Study of resistance pattern and some pathogenic factors of staphylococcus epidermidis isolated from different clinical specimens and healthy controls

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Dr. Nazia Haque 2009
SUMMARY

Staphylococci are versatile pathogen. Strains of Staphylococcus epidermidis that are resistant to methicillin referred to as Methicillin Resistant Staphylococcus epidermidis (MRSE). Now a days, MRSE has been increasing as a serious nosocomial pathogen having the property of multi drug resistance. The present study was conducted to study prevalence, some pathogenic factors and antibiotic resistance patterns of Staphylococcus epidermidis isolated from different clinical specimens and healthy controls. This cross sectional observational study was carried out in the department of Microbiology, Mymensingh Medical College during the period from July 2007 to June 2008. Sixty-two Staphylococcus epidermidis were isolated from 230 specimens. Among them 32 S. epidermidis were isolated from cases and 30 were from controls. A total of 200 clinical specimens were included in this study of which 75 were from surgical wound swabs, 60 from pus of skin infections, 35 from swab of stitch infections, 12 from aural swabs, 11 from burn ulcer exudates and 7 from diabetic ulcer exudates. Out of 200 clinical specimens, 140 (70%) were culture positive. Besides S. epidemidis other isolated bacteria were S. aureus, Pseudomonas spp and Escherichia coli. Staphylococcus epidermidis were identified by standard microbiological technique and their susceptibility was observed by disk diffusion method. Detection of some pathogenic factors (biofilm and hemolysin) was done. Biofilm production was done by modified microtiter plate test and hemolysin production was done in blood agar plate.

Out of 32 S. epidermidis from cases 18 (56.25%) were detected as MRSE by disk diffusion method. Antibiotic susceptibility test was done for all strains of S.
epidermidis isolated from cases and controls. In this study S. epidermidis isolates from cases showed multidrug resistance. Resistance to penicillin was 94% followed by oxacillin 56%, cloxacillin 56%, gentamicin 44%, erythromycin 41%, doxycycline 37%, cephradine 34%, ciprofloxacin 28%, ceftriaxone 28%, fusidic acid 22% and cefuroxime 19%. On the other hand, isolates of S. epidermidis from controls were susceptible to all antibiotics except penicillin, which was only 10% resistant. A remarkable difference was observed in the resistance pattern of S. epidermidis isolated from cases and healthy controls. However in our study no isolate of S. epidermidis was resistant to rifampicin and vancomycin. Among 62 S. epidermidis, biofilm production was observed in 13 (40.62%) from clinical isolates and only 5 (16.66%) from healthy controls. The detection rate of biofilm production by Staphylococcus epidermidis isolated from cases was significantly different from that of control group (p<0.05). But there was no significant difference in hemolysin production of Staphylococcus epidermidis isolated from case and control group.
INTRODUCTION

*Staphylococci* are Gram positive, non-motile, asporogenous bacteria that characteristically divide in more than one plane to form irregular cluster. Nearly all are facultative anaerobes that grow better under aerobic than anaerobic condition (Easmon and Goodfellow, 1990). More than hundred years ago, Sir Alexander Ogston first described *Staphylococcus* and its role in the infectious diseases (Ogston, 1883). *Staphylococci* are widely spread in various niches such as clinical environments and food plants (Euzeby, 1997). Some *Staphylococcal* strains are used for their technological abilities and others are associated with diseases in humans or animals (Blaiotta *et al*, 2003). Species are classified as coagulase positive *Staphylococcus aureus* and coagulase negative *Staphylococci* eg, *Staphylococcus epidermidis, Staphylococcus saprophyticus* (Todd, 2005).

In recent years, *Staphylococcus epidermidis*, one of the *Staphylococcal* species most frequently isolated from the microflora of humans has emerged as a major pathogen in nosocomial infections (Heilmann and Peters, 2000). Few reports of infections with coagulase negative *Staphylococci* (CoNS) were published before the 1970s; clinicians and microbiologists considered them to be contaminants of clinical samples, with *Staphylococcus aureus* being the only pathogenic species within the genus *Staphylococcus* (Kloos and Bannermans, 1995). But Since the early 1980s, it has gained substantial attention because it has been recognized as one of the most important causes of nosocomial infections especially in immunocompromised individuals, neonates and patients with internal prosthetic devices (Vuong and Otto, 2002; Raad *et al*, 1998). Distinguishing, clinically significant pathogenic strain from
contaminant strain is one of the major challenges facing clinical microbiologists (Rasheed and Awole, 2007). This distinction, which has been widely used for clinical diagnosis, represents a challenge in relation to the role of these microorganisms in infectious processes.

