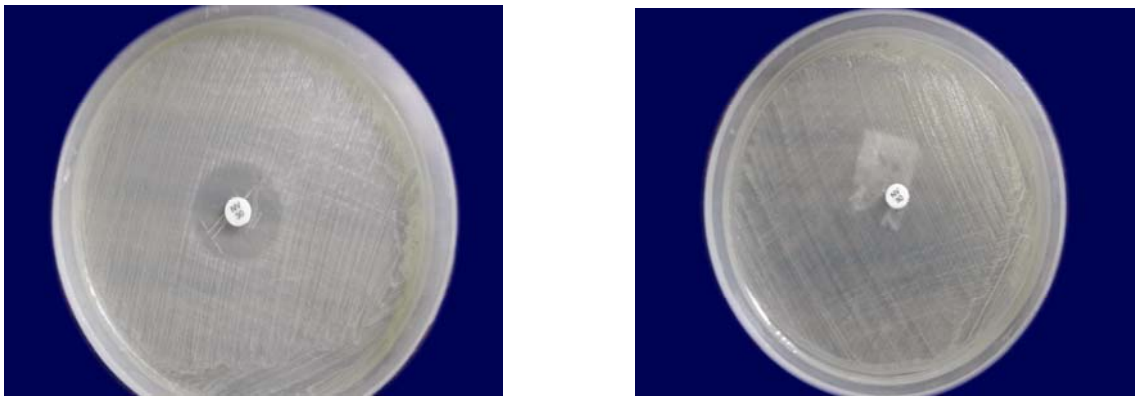


FIGURE 9

BLOOD AGAR PLATE SHOWING BETA HEMOLYSIS

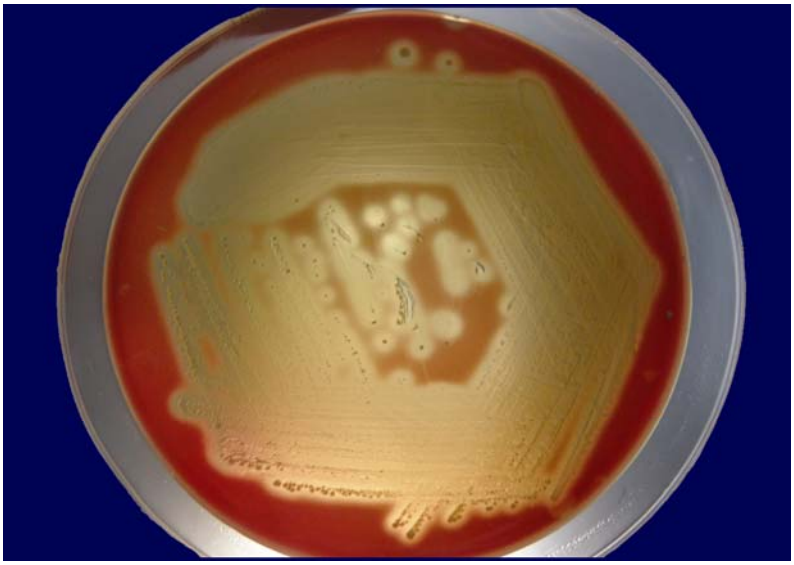


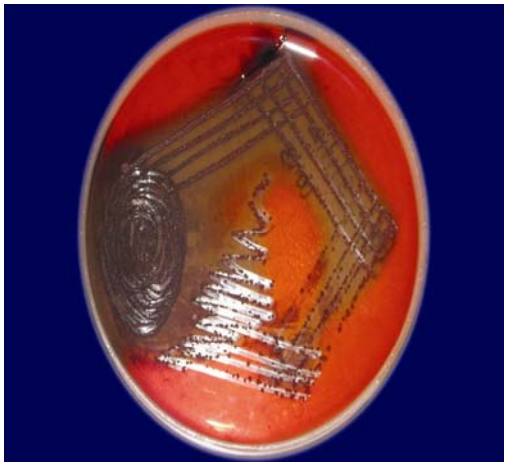
FIGURE 10

ANTIBIOTIC SUSCEPTIBILITY TEST

FIGURE 11

CONGO RED AGAR METHOD

BIOFILM POSITIVE



BIOFILM NEGATIVE

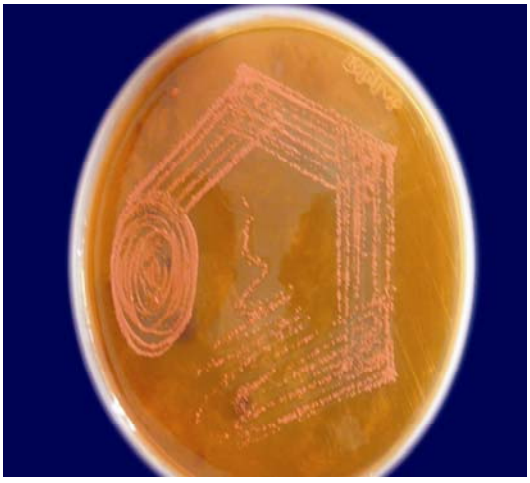


FIGURE 12

TUBE METHOD

BIOFILM POSITIVE **NEGATIVE**

FIGURE 13

TISSUE CULTURE PLATE METHOD

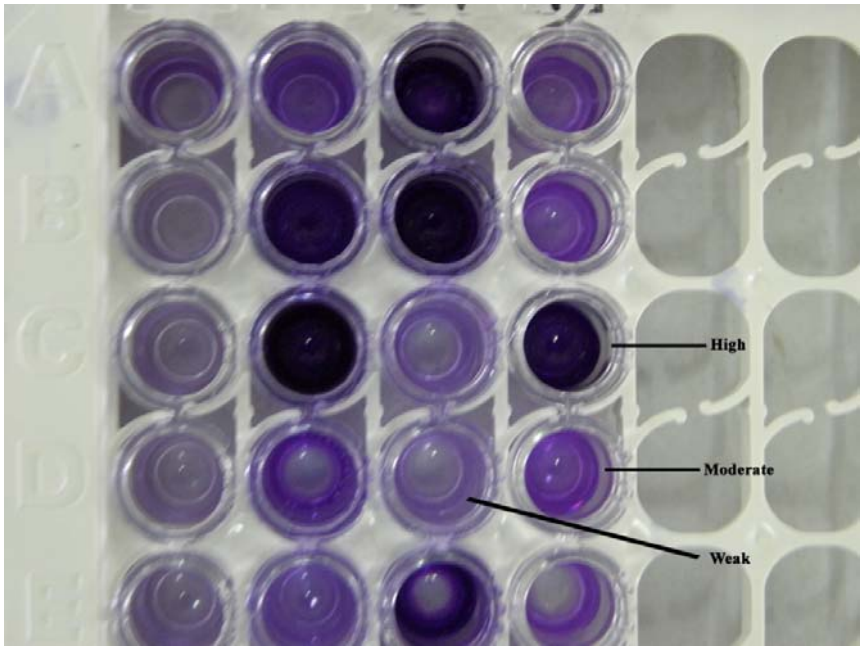


FIGURE 14

GEL DOCUMENTATION OF ICA GENE BY PCR