BACTERIOLOGICAL PROFILE OF CLINICALLY SIGNIFICANT COAGULASE NEGATIVE STAPHYLOCOCCI ISOLATED FROM VARIOUS CLINICAL SPECIMENS WITH SPECIAL REFERENCE TO THE VIRULENCE FACTORS.

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DECLARATION

I, **Dr. B.Padmini** solemnly declare that this dissertation entitled **"BACTERIOLOGICAL PROFILE OF CLINICALLY SIGNIFICANT COAGULASE NEGATIVE STAPHYLOCOCCI ISOLATED FROM VARIOUS CLINICAL SPECIMENS WITH SPECIAL REFERENCE TO THE VIRULENCE FACTORS"** was done by me at Coimbatore Medical College, Coimbatore during the period from June 2007 – July 2008 under the supervision of **DR. ANBU.N. ARAVAZHI, M.D.,** Professor and Head, Department of Microbiology and under the guidance of **DR.K.RAJENDRAN, B.Sc., M.D.,** Professor, Department of Microbiology Coimbatore Medical College, Coimbatore.

This dissertation is submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai towards the partial fulfillment of the requirement for the award of M.D. Degree (Branch- lV) in Microbiology to be held in March 2009.

I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

Place**:**

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CERTIFICATE

This is to certify that the dissertation entitled **"BACTERIOLOGICAL PROFILE OF CLINICALLY SIGNIFICANT COAGULASE NEGATIVE STAPHYLOCOCCI ISOLATED FROM VARIOUS CLINICAL SPECIMENS WITH SPECIAL REFERENCE TO THE VIRULENCE FACTORS"** is a bonafide work done by **Dr. B. Padmini**, Post graduate student in the Department of Microbiology, under the supervision of **DR.ANBU.N. ARAVAZHI, M.D.,** Professor and Head, Department of Microbiology, Coimbatore Medical College and under the guidance of **DR.K.RAJENDRAN, B.Sc., M.D.,** Professor, Department of Microbiology Coimbatore Medical College ,Coimbatore, in fulfillment of the regulations of the Tamilnadu Dr.M.G.R. Medical University towards the award of M.D. Degree (Branch- lV) in Microbiology.

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CONTENTS.

V. PROTOCOL

LIST OF ABBREVIATIONS

1	CoNS	Coagulase Negative Staphylococci
2	MHA	Muller Hinton Agar
3	CLSI	Clinical Laboratory Standards Institute
4	CRP	C Reactive Protein
5	PBP	Penicillin-Binding Protein
6	MR CoNS	Methicillin Resistance CoNS
7	MDR CoNS	Multi drug resistance CoNS
8	PYR	Pyrolidonyl aryl amidase
9	PSA	Polysacharide Adhesin
10	PIA	Polysacharide Intercellular Adhesin
11	FAME	Fatty Acid Modifying Enzyme
12	Ssp	Surface fibrillary protein
13	AtlE	Autolysin adhesion
14	MSCRAMM	Microbial Surface Components Recognising Adhesive Matrix Molecules
15	TCP	Tissue culture Plate method
16	MTP	Micro titre plate method
17	TM	Tube Method
18	CRA	Congo Red Agar
19	AAP	Accumulation Associated Protein
20	RE	Restriction endonuclease

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INTRODUCTION

Coagulase-Negative Staphylococci (CoNS) are ubiquitous microorganisms that predominate the normal skin flora. Due to their ubiquitous nature and relatively low virulence, CoNS have long been considered to be clinically insignificant, and hence when isolated from clinical specimens, the clinicians and the microbiologists discarded them as contaminants.

However, it is noted that under appropriate conditions, CoNS can produce serious human diseases and the incidence of such infections has clearly increased in recent years[1, 9]. Moreover they are seen as one among the most commonly isolated organisms in the clinical microbiological laboratory [1, 3]. According to the National Nosocomial Infections Surveillance System, CoNS are among the five most commonly reported pathogens in hospitals[3].

The postulated reasons for the current prevalence and clinical importance of these organisms include their great numbers on the skin, their selection as a result of wide spread usage of broad-spectrum antibiotics in the hospital, their ability to adhere to and form biofilm on the surfaces of vascular catheters and other medical devices, and their meager nutritional requirements[1].

The emergence of CoNS as important potential pathogens is partly due to the increased usage of transient / permanent medical devices and partly due to the growing appreciation of this group of organisms as opportunistic pathogens [2]. Infection caused by CoNS is increasingly common in patients having indwelling catheters, orthopedic appliances, prosthetic devices and urinary catheters.

Blood stream infections in neonates with IV catheter and orthopedic wound infections with implant devices are usually associated with Staphylococcus epidermidis[3, 5, 27.] Staphylococcus lugdunensis is mainly involved in causation of prosthetic valve endocarditis[27]. Staphylococcus haemolyticus is the second most frequently encountered CoNS species in the clinical laboratory [27.21]. Staphylococcus saprophyticus is the second most common cause of urinary tract infections in females of reproductive age group[1, 3.]

The pathogenicity of this clinically significant CoNS strains is due to the production of extra cellular polysaccharide known as slime which permits these microorganisms to adhere to implants and protects them from host immune system and antimicrobial therapy [4, 7.] It is well documented that biofilms are difficult to eradicate and are often resistant to systemic antibiotic therapy, thus requiring the removal of infected devices [5].

CoNS are becoming the reservoirs of multiple antimicrobial resistant determinants. Because of the enormous increase in emergence of multi drug resistant CoNS strains over the last decades, particularly methicillin resistant strains[2], it is necessary to predict their potential pathogenicity. This could be done by demonstrating biofilm formation in vitro, and evaluating the antibiotic susceptibility of each clinical isolate early in the course of infection, both of which will assist in providing appropriate antibiotic therapy and preventing implant removal [6].

It is essential to detect minor differences between related organisms to recognize the continued presence of an infecting strain or the environmental distribution of an epidemic strain. Combination of various typing systems is most advantageous for

strain identification [8], which will help in establishing improved methods for prevention, diagnosis, and therapy.

Among the various typing systems like phage typing, serological typing, biotyping, and antibiogram, the biotyping has frequently been used (alone or in combination with the antibiogram) as a means to distinguish specific strains of CoNS for clinical and epidemiological purpose[1].

Identification of isolates up to species level increases the knowledge of the pathogenicity of the various species of CoNS[6] and to determine whether any particular species is more likely to cause serious human infection[9]. It also contributes to the predictive value of an isolate being clinically significant[1]. Species identification is important to establish epidemiological trends, to determine the specific cause of infection, and to confirm the treatment failure[6]. Hence ,the study.

AIM:

The aim of the present study is to assess the various microbiological aspects of Coagulase-Negative Staphylococci (CoNS) and its clinical significance with special emphasis on demonstration of virulence factors and methicilin resistance.

OBJECTIVES:

1. To identify CoNS among bacterial isolates from various clinical specimens.
2. Speciation and biotyping of CoNS isolates.
3. To ascertain the significance of pathogenic CoNS in causation of infections, by demonstrating the virulence factors.
4. To evaluate the biofilm production by different methods.
5. To evaluate antimicrobial susceptibility pattern - methicilin resistance and multidrug resistance among CoNS.

REVIEW OF LITERATURE

History

- About 150 years ago, cocci were first observed in diseased tissues and in pus obtained from human abscesses.
  - In 1871, Von Recklinghausen named these organisms as “Micrococci”
  - In 1874, Billroth classified these organisms on the basis of their cell arrangements into Monococcos, Diplococcos, Streptococcos, and Gliococcos.
  - In 1882, Ogston named these pyogenic organisms as “Staphylococci” (Staphyle -“a bunch of grapes” and coccus - “a grain or berry”), an appropriate term derived from the Greek nouns[11].
  - In 1884, Rosenbach was the first to isolate staphylococci, grow it in pure culture, and study its laboratory characteristic[10]. He named the orange and white colony producers as Staphylococcus pyogenes aureus and Staphylococcus pyogenes albus, respectively [10, 11].
  - In 1891, the name Staphylococcus epidermidis albus was proposed for an attenuated form of Staphylococcus pyogenes albus[10].
  - In 1905-1906, Andrewes and Gordon proposed a classification scheme for human staphylococci based on their pigmentation and pathogenicity in guinea pigs. Under this scheme, 4 species were recognized: S.pyogenes - orange, yellow, or white, highly pathogenic. S. epidermidis albus-white,

feebly pathogenic; and two other species which were both white and nonpathogenic.

- In Bergy's Manual of Determinative Bacteriology, from the first edition (1923) till the fifth edition (1939), the number of species listed under genus Staphylococcus varied from none to nine. In the sixth edition (1948), the Staphylococcus genus was deleted and all staphylococci were relegated to the genus Micrococcus. In the seventh edition (1957), the genus Staphylococcus was reintroduced. In addition, two species, S. aureus and S. epidermidis, were recognized on the basis of anaerobic utilization of mannitol and the production of coagulase by the former. In the eighth edition (1974), the genera Staphylococcus, Micrococcus, and Planococcus were included in family Micrococcaceae, and the genus Aerococcus was placed in Streptococcaceae[11].

- In the 1986 edition of Bergy's Manual of Systematic Bacteriology, the family Micrococcaceae included four genera: Planococcus, Somatococcus, Micrococcus, and Staphylococcus [21.]

- In the road map of the latest edition of Bergy's Manual of Systematic Bacteriology,the genus Staphylococci is placed in family Staphylococcaceae, genus planococci in family Planococcaceae, genus Micrococci in family Micrococcaceae, and the only member of genus stomatococci, Somatococcus mucilaginous, is placed in genus Rothia[12, 21.]

PATHOGENESIS

When isolated from clinical specimens, CoNS were hitherto considered as common contaminants. However, in recent years, they are being increasingly recognized as pathogens[12]. Most of the CoNS are common inhabitants of the skin and mucous membranes. CoNS turn pathogenic when alterations in the integument due to wounds or implantation of foreign bodies allow these normal skin inhabitants to gain entry into the body, thus causing infections [9].

Over the last several decades, infection with CoNS has been characterized as related to “medical progress” (Rupp and Archer, 1994). The CoNS infections originate most frequently in the normal skin and mucosal flora of the patient contaminating the introduced implant during the course of surgery, or from the side of the surgical team, or environment or attending personnel. Apparently, the foreign body predisposes the patient to infection with what would otherwise be innocuous bacteria[36].

The organisms have been and continue to be pathogenic in patients with intravenous catheters, orthopedic devices, prosthetic and indwelling foreign devices[16], causing - septicemia, prosthetic joint infection, prosthetic valve endocarditis, peritoneal dialysis catheter-associated peritonitis, cerebrospinal fluid shunt infections[15], and permanent urinary catheter-associated urinary tract infections[42].

The ability of CoNS to form biofilm, mainly on the surface of foreign bodies in humans and also on the surface of tissues, plays the most important role in the pathogenesis of CoNS induced infections[42]. Tissue infections, the pathogenesis of which includes biofilm formation in the absence of foreign body, include urinary tract infection, skin abscesses, post operative wound infection, eye and ear infections[9], and native valve endocarditis. CoNS are common opportunistic pathogens in the immune-compromised. (i.e. intensive care patients, premature newborns, cancer and transplant patients). In these patients, CoNS infections can often be life-threatening[1]

In recent days, CoNS are among the most frequently isolated bacteria in the clinical microbiology laboratory [3, 13]. Frequent isolation of CoNS presents a recurring interpretive challenge to both clinical microbiologists and clinicians[1]. One of the major problems facing the clinical laboratory is in distinguishing clinically significant, pathogenic strains of CoNS from any contaminant strains[3,13].

Epidemiology

As a result of the growing recognition that CoNS are clinically important, many studies have been initiated since 1958 to classify these organisms[3].

- In 1958, Smith and coworkers[3],[71] noted the potential pathogenicity of CoNS by collecting data from patients with septicemia.
- In 1962, Pereira[72] reported that a certain group of CoNS (now known as S. saprophyticus) caused urinary tract infections (UTIs).
- In 1965, Wilson and Stuart[73] reported that CoNS were found in pure culture in 53 of 1,200 (4.4%) cases of wound infections.

- In 1971, Pulverer and Pillich[74] found that in about 50% of all pyogenic lesions observed in hospital patients, CoNS were present in pure culture.