Infections due to *S. epidermidis* can be caused by either community or hospital acquired isolates (Melzer *et al*, 1999). Skin of patients and health care workers, medical equipment, clothing of personnel and environment surfaces can be sources of antibiotic resistant *S. epidermidis* strains (Tammelin *et al*, 2000). Skin contact affords easier transmission between hosts (Ruth *et al*, 2006). The colonization pressure has an impact on both the risk for cross-transmission between patients and the risk for patients to acquire a nosocomial infection (Bonten, 2002).

*Staphylococcus epidermidis* is the most prevalent pathogen involved in hospital-acquired infections. The costs related to infections caused by *Staphylococcus epidermidis* in the hospital setting are enormous and represent a major healthcare burden (Woods *et al*, 2002). It is responsible for a variety of infections such as bacteremia, eye infection (Bannerman *et al*, 1997), urinary tract infection (Nicolle *et al*, 1983) and prosthetic and natural valvular endocarditis (Von *et al*, 2002). Little is known about the factors that have contributed to this development, but the increasing number of immunocompromised patients, the use of indwelling medical devices and a high selective pressure by antibiotics offer bacteria a novel ecological niche (Conlon *et al*, 2002).
As with *S. aureus*, methicillin resistant *S. epidermidis* (MRSE) is a serious concern. Although there are marked geographic variation in MRSE, prevalence in some areas of Europe a high proportion (60%-70%) of *S. epidermidis* are methicillin resistant (Schmitz *et al.*, 1999). The rates of methicillin resistance have increased in the last two decades, according to the National Nosocomial Infectious Surveillance System (NNIS, 2001). In Brazil, a multi-centre study showed that 87.7% of CoNS isolated from blood cultures were resistant to methicillin (Sader *et al.*, 1999). A recent study carried out during the period of December 2003 to September 2004 in Tunisia from different clinical specimens of newborns found the prevalence of MRSE represented 41.6% of the total isolates of *S. epidermidis* (Saida *et al.*, 2006). The 1999 antimicrobial resistance surveillance data by Dr. Celia Carlos reported that 61% *S. epidermidis* isolates from the different sentinel hospitals are oxacillin resistant by the disk diffusion method (Carlos, 2003). However, scant data are available on CoNS responsible for infections in developing countries (Jain *et al.*, 2004). A study carried out at Kasturba Medical College Hospital, Manipal, India from December 2002 to February 2003 reported the prevalence of MRSE as 13.84% of the total isolates of *S. epidermidis* (Shobha *et al.*, 2005). Another survey in India recorded the 62.7% MRSE among clinical isolates collected in Clinical Bacteriology Laboratory of All India Institute of Medical Sciences, New Delhi, between January and April 2004 (Ritu *et al.*, 2006).

Methicillin resistance among CoNS is particularly important due to cross-resistance to virtually all β-lactam agents and other antimicrobial classes. As a result, therapeutic approaches are restricted to glycopeptide and new antimicrobial agents as linezolid (Woods *et al.*, 2002). Therefore, an accurate analysis of methicillin resistance may
allow the provision of better antimicrobial therapy and avoid the selection of vancomycin-resistant strains.

There are many methods of susceptibility testing for identification of MRSE. Most laboratory use disk diffusion method for routine test. In this method, resistance is readily detected for 1μg oxacillin disk at 30 to 35°C for 24 hours recommended by CLSI, 2007.

Pathogenicity of *Staphylococcus epidermidis* has been linked to resistance to antimicrobial agents (Chambers, 1997) and biofilm formation (Pei and Flock, 2001). In these cases, the ability of *Staphylococcus epidermidis* to form biofilms represents the most important virulence determinant. In a biofilm, the bacteria are dramatically less susceptible to antibiotic treatment and attacks by innate host defense. For these reasons, *Staphylococcus epidermidis* biofilm-associated infections are very difficult to eradicate (Vuong and Otto, 2002). The differentiation of *Staphylococci* with respect to its biofilm phenotype might help to elucidate the impact of *Staphylococci* in diagnosis of infections related to biomedical devices and these observation may have utility in the prevention of device related infections (Raad *et al*, 1995).

A number of tests are available to detect biofilm production by *Staphylococcus epidermidis*. Among these tests Modified mirotiter plate method is superior to other tests in terms of objectivity and accuracy because it enables indirect measuring of bacteria attached both to the bottoms and walls of the wells, while in other tests only the dye bound to the bacteria attached to the bottom of the wells is spectrophotometrically registered (Srdjan *et al*, 2000).
Little is known about the importance of hemolytic activity as a pathogenic factor in *S. epidermidis*. Although alpha and gamma hemolysins have been detected in some *S. epidermidis* isolates which may be an important virulent factor (Gemmell, 1996). Alpha hemolysin having been associated with neurotoxic activity and Gamma hemolysin with a severe inflammatory response (Vuong and Otto, 2002).

The worldwide increase in antibiotic resistance is a concern for public health. The fact that, the choice of dose and treatment duration can affect the selection of antibiotic-resistant mutants. Proper use of antibiotics is very important for various reasons. It reduces unnecessary expenses, reduces development of resistance to useful and life saving antibiotics, minimize many side effects. Knowledge of the susceptibility/resistance pattern of *Staphylococcus epidermidis* is a prerequisite for rational use of antibiotics. These resistance patterns vary from one country to another and within a country as evidenced by several recent surveillance studies. In Bangladesh there is no systematic program for studying the antibiotic susceptibility pattern. Proper use of antibiotics is ensured by formulating an antibiotic policy, which can be implemented by continuous monitoring of drug resistance pattern throughout the country.

Considering the importance of *S. epidermidis* as a causal agent of nosocomial infections, in the present work we tried to study the prevalence, resistance pattern and some pathogenic factors of *S. epidermidis* isolated from different clinical specimens and healthy controls.
Aims and Objectives

General objective
To evaluate the prevalence, resistance pattern and some pathogenic factors of *Staphylococcus epidermidis* isolated from different clinical specimens and healthy controls.

Specific objectives
1. To isolate and identify *Staphylococcus epidermidis* from different clinical specimens and healthy controls.
2. To determine the antibiotic susceptibility pattern of *Staphylococcus epidermidis* isolated from different clinical specimens and healthy controls.
3. To compare the resistance pattern of *Staphylococcus epidermidis* isolated from different clinical specimens and healthy controls.
4. Screening of methicillin resistant *Staphylococcus epidermidis* (MRSE) by disk diffusion test.
5. To determine some pathogenic factors (biofilm and hemolysin production) of *Staphylococcus epidermidis* isolated from different clinical specimens and healthy controls.
6. To compare some pathogenic factors (biofilm and hemolysin production) of *Staphylococcus epidermidis* isolated from different clinical specimens and healthy controls.
REVIEW OF LITERATURE

2.1 Evolution of *Staphylococcus epidermidis*

In recent years, *Staphylococcus epidermidis*, one of the *staphylococcal* species most frequently isolated from the microflora of humans, has emerged as a major pathogen in nosocomial infections (Heilmann and Peters, 2000). Few reports of infections with coagulase negative *staphylococci* (CoNS) were published before the 1970s; clinicians and microbiologists considered them to be contaminants of clinical samples, with *Staphylococcus aureus* being the only pathogenic species within the genus *Staphylococcus* (Kloos and Bannerman, 1995). This distinction, which has been widely used for clinical diagnosis, represents a challenge in relation to the role of these microorganisms in infectious processes. Since the early 1980s, it has gained substantial attention because it has been recognized as one of the most important causes of nosocomial infections (Vuong & Otto, 2002; Raad et al., 1998). With respect to nosocomial infections, the glycopeptides have become one of the last remaining options where MRSE are suspected (Michel and Gutmann, 1997). But unfortunately resistance to vancomycin as well as teicoplanin has emerged in some coagulase negative *staphylococci* recently (Sloos et al., 1999).

2.2 Epidemiology of *Staphylococcus epidermidis*

2.2.1 Prevalence
As with *S. aureus*, methicillin resistant *S. epidermidis* (MRSE) is a serious concern. Although there are marked geographic variation in MRSE, prevalence in some areas of Europe a high proportion (60%-70%) of *S. epidermidis* are methicillin resistant (Schmitz *et al.*, 1999). Comparative levels of methicillin resistance among coagulase-negative *staphylococci* have been reported in the USA (Jones *et al.*, 1989). The rates of methicillin resistance have increased in the last two decades, according to the National Nosocomial Infectious Surveillance System (NNIS, 2001). In Brazil, a multi-centre study showed that 87.7 % of CoNS isolated from blood cultures were resistant to methicillin (*Sader et al.*, 1999). The prevalence and significance of coagulase negative *Staphylococci* (CoNS) isolated from blood cultures at the University Hospital of the West Indies (UHWI) Kingston Jamaica, during July to December 2003, found that, more than half of the CoNS (55%) were methicillin resistant *Staphylococcus epidermidis*. Among this MRSE blood isolates of CoNS was highest in the surgical wards (13.2%) and lowest in the obstetrics and gynaecology wards (2.2%). A recent study carried out during the period of December 2003–September 2004 in Tunisia from different clinical specimens of newborns found the prevalence of MRSE represented 41.6% of the total isolates of *S. epidermidis* (Saida *et al.*, 2005). The incidence of methicillin resistance has also risen among nosocomial isolates of CoNS and in the recent SCOPE (Secondary care for osteoporosis in Europe) survey, 79% of CoNS strains were resistant to methicillin (Jones, 1996). The 1999 antimicrobial resistance surveillance data by Dr. Celia Carlos reported that 61% of *S. epidermidis* isolates from the different sentinel hospitals are oxacillin resistant by the disk diffusion method (Carlos, 2003). However, scant data are available on CoNS responsible for infections in developing countries (Jain *et al.*, 2004). A study carried out at Kasturba Medical College Hospital, Manipal, India from December 2002 to
February 2003 reported the prevalence of MRSE as 13.84% of the total isolates of \textit{S. epidermidis} (Shobha \textit{et al}, 2005). Another survey in India recorded the 62.7% MRSE among clinical isolates collected in Clinical Bacteriology Laboratory of All India Institute of Medical Sciences, New Delhi, between January and April 2004 (Singhal \textit{et al}, 2006).