- In the 1980s, the range of infections believed to be caused by CoNS, especially by S. epidermidis, was quite wide. Though S. epidermidis accounts for most CoNS infections, many other species have been identified in association with human infections.[40,41].

- In United States, during the late 1980s and early 1990s, the National Nosocomical Infections Surveillance System identified CoNS as one among the five most commonly reported pathogens (in fifth place at 9 to 9.7%, compared with 10 to 11.2% for Staphylococcus aureus) in hospitals conducting hospital-wide surveillance [3,17].

World-wide, CoNS are the most common cause of blood stream infections, the second most common cause of prosthetic valve endocarditis[76], and second most cause of urinary tract infections (UTI) in females of reproductive age group[21].

In United States, CoNS was the most frequently isolated bacteria from blood cultures[18], as shown in studies[77] by Pfaller (1999) (32%), Richards et al (2000) (38%), Karlowsky et al (2002) (42%).

In United Kingdom, CoNS was found to be the most common cause of peritonitis (29%) by Kavanagh et al[78.] CoNS was also the commonest cause of bacteraemia in the Neonatal Intensive Care Units (NICU) in UK, accounting for two-thirds of positive blood cultures[79.]

In West Indies, Akpaka et al (2003) reported that CoNS was the most prevalent among blood culture isolates (284/854, 33%) [19]

In India, CoNS are now the most common organisms associated with late-onset septicemia, accounting for more than 50% of all cases (Stoll et al., 2002; Nataro et al., 1994; Freeman et al., 1987)[80]. According to Vinodkumar et al(208)., CoNS was the third most common cause (13.4%) of neonatal septicemia[20]. In a study by Lakshmi et al (2006., CoNS was the most common isolate accounting for 36.36% of central nervous system (CNS) shunt infection[81]. In a study by Mohanty et al., CoNS was found to be the second most cause (19.2%) of pyoderma.

Morphology[21, 23]

Staphylococci are gram-positive cocci of about 1µm in diameter. The cocci are arranged in grape-like clusters; however, when examined in pathological specimens or liquid culture, they may occur as

single cells or pairs of cells. They are non-motile and non-sporing. Capsule or slime layer (diffuse capsule) may be present more commonly in vivo - especially in colonization by Staphylococcus epidermidis.

Cell wall contains Ribitol teichoic acid (Polysaccharide A) in Staphylococcus aureus, and Glycerol teichoic acid (Polysaccharide B) in Staphylococcus epidermidis. The guanine plus cytosine (G+C) content of Staphylococci DNA ranges from 30-40 mole% [26].

Cultural characteristics[21,27]

Staphylococci grow readily on media like blood agar, nutrient agar, tryptic soy agar, brain heart infusion agar ,etc. Growth occurs within a temperature range of 10 - 42^{0}C, the optimum being 37^{0}C, and within PH 7.4 - 7.6. They are aerobes or facultative anaerobes.

- In liquid media, uniform turbidity is produced.
- On MacConkey's medium, small pink colonies are produced.
- On blood agar, hemolytic as well as non hemolytic colonies are produced.
- On nutrient agar, tryptic soy agar, and brain heart infusion agar, colonies are large, convex, smooth, shiny, opaque, and easily emulsifiable.
- The typical CoNS colony is non pigmented, smooth, entire, glistening, convex, and opaque

- S. epidermidis colonies are small (1 to 2 mm in diameter depending on the strain), non-pigmented, and the slime-producing strains are sticky.
- S. haemolyticus colonies smooth, butryous, opaque, and may be non-pigmented or cream to orange in color.
- S. hominis colonies mimic the characteristics of S.epidermidis colonies.
- S. lugdunensis colonies are larger, smooth, glossy, cream to orange in colour or non-pigmented, entire-edged, and their center is slightly domed.
- S. warneri mimics the cultural characteristics of S. lugdunensis colonies[27].

- S. schleiferi colonies are non-pigmented, smooth, glossy, and slightly convex with entire edges.
- S. saprophyticus colonies are large, entire, very glossy, smooth, opaque, butryous, more convex, and half of the strains are pigmented.
- CoNS are strongly catalase-positive, and usually oxidase- negative[22, 23].
- S. aureus and S. intermedius are the only Coagulase-positive Staphylococcal species. All other staphylococci are Coagulase- negative (both free and bound Coagulase)

Species

Forty species are currently recognized in the genus staphylococcus; about one-half of these are indigenous to humans [1, 2, 10, 23].

Although, at least 18 staphylococcal species have been isolated from human skin[1], S. epidermidis accounts for more than half of the resident staphylococci with extensive distribution over the body surface. In terms of clinical isolates, S. epidermidis is clearly predominant, comprising more than 75% of CoNS in clinical specimens[29]. Other clinically significant species include S. saprophyticus, which, after Escherichia coli, is the second most common cause of uncomplicated urinary tract infections in women younger than 40 years; it is particularly prevalent in sexually active women who are in their teens and twenties[8]. S.haemolyticus, the second most frequently encountered CoNS species associated with human infections has been implicated in native wall endocarditis, septicemia, peritonitis, wound, bone, and joint infections[3]. S.lugdunensiss has increasingly been recognized

as a cause of invasive infections that include endocarditis, osteomyelitis, and sepsis[9]. Other CoNS species like S. warneri, S. simulans, S. hominis, S. capitis, and S. schleiferi are also implicated in various infections.

According to Gill et al (1983), except for S. saprophyticus and S. simulans, which were found more frequently in urine, the most common source for all other species combined was blood (50%), followed by wounds and abscesses (17%), and I.V. catheter tips (11%)[31].

In a study by Mohan & Jindal (2002), among 192 CoNS isolates, urine was the source of 93 isolates (48.4%), followed by pus with 34 isolates (18%), drain tips / catheter tips / I.V. cannulas with 22 isolates (11.4%), blood with 9 isolates (5%), and aural swabs with 19 isolates (2.7%)[35].

In a study by Goyal et al [2] (2006), of the 102 CoNS strains, wound was the most common source (38.2%), followed by urine samples (28.4%), blood cultures (14.7%), catheters (10.7%), cerebrospinal fluid (3.9%), synovial fluid (1.9%), and ascitic fluid (1.9%)..

Among potentially significant CoNS that were isolated from clinical specimens, 75% were S. epidermidis, while S. haemolyticus and S. hominis were the second and third most frequently encountered species, respectively. The above were also the three most common species isolated from blood cultures by Eng et al[15] (1982), and from wounds and body fluids by Sewell et al [9] (1982)

In a study by Goyal et al [2] (2006), among the clinically significant CoNS isolates, 41% were identified as S. epidermidis, followed by S. saprophyticus (16.6%), S. haemolyticus (14.7%), S. hominis (14.7%), S. lugdunensis (4.9%), S. schleiferi (1.9%), and S. capitis (1.9%). Only 4 isolates were

not identified to the species level; two of which were probably S. capitis subsp. ureolyticus / S. warneri / S. simulans.

Recently, when Lorio et al. (2007)[14] evaluated 198 CoNS isolates from different clinical sites, 69 (34.8%) were S. epidermidis, 44 (22%) were S. haemolyticus, and 25 (12.6%) were S.hominis isolates. The other species identified included S.saprophyticus subsp. Saprophyticus (4%), S.warneri (3.5%), S. lugdunensis (3%), S. capitis subsp. capitis (3%), S.cohnii subsp. urealyticum (2%), S. sciuri subsp.sciuri (2%), S. xylosus (2%), S. capitis subsp. urealyticum (1.5%), and a single S. cohnii subsp. cohnii strain.

In a study by John et al[34], the most common species among the urinary isolates of CoNS was S. epidermidis (53%), followed by S.hominis (12%), S.haemolyticus (10%), and S.saprophyticus (5%). However, Nord et al[67] reported that S. saprophyticus (44%), S. epidermidis (23%), and S. haemolyticus (14%) were the predominating species[34].

VIRULENCE FACTORS

Slime production has been shown to play a major role in the pathogenesis of infections caused by CoNS. According to Koneman et al[21] and Cunha et al[4], CoNS also produce other virulence factors such as hemolysin, lipase, protease, urease, and DNAse. Based on the above considerations, pathogenic CoNS are evaluated by demonstrating the virulence factors[4, 21.]

Virulence factors of Staphylococcus epidermidis [42]

1. Factors SSP-1, SSP-2, AtE and Delta toxin - Play a role in attachment to abiotic surfaces like polystyrene.

2. Factors Fbe /SdrG, Embp, GehD - Aid in attachment to host matrix proteins by fibronectin- , fibrinogen-, and collagen-binding.
3. Factors PSA, PIA - Aid in cell-cell adhesion for accumulation.
4. Exoenzymes like,

    a. lipase for persistence in fatty acid secretion

    b. Cystein protease to induce tissue damage

    c. Metallo protease and Serine protease for biofilm formation and

    d. FAME which detoxifies bactericidal toxin in host

Regulators of Virulence:

agr - Affects lipase and protease production

sar – Affects virulence factors production

sig B – Affects biofilm formation

Virulence factors of staphylococcus saprophyticus [43]

Autolysin Ass, mediates adhesion to fibronectin on uroepithelial surfaces and haemagglutination. The surface fibrillary protein Ssp, functions as intercellular adhesin in formation of multilayered clusters. Other virulence factors include urease (which causes destruction of bladder), lipase, elastase, and FAME[21, 42].

Virulence factors of staphylococcus lugdunensis

The infections caused by S. lugdunensis are more serious. They create bonds with host molecules by producing lipase, elastase, and FAME. S. lugdunensis is not bound to fibrinogen like other CoNS; instead, it produces peptides called S. lugdunensis Synergistic hemolysins (Slush) [42].

Virulence factors of staphylococcus haemolyticus

S. haemolyticus also produces peptides and bind to the host molecules. They also produce Gonococcal growth inhibitor factor(GGI). The reduced susceptibility of S. haemolyticus to teicoplanin is due to substitution of glycine with other amino acids in peptidoglycon.

The above mentioned virulence factors are also produced by other CoNS species[42]. FAME is produced by S. capitis, S. hominis, S. simulans, and S. cohnii. Urease is produced by S. capitis, S. hominis, and S. warneri. Lipase is produced by S. capitis, S. hominis, S. warneri, and S. schleiferi.

Biofilms

CoNS, especially S. epidermidis, frequently infect biomedical implants, transcutaneous devices such as intravascular and peritoneal catheter[16,21], CSFshunts[1], and prosthetic cardiac valves[1,21]. The pathogenesis of biomedical implant infections caused by S. epidermidis can be separated into two distinct phases:

(i) Primary bacterial adherence to the surface of the device and

(ii) the production of a stable biofilm matrix over the surface of the device[37].

I. The primary bacterial adhesion involves rapid adhesion of bacteria to a specific surface followed by their accumulation and proliferation. This results in the creation of multilayer clusters on the surface, whether, of the tissue or of the foreign body, surrounded with extra cellular material[37].This initial phase involves Van der Waals forces, hydrophobic interactions, and polarity changes. Whether the surface of the material is coated ("conditioning film") or uncoated with host molecules is also of importance in adhesion . In case of unmodified surfaces, this interaction is mediated by the surface structures, SSP-1, SSP-2, AtE, and teichoic acid, of the bacteria [43]. In case of modified or conditioned surfaces, adhesion proceeds with the assistance of proteins designated as MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) [4] .

II. Proliferation or production of biofilm matrix, where-in bacteria accumulate to form multilayered cellular clusters, requires intra cellular adhesion which is provided by the polysaccharide intra cellular adhesin (PIA), and the extra cellular protein along with accumulation associated protein (AAP).The genes responsible for the PIA formation are located on the ica operon which consists of icaR (regulating gene) and ica ABCD (biosynthetic gene) [26, 42].

It is now well documented that biofilms are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy and removal of infected device becomes

necessary[9–11]. The differentiation of CoNS with respect to its biofilm phenotype might help to elucidate the impact of CoNS in diagnosis of infections related to biomedical devices and these observations may also enable prevention of device-related infections[12].

In a study by Christensen et al (1982)[51], 60% of clinically significant bloodstream isolates of CoNS produced slime.

In a study by Ishak et al (1985) [52], 92.8% of clinically significant bloodstream isolates of CoNS, as well as 23% of contaminants, produced slime. Among the slime producers, S. epidermidis was the most prevalent constituting 92 % of the isolates.