\subsection{2.2.2 Transmission}

\textit{S. epidermidis} is endogenous to the human skin flora and is therefore easily transmissible. In fact, the dissemination of the resistant counterparts (MRSE) was already described in the hospital environment (Villari \textit{et al}, 2000) as well as in a community. Recent studies have also documented MRSE carriage by students (Silva \textit{et al}, 2001) and young soldiers. The colonization of humans by MRSE and the increasing mobility of individuals suggest that the community can act as a vehicle for MRSE dissemination between distant locations. The geographic dissemination might occur with MRSE strains (Aires \textit{et al}, 2001). The surfaces and materials such as floors and walls of the hospital rooms, stethoscopes, beds, tables for nursing elements, oxygen tube and oxygen masks may play an important role in the spread of infectious agents including antimicrobial resistant strains of \textit{S. epidermidis} (Alcaraz \textit{et al}, 2003).

\subsection{2.2.3 Reservoirs}

Infections due to \textit{S. epidermidis} can be caused by either community or hospital acquired isolates (Melzer \textit{et al}, 1999). Skin of patients and health care workers, medical equipment, clothing of personnel, and environment surfaces can be sources of antibiotic-resistant \textit{S. epidermidis} strains (Tammelin \textit{et al}, 2000).
2.2.4 Risk factors of *Staphylococcus epidermidis* infection

*Staphylococcus epidermidis* is both a human skin commensal and an opportunistic pathogen, causing infections linked to implanted medical devices (Miriam *et al.*, 2007). Immunocompromised patients are particularly at risk of CoNS infections, as are individuals with indwelling catheters or prosthetic devices (Rasheed and Awole, 2007). Beside these cardiac surgery patients, preterm newborns, patients with mechanical ventilation, patients with parenteral nutrition, patients with chest drainage, patients with ventriculo - peritoneal shunt, non removal of foreign body and female patients with membrane rupture >24 hours are high risk patients for developing MRSE infection (Cunha *et al.*, 2006).

2.2.5 Strategy for prevention and control of *Staphylococcus epidermidis* infection

In the past 10 years, there has been a progressive increase in the overall resistance of *Staphylococci* to antimicrobial agents (McGahee *et al.*, 2000). Loudoun County Public School System and the Loudoun County Health Department, address the following areas to mitigate the risk of an MRSE event: the heightened awareness of skin sores and abrasions, the cleaning and covering of such wounds until fully healed; the proper disposal of wound dressings; the availability of alcohol based hand sanitizers; the thorough and consistent cleaning of locker rooms, rest rooms, and the defensive tactics and weight rooms using recommended disinfectant; the proper decontamination of defensive tactics training equipment and the prevention of
sharing such equipment and the proper medical treatment and documentation of any suspected *Staphylococcal* infection (Hammes, 2007).

The best prevention against the development of biofilms is to limit the use of indwelling devices as much as possible. Another strategy is to modify the device to inhibit biofilm growth. For example, the Guidelines for the Prevention of Intravascular Catheter-related Infections published by the Centers for Disease Control and Prevention (CDC) recommend the use of catheters impregnated with antimicrobial or antiseptic substances (CDC, 2002). These include chlorhexidine and silver sulfadiazine, minocycline and rifampin and ionic metals such as platinum and silver. Similarly, catheters for vascular access or urinary drainage are available with antimicrobials impregnated into the catheter material. The use of antimicrobial-impregnated devices has been clinically proven to inhibit biofilm growth (Greenfeld *et al.*, 1995). Similarly, furanone-coated catheters have been shown to reduce biofilm formation from *Staphylococcus epidermidis* by 78% in an animal model (Hume *et al.*, 2004). The use of these devices has been shown to inhibit the adherence of bacteria and appears to provide an effective means of deterring biofilm growth once the device has been implanted.