Deighton et al (1988)[47] found 48% of CoNS isolates from clinical specimens to be slime producers; of this, 53% slime positivity was among S. epidermidis species and 52% among S. haemolyticus.

Kotilainen et al (1990)[49] reported 65% slime production by pathogenic strains and 40% slime production by nonpathogenic strains of CoNS.

Baldassarri et al (1993)[54] noted that the fixation of bacterial films that were produced by a standard slime producing strain of CoNS on plastic tissue culture plates varied with the type of fixative. Bouin reagent is reliable but is potentially explosive, thereby marking it dangerous for routine use.

Rathinam et al (1993) [55] reported that 40% out of 407 CoNS isolates tested positive for slime production. The prevalence rate of slime production among CoNS species were 62% for S. epidermidis, 38.6% for S. saprophyticus, 36% for S. hominis, 25% for S. cohni, and 17% for S. capitis.

Phatak et al (1994) [56] showed that, among the 106 CoNS isolates, 1 out of 24 strains (4.17%) of S. saprophyticus and 34 out of 82 strains (41.46%) of S. epidermidis tested positive for slime productio

Makhija et al (1995)[57] reported that 43 out of the 101 CoNS isolates from clinical specimens were slime producers. The percentage of slime-producing CoNS ranged from 20% in peritoneal fluid to 66% in CSF.

Mohan et al (2002)[35] reported slime producing CoNS from various clinical specimens. Out of 192 strains of CoNS, slime production was exhibited by 77 (48.7%) strains of S. epidermidis and 8 (26.6%) strains of S. saprophyticus.

A number of tests are available to detect slime production by Staphylococci. The methods include Micro titer plate (MTP) method[5, 36], Tube method (TM) [5, 38], Congo red agar (CRA) [39], bioluminescent assay[41], and light or fluorescence microscopic examination[19, 20]. However, these methods often suffer from severe analytical limitations.

The adherence of CoNS to smooth surfaces was assayed by measuring the optical densities of stained bacterial films adherent to the floors of plastic tissue culture / micro titre plates. The optical densities of bacterial films adherent to plastic tissue culture / micro titre plates serve as a quantitative model for the study of the adherence of CoNS to medical devices, a process which may be important in the pathogenesis of foreign body infections[36]. The measurements also agreed with visual assessments of bacterial adherence to culture tubes.

In a study by Mathur et al[5], the modified tissue culture plate method(TCP)was most sensitive (96.2%), specific (94.5%), and accurate (97.3%) in terms of discriminating between biofilm producers and non-producers. In the case of

tube method(TM), though the strong biofilm producers could be easily detected, the difficulty in differentiating between moderate and weak biofilm forming isolates affected its performance in terms of sensitivity (77.9%), specificity (96.0%), and accuracy (86.8 %). The (CRA) congo read agar method showed very little correlation with corresponding methods, and the parameters of sensitivity (7.6 %), specificity (97.2 %), and accuracy (51.3 %) were very low. The Tissue culture plate or MTP method also has the advantage of being a quantitative model to study the adherence of staphylococci on biomedical devices, a finding reported by Christensen et al (1983)[8]

Speciation

In the hospital microbiology laboratory, identification of Staphylococci is often limited to a rapid screening test for S. aureus, while non-S. aureus isolates are simply reported as CoNS [6]. The clinical significance of CoNS continues to increase as strategies in medical practice lead to more invasive procedures. It is crucial to reliably identify these organisms in order to shed light on the clinical significance of infections caused by each Staphylococcal species, and to provide data for control and epidemiological measures.

Identification and speciation of CoNS by the reference method of Kloos and Schleifer involves 36 tests[3]; it is a reliable but relatively cumbersome and time consuming method that requires expensive reagents which are not routinely available in most of the clinical laboratories. Several commercial kit identification systems and automated instruments, which can identify a number of Staphylococcus species accurately, are currently available. However, they are still out of reach of most of the laboratories in developing countries[2, 14]. Hence, convenient, reliable, and inexpensive identification

methods are needed to identify most of the CoNS, which are commonly implicated in majority of infections.

Simplified method using nine tests for CoNS identification, evaluated by Iorio et al (2007)[14], identified 12 clinical species of CoNS, including 8 subspecies, with an accuracy of 98.5%.

Another simple scheme employing minimal number of tests was adapted by Goyal et al (2006)[2] for the rapid identification of CoNS. In this two-step procedure-based scheme, the first step involves identification of species group, and, if identification requires additional tests, a maximum of two tests were selected for second step. The shortcoming of this scheme is the exclusion of the important PYR test which differentiates warneri species.

A rapid 4-hour method (UZA method) has been developed by Leven et al at the Laboratory of Microbiology, University Hospital, Antwerpen, Belgium(1995)[30]. This method, which detects the enzymatic activity of heavy bacterial suspensions in three substrate solutions (non growth tests), correctly identified 97.7% of CoNS isolates within 4 hours.

The five-test simple scheme designed by De Paulis et al (2003)[6], focuses on the species or species groups encountered among clinical isolates. The species groups were defined according to their biochemical reactions rather than their phylogenetic relationships. The addition of one or two tests within each particular species group could complete the strain identification, even the most infrequently isolated species. Of the 201 strains, 200 (99.5%) were identified by the reference method, and the results were similar to those from the simple scheme identification method.

TYPING METHOD

Several different epidemiological typing methods have been applied to study CoNS. Epidemiological typing scheme for CoNS is required for strain identification, which has become important since the recognition of the clinical significance of CoNS. The typing methods include biotyping (biochemical profiles), serological typing, phage typing (bacteriophage susceptibility patterns), DNA typing (plasmid profiling, restriction endonuclease analysis of plasmid and chromosomal DNA hybridization), and antibiogram (antimicrobial susceptibility profiles).

More recently, research groups have been working on a typing scheme that uses a combination of several techniques. Hebert et al[47] used a combination of the API Staph-Ident biochemical profile along with adherence and synergistic hemolysis to define biotypes of CoNS. This scheme, with the addition of five antibiotic discs (Novobiocin, Polymeric B, Bacitracin, Furazolidone, and TaxoA) and pyroglutamyl-3-naphthylamide hydrolysis, allowed separation of strains within the S. lugdunensis and S. schleiferi species into several biotypes.

Herwaldt et al[48] incorporated the API Staph Trac, antibiotic profile, slime production, and synergistic hemolysis for successful strain discrimination.

Ludlam et al[49] achieved 95% of the discriminatory power of the scheme when they used plasmid analysis along with antibiograms or biotyping and phage typing along with antibiograms.

With the ease in obtaining an antibiogram and a biotype, these techniques in combination would provide a moderate degree of strain delineation for those laboratories that are not able to incorporate more advanced techniques. The timely

delineation of specific phenotypic (or genotypic) profiles of CoNS by one or more of the above methods will provide the basis for studies that are designed to answer important epidemiologic and pathogenic questions.

Biotyping

The generation of a biochemical profile or biotype has frequently been used, (alone or in combination with the antibiogram) as a means of distinguishing strains of CoNS[8]. Several different biotyping systems were proposed for both taxonomic and epidemiologic purposes. These systems, based on numerous biochemical and physiological tests, included those proposed by Baird-Parker[11], Bentley et al[44], Holt[45], and several others.

Of the various biotyping schemes, only those of Baird-Parker and Bentley have received much attention. The Baird-Parker system deals primarily with strains from non clinical sources, and, in general, places more emphasis on taxonomy[8,50]. The Bentley et al. system, which includes six stable tests (Maltose, Mannose, Lipase, Phosphatase, Glucuronidase, Gelatinase) was developed specifically for epidemiological purpose and it has often been used in clinical situations[44].

The role of S. epidermidis in severe infections involving the colonization of prosthetic devices is well established. Because of its resistance to treatment, infections by S. epidermidis can present therapeutic problems[21]. To date, the most generally accepted method to distinguish subgroups of S. epidermidis is the biotyping scheme of Baird-Parker. The purpose of this study was to apply this typing scheme to S. epidermidis isolated from patients, and to correlate biotype with clinical source and the production of virulence factors.

Serological typing

Serological typing has been useful as an epidemiological tool in the investigation of a variety of infectious diseases; however, it has not been well developed for use with CoNS[1]. As might be expected, this is largely due to the difficulties in preparing specific antisera and in the standardization of typing methods[10]. Given the availability of other typing systems for CoNS, serotyping does not appear to have much of a role in epidemiological studies of these organisms.

Phage typing

The most established system for epidemiological typing of Staphylococci is the determination of bacteriophage susceptibility patterns (phage typing). Currently, there 4 are major phage-typing sets available for CoNS[1], including those of Van Bovenet al., Pulverer et al. ,Dean et al., and Parisi et al [10] .

According to an international collaborative study, none of the phage typing systems was satisfactory with regard to typability, reproducibility, and discrimination[10]. Despite these problems, phage typing has been used successfully to type CoNS, particularly in epidemic situations.

Christensen et al. (1983)[8] demonstrated that phage typing by itself was a poor strain discriminator in a non epidemic situation; however, when combined with antibiogram and biotyping, phage typing provided adequate discrimination between strains. The authors suggested that the phage typing of CoNS is better reserved for epidemic, rather than endemic, situations[1].

Molecular typing

The application of molecular typing through plasmid pattern analysis, restriction endonuclease (RE) analysis of plasmid and chromosomal DNA, and DNA hybridization, is proving to be a promising mean of identifying strains or a clonal population of Staphylococci.

Plasmid analysis has been shown to be useful in a number of clinical situations and in differentiating contaminants from pathogens which cause septicemias, prosthetic valve endocarditis, intravenous catheter sepsis, urinary tract infections, and osteomyelitis[1.] Further identification of individual plasmids can be achieved by RE treatment of the plasmids followed by gel electrophoresis.The techniques of RE analysis and DNA hybridization provide additional information on the nucleotide sequence within the plasmid, thus enabling the confirmation of the identity between plasmids with a high degree ofconfidence[66]. Genotypic markers, which are more useful for the assessment of strain identity, can at present be carried out by only a few laboratories[66].

Antibiogram

Antibiogram, being the easiest and most frequently used test, has been suggested as the primary tool for the typing of CoNS [3]. Because of the ready availability and pattern variety, clinicians frequently apply antibiograms toward the identification of clinically significant strains of CoNS[8]. The presence of a strain with a unique antibiotic susceptibility pattern can provide a marker for detecting similar isolates; however, CoNS are pathogens associated with multiple antimicrobial resistance mechanisms including, in particular, methicillin resistance [59].

CoNS have historically been more resistant to antimicrobials, including the β-lactam antibiotics, than S. aureus; some hospitals report that the rates of oxacillin resistance in CoNS is approaching 90%[61]. Eighty to 90% of CoNS produce an inducible beta-lactamase. The most important mechanism for resistance to beta-lactam is the production of a low-affinity penicillin-binding protein, PBP2a[70], which is encoded by the mecA gene. Various CoNS species harbor the mecA determinant which confers resistance to the penicillinase-resistant penicillins, such as dicloxacillin, methicillin, nafcillin, and oxacillin[63]. The presence of the mecA gene in a Staphylococcal isolate is considered to be synonymous with oxacillin resistance. Thus, genetic assays for mecA have often been used as the reference method for evaluating new methods of antimicrobial susceptibility testing for staphylococci[68,69].

Most of these methicillin-resistant CoNS were also resistant to multiple additional antimicrobial agents. Multi-resistant CoNS also commonly colonize the skin of hospitalized patients and hospital personnel. Widespread skin colonization serves as a potential reservoir for multi-resistant isolates that can cause infections, particularly infections of indwelling intravascular devices. In addition, these colonizing isolates serve as a reservoir for antibiotic resistance genes that can transfer among CoNS and be acquired by S. aureus[70].

Staphylococcal biofilm formation is quite common in CoNS infections and it reduces the organism's susceptibility to specific antimicrobials. The mechanism of biofilm-associated antimicrobial resistance is likely to be multifactorial. The problem of antibiotic-biofilm interaction has recently become a stimulus to develop antibiotics that can offer some promise of penetration of biofilm to inhibit or kill adherent staphylococci[62].

In a study by John, Gramling, and O'Dell (1978)[60], 24% of the CoNS isolates were sensitive to all the antibiotics tested. The incidence of resistance was greater for penicillin (35%), followed by tetracycline (33%), methicillin (27%), sulfonamide (24%), erythromycin (15%), and clindamycin (13%); only five isolates (4%) were resistant to cephalothin.