Another approach is to use topical antimicrobials to reduce the growth of skin-dwelling microorganisms. A recent in vitro study evaluated four topical antimicrobials used for skin antisepsis prior to catheter insertion. The antimicrobials tested included 70% isopropanol + 2% chlorhexidine gluconate (IPA + CHG), 72% isopropanol (IPA) + 7.5% povidone iodine (PI), 73% ethanol + 0.25% zinc pyrithione, and 62% ethanol + <5% IPA. Each of the topical antimicrobials tested demonstrated strong activity against biofilm formation. The study concluded that an effective, persistent antimicrobial solution should be used to prepare skin sites prior to
vascular catheterization or surgery. In addition, patients should cleanse the surgical skin site with either CHG, IPA + CHG, or alcohol + zinc pyrithione for three to four days prior to surgery. CHG and zinc pyrithione each demonstrate residual antimicrobial activity. The combination of preoperative cleansing, surgical skin site preparation and surgical scrubbing with a persistent topical antimicrobial provides cumulative benefits (Paulson, 2005).

A feared complication of the use of a prosthetic material is the appearance of infection after implant (Soetevent et al, 2004). The causative organisms are predominantly S. epidermidis and S. aureus (Abdulrahman et al, 2002). Most commonly contamination occurs at the time of graft insertion and the most frequent source of infection is from Staphylococci from the patient's skin (Jones et al, 1997). The most important strategies for the prevention of prosthetic infection are asepsis and the perioperative administration of systemic antibiotics (Ghiselli et al, 2002). Moreover, in case of vascular grafts, alternative methods such as antimicrobials bound in high concentrations to prosthetic grafts have been proposed. Antibiotics having good activity against gram-positive bacteria were used in bonding vascular grafts in experimental models (Giacometti et al, 2000).

Nabi Biopharmaceuticals is focused on developing a broad portfolio of vaccines and antibody-based therapies that target Gram-positive bacteria, most notably S. aureus, Staphylococcus epidermidis and Enterococcus. EpiVAX™ (Staphylococcus epidermidis Conjugate Vaccine) is an investigational vaccine in preclinical development for the prevention of S. epidermidis infections. This EpiVAX has been shown to induce antibodies that are protective in animal models and facilitate elimination of bacteria by the same type of immune system response as StaphVAX.
EpiVAX™ (*Staphylococcus epidermidis* Conjugate Vaccine) will probably be used both as a vaccine in order to prevent *S. epidermidis* infections and as a potential therapeutic vaccine to be administrated before onset of antibiotherapy (http://www.ncbi.com.).

### 2.3 Microbiology of *Staphylococcus epidermidis*

#### 2.3.1 Taxonomy

**Scientific classification**

- **Kingdom**: Bacteria
- **Phylum**: Firmicutes
- **Class**: Cocci
- **Order**: Bacillales
- **Family**: Staphylococcaceae
- **Genus**: *Staphylococcus*
- **Species**: *Staphylococcus epidermidis* [Winslow & Winslow 1908; Evans 1916]

#### 2.3.2 General properties of *Staphylococcus epidermidis*

A Scottish surgeon, Sir Alexander Ogstone, first showed in 1980 that a number of pyogenic disease in human were associated with a cluster-forming microorganism. He introduced the name *Staphylococcus* which came from Greek word staphyle = bunch of grapes; kokkos = grain or berry (Humphreys, 2002). *Staphylococci* are Gram positive, non-motile cocci that characteristically divide in more than one plane to form irregular clusters. Their average diameters are 0.5-1.5µm. The size varies from strain to strain. Usually form catalase. Nearly all are facultative anaerobes that grow
better under aerobic than anaerobic conditions; members of the anaerobic species, *Staphylococcus saccharolyticus* are exceptions. The metabolism is respiratory and fermentative and most strains grow in presence of 10% sodium chloride and between 18 and 40°C. The cell wall contains peptidoglycan and teichoic acid; the diamino acid present in the peptidoglycan is L-lysine. Strains contain fatty acids, unsaturated menaquinone, diphosphatidylglycerol and several glyco–and phospholipids as major polar lipids. G+C content of the DNA 30-39 mol % (Easmon and Goodfellow, 1990). Thirty-six validated described species, including 21 subspecies, belong the *Staphylococcus* genus (Euzeby, 1997). The major pathogen with in the genus, *Staphylococcus aureus* is characterized by its ability to clot blood plasma by action of the enzyme coagulase. Other species of *Staphylococci* (*Staphylococcus epidermidis, Staphylococcus saprophyticus*) are lack of this enzyme, hence they are called coagulase negative *Staphylococci* (Dugid, 1996).