Gill et al (1983)[31] reported that the resistance to oxacillin was highest among isolates of S. haemolyticus (76%), followed by S. epidermidis (38%). Sixty four to 76% of isolates of S. haemolyticus showed resistance to oxacillin, cephalothin, cefoxitin, erythromycin, clindamycin, gentamicin, and tobramycin; S. epidermidis showed only 33-51% resistance.

Mary et al (1996)[63], based on PCR-based detection of mecA, reported 49% of methicillin resistance among CoNS isolates.

In a study by Goyal et al (2006)[2], the antibiotic susceptibility pattern of CoNS isolates revealed no resistance to vancomycin; however, 89% were resistant to ampicillin, 59% to cefotaxime, 25% to cloxacillin, 23% to erythromycin, 29% to ciprofloxacin, and 20% were resistant to gentamicin .

In a study by Singh et al (2006)[64], CoNS exhibited maximum resistance against penicillin (80%) and ampicillin (80%), followed by erythromycin (54%), ciprofloxacin (54%), oxacillin (38%), rifampicin (32%), chloramphenicol (32%), and gentamicin (28%); no resistance to vancomycin was seen.

Chaudury et al (2007)[65] reported 68.4% of oxacillin-resistant CoNS, and all the strains were sensitive to vancomycin and linezolid.

When the strains are susceptible to oxacillin, β-lactam drugs are preferred over vancomycin because, β-lactam drugs are easily absorbed into body fluids and tissues, cause fewer complications from treatment, and prevent unnecessary use of vancomycin.

Many studies demonstrate the high prevalence of oxacillin resistance among hospital isolates of CoNS in India[7]. Thus, there has been a pressing need for newer antimicrobial agents with good anti-staphylococcal activity. Vancomycin, along with newer agents like Linezolid and Clindamycin, remains the drug of choice for treating MDR CoNS[61].

METRIALSAND METHODS

Study design

The present study was conducted on CoNS isolates obtained from specimens submitted by in-patients and out-patients at the Diagnostic microbiology laboratory, Dept of Microbiology, Coimbatore Medical College, Coimbatore, during the period of June, 2007 to July, 2008

A total of 135 CoNS Strains were isolated from deep wounds, blood samples, pus, urine samples, body fluids, and IV catheter tips by conventional cultural methods. Of the 135 isolates, 90 were considered as clinically significant. The following criteria were considered while determining the clinical significance of a CoNS isolate[9].

Inclusion Criteria

1. Strains isolated in pure culture from specimens

2. The culture was from body fluids (blood, cerebrospinal fluid, urine, pleural fluid, joint fluid, peritoneal fluid, etc.), or from a purulent, draining wound

3. High colony-count urine samples (>10^5 organisms per ml)

4. I.V. catheter tips > 15 colonies,

5. Significant clinical symptoms of diseases like fever, purulent discharge from wound, signs of general & local Inflammation, and repeated urinary tract infections.

6. Laboratory criteria like increased count of WBC, ESR, CRP, etc.

Exclusion criteria

1. Organism isolated as mixed growth
2. Growth only in broth
3. Growth after 72 hrs.
4. No significant clinical symptoms of disease in patients

The procedures used in this study were approved by the Ethics Committee of the College. The strains collected were initially identified by colony morphology, Gram staining,catalase, slide and tube coagulase (read after 4 & 24 hours). Acid production from glucose in Hugh and Leifson's OF base, and bacitracin (0.04 U) susceptibility were determined to exclude Micrococcus spp.

1. Colony Morphology following culture

Strains were cultured on Nutrient agar plate and Blood agar plate. After 24hrs of incubation at 37^0C, Nutrient agar plates were examined for colony morphology and pigment production, while Blood agar plates were examined for colony morphology and haemolysis.

1. O/F Glucose test (Modified Hugh & Leifson) [82, 84]

Principle

The test is performed to determine the ability of the bacteria to utilize a carbohydrate (Glucose) by oxidative or fermentative metabolism, thereby enabling the differentiation between Staphylococci and Micrococci. Staphylococci produce acid by fermentation both in the anaerobic tube sealed with paraffin and in the aerobic unsealed tube.

Staph OF medium

Tryptone 10.0 grams, Yeast extracts 1.0 gram, Agar 2.0 grams, Bromothymol blue 0.04 gram, and Glucose 10.0 grams, in 1000 ml water.

Method

The test culture was inoculated into two test tubes containing the medium by stabbing down their whole length with a long straight wire. One of the stab-cultured tube was covered with vaspar (1:1 Petrolium jelly and paraffin) or a layer of sterile liquid paraffin, and both tubes were incubated at 37^0C for 48 hours .

Interpretation

The cultures which produced acid by fermentation in both tubes were considered as Staphylococci, and the cultures which produced acid only in aerobic tube by oxidation were discarded as Micrococci.

2. Bacitracin sensitivity [82, 83]

This test is performed to differentiate between Staphylococci from Micrococci.

Procedure

A suspension of test isolate with a 0.5 McFarland standard turbidity was prepared. The test culture was swabbed on the Muller Hinton Agar and plates. Using heated forceps, a bacitracin disc impregnated with 0.05 µg of bacitracin was placed over the surface of the inoculated media and incubated for 24 hours.

Interpretation

Organisms showing less than 10 mm of inhibitory zone were considered as Staphylococci, and organisms showing more than 10 mm of inhibitory zone were discarded as Micrococci

3. Coagulase test

Principle

The test is performed to determine the ability of an organism to clot plasma by the action of the enzyme coagulase, and is used to differentiate between S. aureus and CoNS.

a. Slide Coagulase test [82, 85] **(**Williams and Harper 1946)

This test detects bound coagulase.

1. A single colony was emulsified in a drop of saline on a clean glass slide with a minimum of spreading. If the isolate did not form a smooth milky suspension, the test was aborted at that step.

2. Similar suspensions were made for positive and negative control strains to confirm the proper reactivity of the plasma.

3. A straight inoculating wire, flamed and subsequently cooled, was dipped into the undiluted plasma at room temperature, withdrawn, and the adhering traces of plasma (not a loopful) was stirred into Staphylococcal suspension on the slide. The procedure was repeated for the control suspensions.

4. The test was read as positive if visible coarse clumping occurs within 10 seconds, and read as negative if clumping was absent or if any of the reactions took more than 10 seconds to develop. However, any slow reacting strains were re-examined by the tube test.

b. Tube Coagulase test [82, 84] (modified from Gillespie 1943)

This test detects both bound and free coagulase.

1. A 1 in 6 dilution of plasma in saline (0.85% Nacl) was prepared, and subsequently 1ml volumes of the diluted plasma were aliquoted into small tubes.

2. A staphylococcal colony being tested was emulsified in a tube of diluted plasma.

3. For each batch of tests, known Coagulase-negative and coagulase-positive cultures were included as controls. In addition, a tube of unseeded diluted plasma was included to confirm that it does not coagulate spontaneously.

4. The tubes were incubated at 37^0C for up to 4 hrs.

5. The test samples were examined at 1, 2 and 4 hour intervals for clot formation, by tilting the tube through 90^0.

6. Negative tubes were left at room temperature overnight and re-examined the next day.

7. The test was read as positive if any degree of clot formed, and read as negative if the plasma remained wholly liquid showing only a flocculent or ropy precipitate.

Speciation

The collected CoNS isolates were speciated by using five tests simple scheme method[6] (appendix- 1). The scheme combines one susceptibility test (novobiocin resistance test) with tests for urease, PYR, Ornithine decarboxylase, and aerobic acid from mannose. These tests were performed according to the reference method. Only one or two additional tests were used to resolve each species group: trehalose and mannitol for the S. epidermidis group, acetoin production and lactose for the S. haemolyticus group, trehalose for the S. saprophyticus group, anaerobic thioglycolate growth for the S. warneri group, and xylose for the S. cohnii group.

1. PYR Test [39]

Principle

This test is a useful phenotypic test for speciation of CoNS. The L-Pyrrlidonyl-ß-naphthylamide is cleaved by the aryl amidase, which is produced by the organism, to L-pyrolidone and free β-naphthylamide. These, then combine with the PYR reagent (p-dimethyl amino cinnamaldehyde) to produce red colour.

PYR Agar (Himedia)

Beef heart, infusion 500 gms/litre from 20 gms/litre of Peptic digest of animal tissue, Dextrose 2.0 grams/litre, Sodium chloride 2.0 grams/litre, Disodium phosphate 0.40 grams/litre, Sodium carbonate 2.5 grams/litre, Chromogenic mixture 0.10 grams/litre, Agar 5.0 grams/litre.

PYR Reagent

N, N–Dimethyl amino cinnamaldehyde 1.0 gram, Hydrochloric acid (concentrated) 1.0 ml**,** Distilled water 99.0 ml.

Method

The test isolate was inoculated on the PYR agar plate and incubated for 24 hrs. Chromogenic mixture in the agar provides substrate for PYR enzyme. After incubation, 1 drop of PYR reagent (R043) was added directly to any suspected surface growth on plate, and subsequently observed for colour change after 2 minutes.

Interpretation

The appearance of red colour within 2 minutes was considered as positive reaction, while orange or pink colour appearance was considered as negative reaction.

2. Ornithine Decarboxylase test (ODC) [82] (Kloos & Lamble 1991)

The production of Ornithine Decarboxylase identifies S. lugdunensis.

Medium

Peptone 5.0 grams, Yeast extract 3.0 grams, L-Ornithine hydrochloride 0.5%**,** Glucose 1grams, and Bromocresol purple 0.2%, in 1 litre of water.

Method

The test isolate was stab-inoculated into the medium, overlaid with a layer of sterile liquid paraffin, and incubated at 37^0C for 24 hrs. The production of the enzyme decarboxylase by the organism in the tube was tested.

Interpretation

The production of violet colour was read as production of the Decarboxylase, while a yellow colour was read as a negative reaction

3. Voges – Proskauer Test: [84, 85, 87]

Principle

The test is performed to determine the ability of the micro organisms to ferment glucose with the production of acetyl methyl carbinol (acetoin). This test helps to speciate CoNS.

Medium

Glucose phosphate peptone water.

Method

Five ml of the media was inoculated with the test isolate and incubated at 37^0C for 48 hrs. Subsequently, 1 ml of 40% potassium hydroxide and 3 ml of 5% solution of α-naphthol in absolute alcohol were added and the contents were shaken vigorously for 30 seconds.

Interpretation

Reddening of the supernatant within 5-10 mins due to acetoin production was read as positive reaction. A negative reaction was indicated by the development of yellow colour.

4. Carbohydrate utilization test [82,84]

Principle The test is used to determine the ability of an organism to ferment a specific carbohydrate which is incorporated in a basal medium, and to produce acid or acid with visible gas. Sugars tested included Mannitol, Trehalose, Lactose, and Xylose; these sugar tests help in further speciation of CoNS.

Medium

Sugar 1.0 gm, Nutrient broth base 100 ml, Bromothymol blue indicator1.2 ml.

Method

The test media was inoculated with the cultural isolate and subsequently incubated at 37^0C for 24 hrs.

Interpretation

A positive test was shown by yellow colouration of the medium due to acid production; no colour change, due to inability of the microorganisms to ferment sugars, was read as negative reaction.

5. Novobiocin sensitivity test [21]

This test differentiates S. saprophyticus from other CoNS.

Method

A suspension of staphylococcal isolate, equivalent to 0.5 McFarland standards in turbidity was prepared and subsequently spread over MHA plate by using a sterile swab. A 5 µg Novobiocin disc was placed over the media by using heated forceps, and the plate was incubated for 24 hours.

Interpretation

The isolates were distinguished as saprophyticus if the inhibition zone was less than 12 mm in diameter, and other CoNS if the inhibition zone was more than 12 mm in diameter

6. Urease Test [82, 84]

Principle

The test is performed to determine the ability of microorganisms to produce the enzyme urease. The occurrence of this enzyme can be tested for by growing the organism in the presence of urea and subsequently testing for alkali (NH3) production by using a suitable pH indicator.

Medium

Christensen's urease medium:

Peptone 1.0 gram, Glucose (0.1%) 1.0 gram, Sodium chloride 5.0 grams, Mono potassium phosphate 2.0 grams, Phenol red 0.012 gram, Agar 15-20 grams, Distilled water 1000 ml.

Method

A drop of 4-6 hours old bacterial broth culture was inoculated heavily over the entire slope surface. The sample, incubated at 37^0 C, was examined after 4 hrs or after overnight incubation.

Interpretation

The test was read as positive reaction when the indicator turned purple–pink , and negative reaction when no change in colour occurred.

7. **Growth in Thioglycolate broth**

The test is performed to determine the ability of microorganisms to grow anaerobically.

Medium

Thioglycolate broth**:** Casein 15.0 grams, Yeast extract 5.0 grams, Sodium chloride 2.5 grams, Dextrose 5.5 grams**,** L-Cystine 0.5 gram, Sodium thioglycolate 0.5 gram, in 1000 ml of water.

Method

The test culture was inoculated in to the broth and incubated at 37^0C for 24hrs.

Interpretation

The presence of turbidity indicates the growth of the organism.

BIOTYPING

Subgroups of S. epidermidis were distinguished by the biotyping scheme of Baird-Parker[11]. The production of acid from lactose, maltose, and mannitol under aerobic conditions, and the production of acetoin as described earlier in carbohydrates utilization tests and Phosphatase test, were used to classify each culture into one of the four biotypes of Baird Parker.

Phosphatase test [82, 84]

Principle

The test is used to determine the ability of microorganisms to produce sufficient phosphatase enzyme for splitting phenolphthalein di phosphate.

Medium

Phenolphthalein phosphate Agar.

Method

The test and control cultures were spot-inoculated on to the plates, and the plates were incubated overnight at 37°C. The plates with culture were inverted over the lid after adding few drops of ammonia solution in the lid.

Interpretation

The assay was read as positive if the colonies turned pink, and negative if the colonies were colourless.

DEMONSTRATION OF VIRULENCE FACTORS

The production of slime, lipase, lecithinase, DNAse, and hemolysin by the different isolates were determined.

Hemolysin production [4,87]

Principle: To determine the production of hemolysin by CoNS.

Medium: Blood agar plates containing of 5% sheep blood.

Method: The test culture isolates were inoculated on to Blood agar plates, and the plates were incubated at 37°C for 24 hrs.

Interpretation: A positive result was indicated by the formation of hemolytic zones around the isolated colonies.

Lipase production[44]

Principle: To determine the ability of CoNS isolates to produce the enzyme Lipase.

Medium: Sterile lipase reagent (HIMEDIA) was added (30% W/V) to spirit blue agar (HIMEDIA) which had been autoclaved at 121^0C for 15 min.

Method: The test culture isolates were spot-inoculated on to the spirit blue agar plate along with known positive and negative controls. The inoculated plates were incubated at 37°C for 48 hrs.

Interpretation: Lipolytic strains gave a dark blue clear zone beneath and around the colonies.

Lecithinase production [87,5,50]

Principle: To determine the ability of CoNS isolates to produce the enzyme lecithinase.

Medium: BHI agar 100 ml; Yolk of 1 egg (Egg yolk is added to BHI agar which had been autoclaved at 121^0C for 15 mins and cooled to 45^0C)

Method: Test culture isolates were spot-inoculated on to the egg yolk agar plate, along with known positive and negative controls, and the contents were incubated at 37°C for 48 hrs.

Interpretation: Positive reactions showed a glistening or opaque zone, or both, around the colonies.

DNAse production[44,87]

Principle: To determine the ability of CoNS isolate to produce the enzyme DNAse.

Medium: (Himedia) DNAse test medium that had been autoclaved for 15 mins at 121^{0}C.

Method: The test culture isolates were spot-inoculated on to DNAse agar plate with known positive and negative controls, and incubated at 37°C for 48 hrs. Plates were flooded with1%Toluidine blue and allowed to stand for 15 mins.

Interpretation

Any degree of clearing of the opaque media was read as a positive reaction.

Slime production

Principle

To determine the ability of micro organism to form biofilm, and also to evaluate the reliability of these methods for determining the most suitable screening method.

Microtitre plate method (MTP) [5,36]

• Isolates from fresh agar plates were inoculated into trypticase soy broth (TSB) with 1% glucose, and the contents incubated for 48 hrs at 37^{0}C.

• The broth culture was diluted 1 in 100 with fresh medium. •

Individual wells of sterile, polystyrene, 96 well-flat bottom Microtitre plates were filled

with 0.2 ml aliquots of the diluted cultures; un-inoculated broth served as a control to check for sterility and non-specific binding of media.

• The tissue culture plates were incubated for 24 hours at 37°C.

• After incubation, contents of each well were removed by gently tapping the plates.

• The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS, pH 7.2) to remove free-floating 'plank tonic' bacteria.

• Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and then stained with crystal violet (0.1% w/v).

• Excess stain was rinsed off by thorough washing with deionized water, and the plates were kept for drying.

•Optical density (OD) of stained adherent bacteria were determined with a micro ELISA autoreader (model680, Biorad) at a wavelength of 570 nm (OD_{570}nm).

• These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

• To compensate for background absorbance, OD readings from sterile medium, fixative, and dye were averaged and subtracted from all test values. • The mean OD value obtained from media control well was deducted from all the test OD values[88] (appendix-2). S.epidermidis ATCC 31432 strain is used as control.

Tube method (TM) (Christensen et al) [5, 36]

A semi qualitative assessment of biofilm formation was determined as

• TSB supplemented with 1% glucose in tubes were inoculated with loopful of microorganism from overnight culture plates, and the contents were incubated for 24 hours at 37°C.

• The tubes were decanted and washed with PBS (pH 7.3), and dried.

• Dried tubes were stained with crystal violet (0.1%).

• Excess stain was removed and the tubes were washed with deionized water.

•Tubes were then dried in inverted position and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as absent/weak, moderate, or strong.

Congo red Agar method (CRA) [5, 39]

An alternative method for screening biofilm formation, as described by Freeman et al., for Staphylococcal isolates

Medium

BHI (37.0 grams/L), sucrose (50.0 grams/L), agar (10.0 grams/L) and Congo red stain (0.8 grams/L).

Method

The test culture isolates were inoculated on to CRA plates and incubated aerobically for 24 - 48 hours at 37°C.

Interpretation

The test was read as positive if there were black colonies with a dry crystalline consistency. Weak slime producers were indicated by pink colonies with occasional darkening at the centers of colonies. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

ANTIBIOTIC SENSITIVITY TESTING

Antimicrobial susceptibility testing employs the Kirby Bauer method of agar disk diffusion performed with Mueller-Hinton agar plate. Commercially available antibiotic discs (Himedia) were used. Various groups of 18 antibiotic discs (Appendix 3) were used to assess the antibiotic sensitivity pattern, MR and MDR among CoNS isolates. S.epidermidis ATCC 14990 strain is used.

Procedure

Using a sterile wire loop, 3-4 well-isolated colonies of similar appearance from an agar plate culture were inoculated into 2-3 ml of suitable broth medium, and subsequently emulsified. The turbidity was adjusted so as to correspond to the 0.5 Mc farland standards. The test cultures were swabbed on the Muller Hinton agar plates and dried for not more than 15 mins. The discs were applied on to the surface of the agar; three plates were used for one isolate due to the increased number of discs.

Interpretation

Results were read after incubation at 37^0C for 18-24 hrs. The sizes of zone of inhibition were interpretated by referring to the CLSI standards.

(Detailed procedure about inoculum preparation, turbidity standard for inoculums preparation, inoculation of test plates and interpretation of zone sizes are explained in Appendix 4)

RESULTS

The present study was conducted on 90 clinically-significant CoNS isolates. All strains were negative for both clumping factor and tube coagulase.

The most common source for all CoNS species combined was wound infections and pus, constituting 39 out of 90 strains (43%), followed by 21 (23%) from blood cultures, 14 (15.5%) from urine samples, 6 (6.6%) from catheters, 2 (3.9%) from cerebrospinal fluid, 4 (1.9%) from ascitic fluid, and 5 from other sources (Table & Chart 1).

Speciation was done by five-test simple scheme method which is based on the biochemical reactions, and the results are shown in Table & Chart 2. All species groups were resolved to the species and sub-species level by using additional tests. All 54 trehalose- and mannitol-negative isolates from S. epidermidis group were identified as S. epidermidis. From the S. haemolyticus group, 11 isolates produced acetoin, and hence were identified as pure S. haemolyticus. All 12 isolates in the S. saprophyticus group were trehalose-positive, and hence were determined to be subsp.saprophyticus. Out of the 7 S.warneri group isolates, 3 were identified as S. warneri sp (anaerobic thioglycolate growth-positive), and 4 as S. hominis subsp. hominis (anaerobic thioglycolate growth-negative). Of the remaining 6 isolates, 4 were identified as S. lugdunesis sp, while 2 were determined to be S. capitis subsp. capitis.

Table & Chart 3 lists the incidence of CoNS species among the clinical samples. Of the isolates from 40 exudate samples (wound swabs and pus), 24 isolates were identified as S. epidermis, 4 isolates were S. haemolyticus, 2 isolates were S. lugdunesis, 3 isolates were S. hominis, 2 isolates were S. warneri, and 2 were S. capitis. Of the 21 blood samples, 14 harbored S.epidermidis, 4 harbored S. haemolyticus, and 1 each harbored either S. lugdunensis or S. hominis or S. capitis. Of the isolates from 14 urine samples, majority (12) were S.saprophyticus, while only 2 were S. epidermidis. However, S. epidermidis was the most common isolate from wound & pus (26/39), blood (14/21), catheters (5/6), peritoneal fluid (2/3), CSF (2/2), and others (3/5); S. haemolyticus came second with 1 isolate in each type of specimen.

Biotyping of the 54 S. epidermidis isolates by modified Baird-Parker system revealed that the majority, 32 (59%), of the isolates belonged to biotype II, while 14 (25.9%) were of biotype I, 5 (9.2%) were of Biotype III, and 3 belonged to biotype IV (5.5%) (Table & Chart 4).

The virulence factors of each species of CoNS isolates are listed in Table & Chart 5. Of the 54 clinically significant S. epidermidis isolates, 27 produced hemolysin, 6 produced lipase, 3 produced DNAse, 36 produced slime; none of the strains produced lecithinase. Among 11 S. haemolyticus isolates, 8 produced haemolysin, 4 produced lipase, 1 produced DNAse, and 3 produced slime; however, none of the isolates produced lecithinase.

Among the 12 S. saprophyticus isolates, 2 produced haemolysin, 3 produced lipase, 1 produced DNAse, and 6 produced slime; however, none of the isolates produced lecithinase. Of the 4 S. lugdunensis isolates, 2 produced either hemoysin or lipase, 2 produced slime, and none of the isolates produced DNAse and lecithinase. Of the 3

S.warneri isolates, whi 2 produced haemolysin, none of the isolates produced slime or lipase or DNAse or lecithinase. No virulence factors could be demonstrated among the 4 S.hominis and 2 S.capitis isolates.

Table & Chart 7 lists, maximum slime positivity by S.epidermidis (36 =10 heavy + 26moderate), followed by S.haemolyticus (6 = 2 heavy + 4 moderate), S. saprophyticus (3 = 1 heavy + 2 moderate), and S.lugdunensis (2 moderate).

Table & Chart 8 shows the relationship between the biotypes and slime production. A majority, 26 out of 36 (76%), of biotype II isolates produced slime, and 9 out 10 (90%) among them were heavy slime producers. Among the biofilm-producing S. epidermidis isolates, 7 biotype I isolates (50%), 2 biotype III isolates (40%), and 1 biotype IV (30%) isolate produced slime.

Of the 90 isolates tested by MTP method for biofilm formation, 13 (14%) were strong biofilm producers, and 34 (37.7%) were moderate biofilm producers. Results from the TM showed good correlation with those obtained with the MTP method – a total of 12 (11.8%) isolates were picked up as strong biofilm producers, while 29 (32%) were moderate biofilm producers. In contrast, results obtained by using the CRA method presented a different picture. Majority of the strains produced red (pink to orange) colonies on medium, which is a typical attribute of biofilm-producing staphylococcal isolates. Though 1 (1.97%) isolate produced occasional blackening of colonies, it did not display the dry crystalline morphology. After 24-48 hours only 2 (2.2%) isolates produced red colonies with dry crystalline morphology. With regards to the colony morphology on CRA, no correlation was observed between the results from CRA and MT P assays.