### 2.3.3 Cultural characters of *Staphylococcus epidermidis*

*Staphylococcus epidermidis* are facultative anaerobe, which are best produced aerobically. Temperature for growth range 10-42°C, optimum temperature 37°C and optimum pH 7.5 (Cheesbrough, 2000).

Growth characters in common bacteriological media are as follows:

#### 2.3.3.1 Nutrient agar (NA)

Colonies of *Staphylococcus epidermidis* are white coloured, circular, 2-4 mm in diameter, convex with shining surface (Cheesbrough, 2000).
2.3.3.1.2 Blood agar (BA)

Little is known about the importance of hemolytic activity in *S. epidermidis*. Although alpha and gamma hemolysins have been detected in some *S. epidermidis* isolates which may be another important virulent factor (Vuong and Otto, 2002).

2.3.3.1.3 MacConkey agar (MA)

*Staphylococcus epidermidis* may not grow in MacConkey agar (Dugid, 1996).

2.3.3.1.4 Biochemical behavior of *Staphylococcus epidermidis*

*Staphylococcus epidermidis* are coagulase negative, catalase positive, oxidase negative, weakly Dnase positive, urease positive, novobiocin (5µ gm) sensitive, Polymyxin B resistant, ferment sucrose, D-manose and maltose but not D-mannitol, D-trehalose, D-xylene and D-cellobiose, do not form acid but produce acetoin and phosphatase and reduce nitrate (Cheesbrough, 2000; Tammy *et al*, 2007). Following table shows differentiation of *Staphylococcus* species
<table>
<thead>
<tr>
<th>Species</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td></td>
<td>Large colonies</td>
</tr>
<tr>
<td>S. aureus</td>
<td>+</td>
</tr>
<tr>
<td>S. epidermidis</td>
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<tr>
<td>S. saprophyticus</td>
<td>-</td>
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</tbody>
</table>

+ indicates 90% or more strains positive  
- indicates 90% or more strains negative  
d indicates 11-89% strains positive  
ND indicates not determined  

(Tammy et al, 2007)
2.3.4 *Staphylococcal* component and product

2.3.5 Genome

*Staphylococcus epidermidis* genomes have been sequenced: those of the non-biofilm-forming, non-infection-associated strain ATCC 12228 and the infectious biofilm-forming strain RP62A (ATCC 35984). *Staphylococcus epidermidis* ATCC 12228 consists of 2419 gene, 2499279 bp of DNA and number of G+C bases: 802148 bp. *Staphylococcus epidermidis* RP62A consists of 2665 gene, 2643840 bp of DNA and number of G+C bases: 849969 bp (Zhang et al., 2003). There is also an assortment of extra chromosomal accessory genetic elements: conjugative and non-conjugative plasmids, mobile elements, prophages and other variable elements (Ito et al., 2003). These genes are transferred between *Staphylococcal* strains, species of other gram-positive bacterial species through the extrachromosomal elements (Schaberg and Zervos, 1986).

2.3.6 Cell wall

The cell wall of *Staphylococcus epidermidis* is mainly peptidoglycan, composed of repeating disaccharide N-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) units with attached teichoic acids (Navarre and Schneewind, 1999). Glycan chains are crosslinked by tetrapeptides consisting of L-alanine, D-glutamate, L-lycine, and L-alanine to pentaglycine interbridge, linked to wall peptide. The main function of peptidoglycan is to provide a rigid envelope for the cell contest. Peptidoglycan also has endotoxic properties and has been reported to cause organ dysfunctions in experimental animals (Holtfreter and Broker, 2005). Teichoic acids, another cell wall component together with peptidoglycan contribute to the severity of *staphylococcal* sepsis (De Kimpe et al., 1995). The cell wall assembly is catalysed by high molecular
weight bifunctional enzymes, penicillin-binding proteins (PBP). Four native forms of PBPs (1-4) promote the polymerization of glycan from its disaccharide precursor and from the transpeptidation of wall peptides (Navarre and Schneewind, 1999).

2.3.7 Virulence factors of \textit{Staphylococcus epidermidis}

\textit{Staphylococci} are major human pathogen that produces a wide array of toxins and enzymes, thus causing various types of diseases (Naomi and Avr, 2000). Virulence mechanisms of the leading nosocomial pathogen \textit{Staphylococcus epidermidis} are poorly understood (Yao \textit{et al}, 2005). The virulence factors produced by CoNS and how they contribute to the pathogenicity of infections associated with foreign bodies are currently under investigation (Vogel \textit{et al}, 2000). Differentiation between virulent and non-virulent strains has been difficult since the virulence factors of these microorganisms are still not well defined (Gemmell, 1987).