As evident from Table & Chart 10, antimicrobial susceptibility testing revealed wide-spread (54%) methicillin resistance among the CoNS isolates. MDR was also prevalent among the CoNS isolates, with a higher incidence among the methicillin-resistant isolates than the methicillin-sensitive isolates. Though resistance to methicillin was highest (64%) among the S. haemolyticus isolates, the MR isolates exhibited only 36% of MDR and 57% of MDR among MR.

Among the 63% methicillin-resistant S. epidermidis isolates, 88% exhibited MDR; however, overall MDR among S.epidermidis was 55%. Fifty percent and 42% of S. lugdunensis and S.saprophyticus exhibited methicilin resistance, respectively; both exhibited 25% MDR. Among the MR-isolates, 50% of MDR-S.lugduensis and 60% of MDR-S.saprophyticus were methicillin-resistant. Of the 33% MR- S. Warneri isolates, all were MDR. None of the S. hominis and S. capitis isolates exhibited MR and MDR.

Analysis of the antibiotic susceptibility pattern of S. epidermidis and other CoNS isolates, shown in Table & Chart 11, revealed no resistance to Linezolid. Ninety eight percent of S. epidermidis and 94% of the other CoNS isolates were Vancomycin-sensitive. Maximum resistance was observed for Amoxicillin (S. epidermidis 81.5%, other CoNS 56%), followed by Cloxacillin (S. epdiermidis 74%, other CoNS 33%), Resistance to antibiotics was more among S. epidermidis isolates than other CoNS isolates. More than 80% of other CoNS isolates were sensitive to Amikacin, Ofloxacin, Levofloxacin, Azithromycin, and Clindamycin.

Table –1 Distribution of CoNS among the clinical specimens

No.	Specimen	No. of CoNS	Percentage
1	Wound & Pus	39	43%
2	Blood	21	23%
3	Urine	14	15.5%
4	Catheters	6	6.6%
5	Peritoneal fluid	3	3.3%
6	CSF	2	2.2%
7	Others	5	5.5%
	Total	90	-

Table – 2. Species distribution among CoNS isolates

No.	Species	No. of isolates	Percentage
1	S.epidermidis	54	60
2	S.haemolyticus	11	12.2
3	S.saprophyticus	12	13.3
4	S.lugdunensis	4	4.4
5	S.hominis	4	4.4
6	S.warneri	3	3.3
7	S.capitis	2	2.2
	Total	90	-

Table-3.

Frequency of species of CoNS isolates from clinical specimens

Species	% Wound & Pus	% Blood	% Urine	% Catheters	% Peritoneal fluid	% CSF	% Others
S.epidermidis **54**	26 (48)	14 (26)	2 (3.7)	5 (9.2)	2 (3.7)	2 (3.7)	3 (6.5)
S.haemolyticus **11**	4 (36)	4 (36)		1 (9)	1 (9)		1 (9)
S.saprophyticus **12**			12(100)				
S.lugdunensis **4**	2 (50)	1 (25)					1 (25)
S.hominis **4**	3 (75)	1 (25)					
S.warneri **3**	2 (67)	1(33.3)					
S.capitis **2**	2 (100)						

Table-4. Biotypes of S.epidermidis - Baird-Parker system

Specimen	Biotype 1	Biotype2	Biotype3	Biotype4
Wound & Pus **26**	7	16	2	1
Blood **14**	4	7	2	1
Urine **2**	-	2	-	-
Catheters **5**	2	3	-	-
Peritoneal fluid **2**	-	2	-	-
CSF **2**	1	1	-	-
Others **3**	-	1	1	1
Total **54**	14	32	5	3

Table-5. Frequency of virulence factors among CoNS isolates

Species	**Hemolysin**		**Lipase**		**DNAse**		**Lecithinase**	**Slime**	
	n	**%**	**n**	**%**	**n**	**%**		**n**	**%**
S.epidermidis	27	(50)	6	(11)	3	(5.5)	0	36	(67)
S.haemolyticus	8	(72)	4	(36)	1	(9)	0	3	(27)
S.saprophyticus	2	(17)	3	(25)	0		0	6	(50)
S.lugdunensis	1	(25)	1	(25)	0		0	2	(50)
S.hominis	0	(0)	0		0		0	0	
S.warneri	2	(67)	0		0		0	0	
S.capitis	0	(0)	0		0		0	0	

Table-6. Production of Slime with regards to specimen

No.	Specimen	Heavy slime Producer	Mod. slime Producer	Total n %
1	Wound & Pus 39	6	16	22 (56.4)
2	Blood 21	2	6	8 (38)
3	Urine 14	1	2	3 (21.4)
4	Catheters 6	2	4	6 (100)
5	Peritoneal fluid 3	1	2	3 (100)
6	CSF 2	-	2	2 (100)
7	Others 5	1	2	3 (60)

Table -7

Distribution of Slime production among species of CoNS isolates

Species	Heavy slime Producer		Mod. Slime Producer		Total	
	n	%	n	%	n	%
S.epidermidis 54	10	(18.5)	26	(48.2)	36	(66.8)
S.haemolyticus 11	2	(18)	4	(36.2)	6	(54.2)
S.saprophyticus 12	1	(8.3)	2	(16.6)	3	(24.9)
S.lugdunensis 4	--		2	(50)	2	(50)
Total	13		34		47	

Table-8

Slime producing S.epidermidis isolates according to biotypes

Biotypes	**No. of slime producers**		**Total**
	Heavy	**Moderate**	
Biotype I (14)	1	6	7(50%)
Biotype II (32)	9	17	26 (81%)
Biotype III (5)	0	2	2 (40%)
Biotype IV (3)	0	1	1(33%)
Total (54)	10	26	36(66%)

Table-9

Comparison of biofilm formation by MTP, TM and CRA method

Biofilm formation	**MTP**	**TM**	**CRA**
High (13)	13	12	0
Moderate(34)	34	29	3
Non biofilm producers(43)	43	49	87

Table-10. Frequency of MR CoNS and MDR CoNS

Sl. No	Species	No. of MR Isolates :	No. of MDR Isolates: %	
		n %	MDR among CoNSspecies	MDR among MR isolates
1	S.epidermidis (54)	34 (63)	30 /54 (55%)	30/34 (88%)
2	S.haemolyticus (11)	7 (64)	4/11 (36%)	4 /7 (57%)
3	S.saprophyticus (12)	5 (42)	3/12 (25%)	3/5 (60%)
4	S.lugdunensis (4)	2 (50)	1 /4 (25%)	1 /2 (50%)
5	S.hominis (4)	0 (0)	0/4 (0%)	0 (0%)
6	S.warneri (3)	1 (33)	1/3 (33%)	1/1 (100%)
7	S.capitis (2)	0 (0)	0 /2 (0%)	0 (0%)

Table -11. Antibiotic sensitivity pattern of S.epidermidis and other CoNS isolates.

	Antibiotics	No. of susceptible isolates	
		S.epidermidis (54) %	Other CoNS (36) %
1.	Oxacillin	20 (37)	21 (58)
2	Amoxicillin	10 (18.5)	16 (44)
3	Cloxacillin	14 (26)	24 (67)
4	Piperacillin	38 (70)	28 (78)
5	Amikacin	42 (78)	32 (89)
6	Gentamicin	14 (26)	20 (56)
7	Ciprofloxacin	34 (63)	27 (75)
8	Ofloxacin	40 (74)	29 (80.5)
9	Levofloxacin	43 (80)	32 (89)
10	Erythromycin	24 (44)	28 (78)
11	.Azithromycin	30 (56)	29 (80.5)

12	Clindamycin	33 (61)	29 (80.5)
13	Vancomycin	53 (98)	34 (94)
14	Linezolid	54 (100)	36 (100)
15	Cephalexin	18 (33)	21 (58)
16	Cefoxitin	22 (41)	20 (56)
17	Cephotaxime	15 (28)	23 (64)
18	Cefepime	38 (70)	26 (72)

DISCUSSION

Historically, due to their ubiquitous nature, the coagulase-negative staphylococci have been regarded as saprophytes with little pathogenic potential. However, recently, CoNS have emerged as significant pathogens, particularly in infections associated with medical devices and in immuno-compromised patients. CoNS are among the most frequently isolated bacteria in the clinical microbiology laboratory.

In the present study, 135 CoNS were isolated from different clinical specimens from different sources; Ninety (66%) among them fulfilled the criteria, and hence were considered as clinically-significant and included in this study. This level of clinical significance (66%) is comparable to that reported by Ishak et al (1985) [52](52%), Maria de Lourdes Ribeiro de Souza da Cunha et al (2006)[4] (51%) ,and by Mohan et al (2002)[35] (44.3%). In contrast, Sewell et al(1982) [9] reported that only 21% of CoNS isolates were clinically significant; this discrepancy might have arisen due to their inclusion criteria that considered organisms from closed sources such as blood, sub-arachnoid space, joint, or pleural space to be more pathogenic than the organisms which were isolated from an open source such as a wound. Accordingly, if the inclusion criteria used by Sewell et al is employed in the current study, only 26.9% of CoNS will account as clinically significant.

In the present study, majority (43%) of the isolates were from wound infections & pus, while 23% were from blood cultures, 15.5% were from urine samples, 6.6% were from IV catheters, 3.9% were from cerebrospinal fluid, 1.9% were from ascitic fluid, and 2% were from other sources. Intriguingly, the above data correlates with the results published by Sewell et al [9], who reported drainage from wounds as the most frequent source of CoNS(43%), followed by blood (26%),

urine(12%), peritoneal fluid (9%), cerebrospinal fluid (8%), joint fluids (1%), and pleural fluid (1%). In addition, results from this study also correlate well with the study by Goyal et al [2] (2006) which identified wounds as the most frequent source of CoNS (38.2%) followed by urine samples (28.4%), blood cultures (14.7%), catheters (10.7%), cerebrospinal fluid (3.9%), each from synovial fluid (1.9%), and ascitic fluid (1.9%).

Contrasting results were published by Vee j Gill et al (1983)[31] who reported that blood was the most common source for CoNS isolates (50%), followed by wounds and abscesses (17%), and I.V. catheter tips(11%). However, the above study included only the organisms from wounds and abscesses that displayed moderate to heavy growth. Two additional reports further highlight the prevalence of variability in these type of studies - Mohan et al [35] (2002) determined urine as the most common source of CoNS (48.4%), followed by pus (18%), drain tips/ catheter tips/I.V.cannulas (11.4%), blood (5%), and aural swabs (2.7%); De paulis et al(2003)[6] also identified urine as the most common source of CoNS (35.8%), followed by pus (15%), blood (13%), central venous catheters (11%), aural swabs (2.7%), peritoneal dialysis fluids (9.5%), and cerebrospinal fluids (1%).

The differences observed in the distribution of CoNS among clinical specimens between different studies may have resulted from the use of non-standard criteria for defining the clinical significance and for selection of clinically significant CoNS isolates.

Several methods exist for determining CoNS species. For example, the method of Kloos and Schleifer is a reliable technique for identification and speciation of CoNS. However, it is a relatively cumbersome, and time consuming method requiring the use of expensive reagents. Alternative approaches include the

use of the several commercially-available identification kits and automated instruments; however, several factors precluded their use in this study. Hence, convenient, reliable, and inexpensive identification methods were opted. Among the different approaches, the five-tests simple scheme by DePaulis et al (2003) [6], which identified 99.5% of CoNS isolates, was chosen. This level of accuracy is not possible with other approaches that were considered, including the nine tests scheme by Iorio et al(2007)[14] (98.5% accuracy), and rapid 4 h method (UZA method) developed by Ieven et al(1995)[30] (97.7% accuracy). In the case of the simple scheme, a two-step procedure, used by Goyal et al (2006) [2], it does not include the important PYR test to differentiate the warneri species.