One of the key factors enabling \textit{Staphylococcus} to survive, colonize, proliferate and cause human infections is the expression of virulence factors (VFs). This can be divided into three groups. 1) Bacterial attachment, 2) evasion of host defenses and 3) tissue invasion. Single virulence factor alone is not sufficient to cause infection (Projan and Novick, 1997).

2.3.8 Capsule

Capsular polysaccharide of \textit{Staphylococcus epidermidis} contains poly-N-succinyl β-1-6 glucosamine (PNSG). It mediates adherence of coagulase-negative \textit{staphylococci} (CoNS) to biomaterials, serves as the capsule for strains of CoNS that express
polysaccharide/adhesin and is a target for protective antibodies (Mckenney et al, 2000).

2.3.9 Extracellular matrix binding protein (ECMBP₅)

Access and adherence to host tissues or implanted material is mediated mainly by ECMBP₅ from *S. epidermidis* are attached to the bacterial cell wall surface and interact with target structures on the eukaryotic cells. Many *Staphylococcal* surface proteins have certain structural features in common. These features include a secretory signal sequence at the N-terminal, positively charged amino acids that extend into the cytoplasm, a hydrophobic membrane spanning domain and a cell wall anchoring region. Most ECMBP₅ are attached to the *Staphylococcal* cell wall by a common mechanism (Mazmanian et al, 1999). Only a few *S. epidermidis* cell-wall-associated proteins with potential adhesin function have been identified namely SdrG (Fbe) (Davis et al, 2001; Nilsson et al, 1998), Embp (Williams et al, 2002), GehD (Bowden et al, 2002), AtlE (Heilmann et al, 1997) and Ebps (Park et al, 1996). These adhesins bind to fibrinogen, fibronectin collagen, vitronectin and elastin respectively. Most of the ECMBP₅ have specific biological functions and are proposed to contribute to successful colonization and persistence at various sites within the host (Peacock et al, 2002).

2.3.10 Biofilm formation

One of the great challenges of modern medicine is the increasing use of invasive medical procedures such as device implantation. Biofilm production by *S. epidermidis* can occur on almost any kind of catheter and in a variety of other medical devices and implants. Once a biofilm is formed, a chronic infection is generally established and in
some cases the patient has to undergo surgical intervention for the removal of the implant. Consequently, the extensive use of such indwelling devices has led to the emergence of new routes of infection associated with biofilm production on the surfaces of such foreign bodies (Gotz, 2002). Species of *Staphylococcus*, particularly *S. epidermidis* and *S. aureus* are the bacteria most frequently isolated in this infectious process worldwide (Von *et al*, 2005). The process of biofilm production in *S. epidermidis* has not yet been totally clarified but seems to occur in two important steps: adherence to the inert surface and biofilm accumulation. Many bacterial products are thought to be involved in the initial phase of adherence including the AtlE protein, teichoic acid and also *staphylococcal* adhesins proteins which play an important role in plasma-coated biomaterial (Takahashi *et al*, 2002). In the second phase, bacteria connected directly or indirectly to the surface of the polymer produce and accumulate an extracellular, amorphous and mucoid polysaccharide material: the biofilm. This is thought to be the main mechanism of bacterial adherence to plastic surfaces and of auto-aggregation (Von *et al*, 2002). The biofilm of *S. epidermidis* consists of clusters of cells that are embedded in extracellular slime substance that is up to 160 micrometers thick, exceeding 50 cells (Nilsson *et al*, 1998). Biofilm formation is regulated by expression of polysaccharide intracellular adhesin (PIA), which mediates cell-to-cell adhesion and the gene product of *icaADBC* (Heilmann *et al*, 1996). Biofilms act as a diffusion barrier to antibiotics and host defense (Nilsson *et al*, 1998). Studies have indicated that the mature biofilm facilitates colonization and persistence of bacteria in the host (Gotz, 2002).
2.3.11 Toxins and enzymes

In case of *Staphylococcus epidermidis*, differentiation between virulent and non-virulent strains has been difficult since the virulence factors of these microorganisms are still not well defined (Gemmell, 1987). According to Chunha (2006), Koneman and Winn (1997), CoNS produce other virulence factors, such as hemolysins, lipases, proteases, and toxins.