In the present study, the most common species isolated from all the sources were S. epidermidis (60%), followed by S. saprophyticus (13.6%), S. haemolyticus (12%), S. hominis (4.4%), S. lugdunensis (4.4%), S. warneri (3.3%), and S. capitis (2.2%). This distribution pattern correlates well with the results published by De Paulis et al(2003)[6] which pegged the incidence of S. epidermidis at 50.5%, S. haemolyticus at 18.5%, S. saprophyticus subsp.saprophyticus at 16.0%, S.lugdunensis at 6.0%, andS.warneri at 2.5% among the CoNS isolates. Another study conducted by Goyal et al (2006)[2] reported similar incidence rates for S.epidermidis (41%), S.saprophyticus (16.6%), S. haemolyticus (14.7%), S. hominis (14.7%), S. lugdunensis(4.9%), S.schleiferi (1.9%), and S.capitis (1.9%) among isolates; S.warneri could not be differentiated in this scheme of speciation. Recently, Iorio et al (2007)[32] also reported similar distribution of staphylococcal isolates from different clinical sites- S.epidermidis(34.8%),Shaemolyticus(22.2%), S.hominis(12.6%) S.saprophyticus subsp. saprophyticus (4%), S. warneri (3.5%) ,S.lugdunensis (3%), S. capitis subsp. capitis (3%), S. cohnii subsp. urealyticum

(2%), S.sciuri subsp. sciuri (2%), S.xylosus (2%),S.capitis subsp urealyticum (1%), and S. cohnii subsp. cohnii (1strain).

In the current study, majority of the isolates from the 40 exudate samples (wound swabs & pus) were S. epidermidis (67%), followed by S. haemolyticus (10.2%), S.hominis (7.6%), S.lugdunensis (5%), S.warneri (5%) and S.capitis (5%). Similar findings were reported by Sewell et al (1982)[9] and Kleeman et a l(1993)[13]. In contrast, Shubhra Singh(2008)[64] reported 28.57% of saprophyticus among wound isolates.

In the present study, among 21 blood samples, 66% were S.epidermidis, 19% were S.haemolyticus, and 4.7% each were S.lugdunensis or S.hominis or S.capitis; this data correlates well with the findings by Eng et al(1982) [15]. Both Vee j. Gil et al(1983)[31] and Maria de Lourdes Ribeiro de Souza da Cunha et al(2006)[4] , reported S. epidermidis, S.haemolyticus, and S. hominis as the first, second, and third most frequently encountered species, respectively, followed by warneri. In contrast, Shubhra Singh(2008)[64] reported 57.14 % of saprophyticus among blood isolates, while De Paulis et al(2003)[6] identified 1 S.saprophyticus out of 26 blood isolates(3.8%).

In this study, majority of the isolates from the 14 urine samples were S. saprophyticus (86%), followed by S.epidermidis (14%). Similar results were obtained in a study by Goyal et al(2006)[2.] In contrast, Gill et al (1983) [31] reported that 63% of the urinary isolates were S. epidermidis, while 11% were S. saprophyticus. Intriguingly, Shubhra Singh (2008) [64]reported an incidence of 15% S. epidermidis and 14% S. hominis among the urinary isolates, while S. saprophyticus could not be isolated.

In the current study, when the 54 S. epidermidis isolates were biotyped by modified Baird-parker system, majority of the isolates belonged to biotype ll (59%), followed by biotype l (25.9%), Biotype III (9.2%), and biotype lV (5.5%). Similarly, NiranjanNaik et al (2007)[60] reported that 46.3%, 28%, 15% and 10.5% of the isolates belonged to Biotype ll, I, lll and lV, respectively. However, Bonnie M. Males et al (1975) [29] found Biotype l to be the predominant group in their studies; this difference could be due to the fact that most of the isolates were from urine, and also because of the use of the tube phosphatase test instead of plate method.

The pathogenisity of CoNS are evaluated by demonstrating the virulence factors [4,21] . Results from the present study are as shown in Table&chart 5 - haemolysins were produced by isolates of S. epidermidis, S. haemolyticus, S. saprophyicus S. lugdunensis, and S. warneri, but not by strains of S. hominis and S. capitis. Similar results have been reported by Kloos and Schleifer [32](1975), Lambe et al[94] (1990), and Cunha et al[88] (2004). In the present study, 72% of S. haemolyticus and 54% of S. epidermidis were found to produce haemolysin; however, Pairisi et al[29] (1975) reported that 79% of S. epidermidis produced hemolysin.

Lambe et al (1990)[89] reported that most S.epidermidis *S.* warneri, and S.hominis strains included in their study produced lipase and DNAse. However, the present study found most of the clinically significant S. epidermidis, S. haemolyticus and S. lugdunensis strains to produce lipase. The findings from the current study find validation in a previous publication by Ribeiro de Souza da Cunha et al[4] (2006), which reported very similar observations. In the case of DNAse, while the present study found only S. epidermidis and S. haemolyticus to produce

DNAse, Ribeiro de Souza da Cunha et al[4] (2006) et al found that, in addition to the two strains , S. lugdunensis and S. simulans also produced DNAse.

The production of Lecithinase by 1 S.epidermidis and 1 S. warneri, was reported by Ribeiro de Souza da Cunha et al[4]. But in the present study, none of the strains were found to produce lecithinase.

The most important colonizing and virulence factor, i.e. biofilm production, in CoNS was evaluated. Forty seven out of 90 (52%) isolates produced slime in this study. Comparable results have been previously published by Deighton et al (1988)[47], Kotilainen et al (1990).[49], and Mathur et al (2006) [5] who found that 48%, 65%, and 53.9%, respectively, of the CoNS in their studies produced biofilm. In contrast, Ishak et al (1985) [52] found 92.8% of clinically-significant bloodstream isolates of CoNS producing slime; however, it should be noted here that all the isolates used by Ishak et al were from blood. Among the species included in the present study, S. epidermidis and S. haemolyticus were the only two species that produced almost all the virulence factors.

When specimen-related slime production was assessed (Table& chart 6), 100% slime production was seen among isolates from IV Cat**h**eters, Peritoneal fluid, and CSF, followed by, 56.4% from wound & pus, 38% from blood , 21.4% in Urinary isolates and 60%s from other sources . It is interesting to point out here the findings by Makhija S.K. et al (1995)[57], where 66% and 20% slime production was demonstrated in CSF and peritoneal fluid, respectively.

In the present study, out of 90 CoNS isolates (Table&chart 7), S. epidermidis was the major species that produced slime (67%), followed by S.haemolyticus (27%), S.saprophyticus (50%), and S.lugdunensis (50%).

Comparable pattern of slime production by S.epidermidis species was observed by Rathinam. K. et al (1993)[55] (62%), Kleeman et al (1993)[8](64.5%), Agarwal et al [66] 1987 (48.7%), Deighton et al[47] 1988 (53%) and Ishak et al[52] (1985 reported that 92% of S.epidermidis produced slime; however, only blood isolates were included in that study.

Riley and Schneider (1992) suggested that slime production does not seem to be an important virulence factor of S. saprophyticus that were isolated from women with urinary tract infection[42]; however, in the present study, the S. saprophyticus isolated from urine samples produced slime. The reason might be because, most samples were from patients with urinary catheters which functions as a foreign body assisting in the formation of slime as has been previously reported by Longaurova et al (2006) [42] .

When the slime-producing S. epidermidis isolates were assessed according to biotypes (Table & Chart 8), 26 out of the 32 biotype II isolates exhibited slime productivity, followed by 7 out of the 14 biotype l isolates, 2 out of the 5 biotype lll isolates, and 1 out of the 3 biotype lV isolates produced slime. Such maximum slime production by biotype II isolates has previously been reported by Niranjan nayak et al (2007)[60] and Christesen et al(1983)[8] . In contrast, Bonnie M. Males et al (1986)[29] found maximum slime production among the Biotype l isolates; however, the caveat in that study was that the majority of the isolates used belonged to biotype l [29.]

When the biofilm production was compared by 3 different methods, MTP method was the most sensitive as it could detect all the slime producers, whereas TM correlated well with the MTP method for strong biofilm producing strains; weak slime producers were difficult to discriminate from biofilm-negative isolates. CRA does not correlate well with either of the above two methods for detecting biofilm formation in staphylococci. The MTP method was found to be the most sensitive, accurate and reproducible screening method for detection of biofilm formation by staphylococci. It also has the advantage of being a quantitative model to study the adherence of staphylococci on biomedical devices, a finding that correlates very well with the reports by Christensen et al(1983)[8] and Mathur et al(2006)[5].

Antimicrobial susceptibility testing revealed wide spread methicillin resistance (54.4%) among the CoNS Isolates (Table & chart 10). Comparable levels have previously been reported by Chaudury et al (2007)[65](68.4% oxacillin resistant CoNS) and Marry et al (1996).[63] (49% methicillin resistant CoNS). In a study by Shubhra Singh et al (2006)[64], 38% of the CoNS exhibited oxacillin resistance.

In the present study, S. haemolyticus showed highest methicillin resistance (64%), followed by S.epidermidis (63%); these values are similar to those reported by Vee J. Gill et al (1983)[31].

The antibiotic susceptibility pattern of S. epidermidis and other CoNS isolates, shown in Table &chart 11, revealed no resistance to Linezolid. Ninety eight percentage of S.epidermidis and 94% of the other CoNS isolates were sensitive to Vancomycin. More than 70% of S.epidermidis isolates were sensitive to Piperacillin, Amikacin, Ofloxacin, Levofloxacin and Cefepime. More than 80% of the other CoNS isolates were sensitive to Amikacin, Ofloxacin, Levofloxacin Azithromycin and

Clindamycin. These findings correlate well with similar observations made by Archer et al(1994)[70] and Diekema et al (2001)[90]

MDR was also prevalent among the CoNS isolates; the prevalence was more in the methicillin-resistant isolates than in the methicillin-sensitive isolates. These findings are very similar to those reported by Archer et al (1994)[70] and Diekema et al[90] (2001).

SUMMARY

- A total of 135 CoNS were isolated from deep wounds, blood samples, pus, urine samples, body fluids and IV catheters from the patients at Diagnostic microbiology laboratory, Dept of Microbiology, Coimbatore Medical College, Coimbatore, during the period of June, 2007 to July, 2008.

- Nintey out of 135 (66%) isolates which fulfilled the criteria were considered as clinically significant and were included in the present study.

- Majority (43%) of the isolates were from wound and pus followed by blood (23%), urine (15.5%), IVcatheters (6.6%), CSF (3.9%) ascitic fluid (1.9%) and from other sources (5.5%).

- When the 90 isolates were speciated by the five tests simple scheme method 60% were S.epidermidis, followed by S.saprophyticus 13.6%, S. haemolyticus 12%, S. hominis 4.4%, S. lugdunensis 4.4%, S.warneri 3.3% and S.capitis 2.2%.

- S.epidermidis was the most common species isolated from wound & pus, blood, IVcatheters, peritoneal fluid, CSF, and other sources.

- S.saprophyticus (12) was solely isolated from urine which constituted 86% of urinary isolates..

- Among the 54 S.epidermidis isolates ,59%, 25.9%, 9.2% and 5.5% belonged to Baird-parker biotype - II , I, III, and IV respectively.

- When the pathogenisity was evaluated by determination of the virulence factors , haemolysin, lipase and slime were produced by S. epidermidis,

S. haemolyticus , S.saprophyticus and S.lugdunensis. S. warneri produced only haemolysin.

- DNAse was produced by only 3 of the 54 S. epidermidis isolates and 1 of 11 S. haemolyticus isolates.
- Lecithinase was produced by none of the isolates.
- All (100%) isolates from IVCatheters, Peritoneal fluid ,CSF exhibited slime, while 56.4% isolates from wound & pus , 38% isolates from blood , 21.4% isolates from urine and 60% isolates from other sources produced slime.
- S.epidermidis was the predominant slime producing species (75%) followed by S. haemolyticus (13%), S. saprophyticus (6%) and S. lugdunensis (4%).
- Among the biotypes of S.epidermidis isolates, maximum number of biotype ll isolates produced slime (72%) followed by biotype l

isolates (50%), biotype lll isolates(40%) and biotype lV isolates (30%)

- Among the 3 different methods used for detecting the biofilm production , MTP method Identified all slime producers and was sensitive enough to differentiate strong, moderate & non slime producers.
- TM method identified 87% of slime producers and it correlated well with the MTP method for strong biofilm producers, where as weak slime producers were difficult to discriminate from non biofilm producers.

- By CRA method only 3 isolates were identified as moderate slime producers. No correlation was observed between colony morphology on CRA and MTP results.
- Methicillin resistance was 54.4% among the CoNS Isolates.
- Highest methicillin resistance was exhibited by S. haemolyticus (64%) followed by S.epidermidis (63%).
- MDR was also prevalent among CoNS isolates and the prevalence was more in the methicillin-resistant isolates compared with methicillin sensitive isolates.
- MDR among the CoNS isolates was 55% in S.epidermidis, 36% in S. haemolyticus, 33% in S. warneri, 25% in S. saprophyticus and S.lugdinensis.
- The antibiotic susceptibility pattern revealed 100% sensitivity to Linezolid. Ninety-eight percent of S.epidermidis and 94% of other CoNS isolates were sensitive to Vancomycin.
- Maximum resistance was observed for Amoxicillin (S. epidermidis 81.5% ,other CoNS 56%) followed by Cloxacillin , Gentamicin, Cephotaxime ,Cephelexin,Oxacillin , Cefoxitin and Erythromycin.

CONCLUSIONS

The study of CoNS isolated from various sources indicated 66% of isolates as clinically significant and S. epidermidis as the most frequently isolated species. The speciation was done by five tests simple scheme method, which is reliable, simple, inexpensive, and accurate procedure and will prove useful in microbiology laboratory as simplicity and speed are very essential in diagnostic and therapeutic purpose. The most important virulent factor biofilm production in CoNS was associated mostly in prosthetic device related conditions like orthopedic wounds, PDF, CSF and IV catheters and biofilm on implanted foreign material can cause major medical and economic sequel. Antimicrobial susceptibility testing revealed the wide spread methicillin resistance and MDR among CoNS and is particularly notable with S. epidermidis. The prevalence of MDR was more in the methicillin-resistant isolates and in slime producers as cells in biofilms are normally more resistant to antibiotics. It is necessary to predict the potential pathogenicity by biofilm demonstration in vitro which can be easily done by MTP method and antibiotic susceptibility of each clinical isolates, early in the course of infection, which will be helpful to provide appropriate antibiotic therapy and prevent implant removal. Taking into consideration that the etiological importance of CoNS has often been neglected, the present investigation confirmed that these microorganisms should not be ignored or classified as mere contaminants.

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Appendix-1:

Presumptive identification of CoNS by simple scheme

Species	Novobiocin (5µg)	Urease	Acid from-mannose	PYR	ODC
S. epidermidis group	S	+	+	-	d
S. haemolyticus group	S	-	-	+	-
S. saprophyticus	R	+	-	-	-
S. lugdunensis	S	D	+	+	+
S. warneri group	S	+	-	-	-
S. schleiferi subsp.schleiferi	S	-	+	+	_
S. simulans	S	+	d	+	-
S. capitis subsp. capitis	S	-	+	-	-
S. cohnii subsp. cohnii	R	-	D	-	-
S. cohnii group	R	+	+	d	-

(+ Positive; - negative; d, positive or negative; R, resistant, S, susceptible) When Ornithine decarboxylase is negative:

Trehalose negative; mannitol negative - S.epidermidis;

Trehalose positive; mannitol negative - S.caprae;

Trehalose negative; mannitol positive - S. capitis subsp. ureolyticus

Acetoin production positive - S. haemolyticus ;

Acetoin production – negative; lactose negative - S. auricularis;

Acetoin production negative ; lactose positive- S. casseolyticus

Trehalose positive - S.saprophyticus subsp. saprophyticus;

Trehalose negative, S.hominis subsp.novobiosepticus

Anaerobic growth positive - S. warneri;

Anaerobic growth negative- S. hominis subsp. hominis

Xylose positive - S. xylosus;

Xylose negative -S. cohnii subsp. urealyticum is suspected.

Appenix-2 :

Classification of bacterial adherence by MTP method [5,28]

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

Chose the number 0.120 for a guideline because it was three standard deviations (0.023) above the mean OD (0.050) of a clean MTP plate stained by the previously described procedure [28].

Biotyping of S.epidermidis isolates by Baird - Parker system

TESTS		BIOTYPES			
		1	2	3	4
ACETOIN		+	-	+	+
PHOSPHATASE		+	+	-	--
ACID AEROBICALLY FROM	LACTOSE	+	D	-	D
	MALTOSE	+	-	D	D
	MANNITOL	-	-	-	+

(+ POSITIVE , - NEGATIVE , D – VARIABLE)

Acetoin production - Voges – Proskauer Test

Phosphatase - Phosphatase test by plate method

Sugar fermentation tests for Maltose, Mannitol, Lactose.

Appendix - 3 .

List of Antibiotic discs used and zone size interpretative chart.

Antibioticdiscs	Disc content (mcg)	Resistant mm	Intermediate mm	Sensitive mm
Oxacillin	1	17	-	18
Amoxicillin	10	19	-	20
Cloxacillin	5	17	-	18
Piperacillin	100	17	-	18
Amikacin	30	14	15-16	17
Gentamicin	10	12	13-14	15
Ciprofloxacin	5	15	16-20	21
Ofloxacin	5	12	13-15	16
Levofloxacin	5	15	16-18	19

Erythromycin	15	13	14-22	23
Azithromycin	15	13	14-17	18
Clindamycin	2	14	15-20	21
Vancomycin	30	-	--	15
Linezolid	30	-	-	21
Cephelexin	30	14	15-17	18
Cefoxitin	30	14	15-17	18
Cephotaxime	30	14	15-22	23
Cefepime	30	14	15-17	18

Appendix-4

Antibiotic sensitivity testing by Kirby-Bauer disc diffusion method.

Inoculum preparation:

Using a sterile wire loop, touched 3-4 well isolated colonies of similar appearance from an agar plate culture and emulsified in a tube containing 2-3 ml of suitable broth medium.

Adjusted the turbidity so as to correspond to the 0.5 Mcfarland standards. To perform this step properly, adequate light is needed to visually compare the inoculums' tube and the 0.5 Mc Farland standards against a card with a white background and contrasting black lines.

Turbidity standard for inoculums preparation:

• To standardize the inoculums density for a susceptibility test,a Barium sulfate turbidity standard, equilent to a 0.5 McFarland standard was used.

• Preparation of Barium sulfate 0.5 Mc Farland standard:

• A 0.5 ml of aliquot of 0.048 mol/l (1.175% w/v $Bacl_2.2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO4 (1% v/v) with constant stirring to maintain a suspension.

• The correct density of the turbidity standard was verified and transferred the Barium sulfate suspension in 5-6 ml aliquot into screw cap tubes.

•These tubes were tightly sealed and stored in ambient temperature in the dark.

• The barium sulfate turbidity standard was vigorously agitated before each use and inspected for a uniform turbidity appearance.

Inoculation of test plates: After adjusting the turbidity of the inoculum's suspension, a sterile cotton swab was dipped in to the suspension .Excess fluid was removed by pressing and rotating the swab against the side of the tube above the level of suspension.

• Streaked the swab evenly over the surface of Muller Hinton agar plate in three directions, rotating the plate approximately 60^0 to ensure the even distribution.

• With the Petri dish in place allowed 3-5 minutes for the surface of agar to dry.

• Using sterile forceps the appropriate antimicrobial discs were evenly distributed over the inoculated plate. The disc should be about 15mm from the edge of the plate and no closer than about 25mm from disc to disc. Maximum of 6 discs can be placed in a 90mm Petri dish plate. Each disc should be lightly pressed down to ensure its contact with agar. Three plates were used for one isolate due to the increased number of discs.

• The plates were incubated at 37^0C for18-24 hrs

Interpretation of zone sizes:

After overnight incubation , examined the plates to ensure the confluent or near confluent growth .Using a ruler held on the underside of the plate measured the diameter of each zone of inhibition in mm. The end point of inhibition is where growth starts. The sizes of zone of inhibition were interpretated by referring to the CLSI standards, and the organisms are reported as susceptible, intermediate, or resistant to the agents that have been tested. In place of Methicillin, Oxacillin 1µgm discs were used.

IV PROFORMA

NAME:

AGE/SEX:

ADDRESS:

OCCUPATION / INCOME:

IP/OP NO: LAB.NO:

WARD:

SAMPLE:

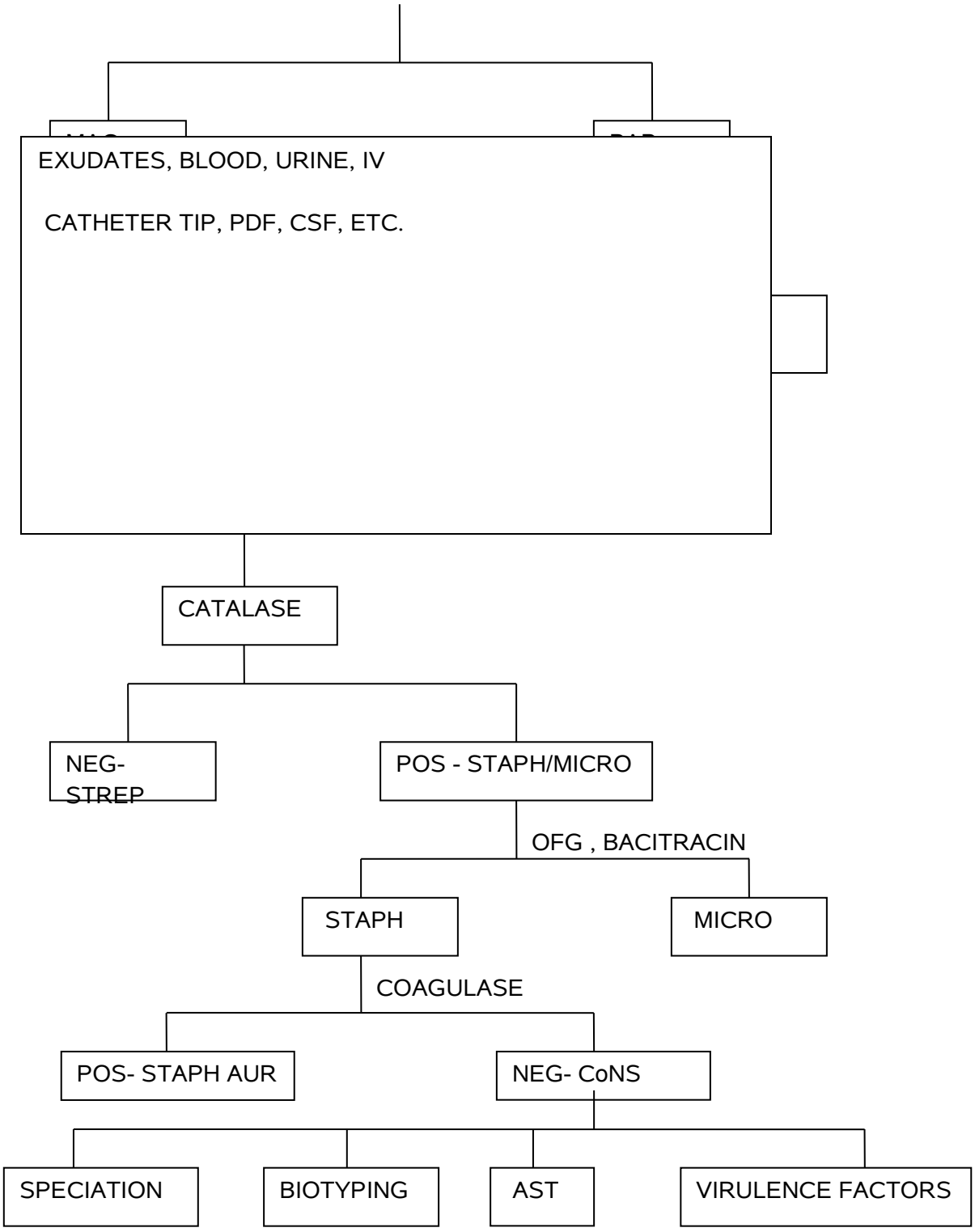
Clinical significance:

Fever / Purulent Discharge from wound /

prosthetic device / IV catheters/urinary catheters/peritoneal dialysis catheters

WBC / ESR / CRP / OTHERS

V PROTOCOL


EXUDATES, BLOOD, URINE, IV
CATHETER TIP, PDF, CSF, ETC.
CATALASE
NEG- STREP
POS - STAPH/MICRO
OFG , BACITRACIN
STAPH
MICRO
COAGULASE
POS- STAPH AUR
NEG- CoNS
SPECIATION
BIOTYPING
AST
VIRULENCE FACTORS