2.3.12 Alpha-Hemolysin (Alpha-Toxin)

In some *Staphylococcus epidermidis* strains, hemolytic activity has been detected (Dingers *et al*, 2000). Alpha-hemolysin is most potent cytotoxins of *S. epidermidis* (Gray and Kehoe, 1984). It is particularly active against rabbit erythrocytes and it is also dermonecrotic and neurotoxic (Bhakdi *et al*, 1991). The toxin acts on cells without requiring bacterial internalization. The toxin is secreted as a hydrophilic monomer that forms large size ionic pores in the cytoplasmic membranes of eukaryotic cells (Gouaux *et al*, 1994; Valeva *et al*, 1997). These pores behave like an ionophore for ions such as calcium (Song, 1996; Valeva, 1996). Alpha toxin has been shown to cause the adhesion of polymorphonuclear leukocytes to endothelial cells, which is an essential step in inflammatory reaction. In vivo, Alpha-toxin also damages the primary host defense system by reducing phagocytic activity of macrophages and by inducing the over secretion of IL-1-alpha and IL-6 but not TNF-alpha, via a mechanism that requires the synergistic action of bacterial components (Onogawa, 2002).
2.3.13 Gamma-Hemolysin

Gamma-toxin, which is known also as Gamma-hemolysin, is one of the exotoxins produced by most isolates of Staphylococcus epidermidis, which has a severe inflammatory response (Vuong and Otto, 2002). Gamma-toxin is a bi-component pore-forming cytotoxin (Sugawara et al, 1997) composed of non-associated secreted proteins, referred to as S and F components (for slow- and fast-eluting proteins in an ion-exchange column) (Finck, 1991; Woodin, 1960). Gamma toxin is lytic for erythrocytes from different mammalian species and also cytotoxic for leukoctes (Ferreras et al, 1998).

2.3.14 Catalase

Staphylococcus epidermidis produce catalase, which converts hydrogen peroxide into water and oxygen (Cheesbrough, 2000).

2.3.15 Protease

Protease is produced by Staphylococcus epidermidis, which is involved in tissue damage and the inflammatory response of the host (Goguen et al, 1995). The general function of all proteases is to cleave proteins and in doing so, this may inactivate key proteins and antimicrobial peptides involved in host defenses (Miedzobrodzki et al, 2002).

2.3.16 Lipase

Extracellular lipases have been shown to be a pathogenicity factor of various microorganisms, including S. epidermidis (Gemmell, 1996), contributing for the survival of microorganisms in environments containing high concentrations of lipids.
and affecting the capacity of the microorganism to penetrate the skin and invade epidermal tissues. No differences on the percentage of lipolytic isolates were found when comparing clinical and healthy isolates, indicating that lipolytic activity is important not only during pathogenic processes but also for *S. epidermidis* skin colonization (Goguen *et al*, 1995).

### 2.3.17 Beta lactamase

Beta lactamase is an enzyme that inactivates penicillin as well as beta lactam drugs. Panicillin-binding proteins are enzymes located in the cytoplasmic membrane that are involved in cell wall assembly. A novel penicillin-binding protein is responsible for *Staphylococcal* resistance to the penicillinase – resistant penicillins and cephalosporins (Kemodle, 2000).

### 2.3.18 Staphylokinase

Staphylokinase is a 136 aa long bacteriophage encoded protein. The role of staphylokinase during bacterial infection is based on its interaction with the host proteins, α-defensins and plasminogen. α-defensins are bactericidal peptides originating from human neutrophils. Binding of staphylokinase to α-defensins abolishes their bactericidal properties, which makes staphylokinase a vital tool for *staphylococcal* resistance to host innate immunity. Complex binding between staphylokinase and plasminogen results in the formation of active plasmin, a broad-spectrum proteolytic enzyme facilitating bacterial penetration into the surrounding tissues. Our observations indicate that staphylokinase favours symbiosis of *staphylococci* with the host that makes it an important colonization factor (Bokarewa *et al*, 2006).
2.3.19 Regulation of virulence factors

The expression of surface-associated and secreted *Staphylococcal* exoproteins is controlled by a complex regulatory network (Novick, 2003). The best characterized part of this network is accessory gene regulator *agr* (Peng *et al.*, 1988; Recsei *et al.*, 1986). Two transcripts are divergently produced from the *agr* locus promoters P2 and P3. Products of the P2 transcript, Agr A, B, C and D, act as a two-component regulatory system: Agr D is processed and transported out of the cell by AgrB. The processed peptide binds to the transmembrane AgrC causing its auto phosphorylation and leading to phosphorylation of intracellular AgrA. AgrA further activates the promoters P2 and P3. The transcript of P3, RNAIII, is the effector upregulating genes of many extracellular proteins and down regulating genes of many surface proteins by an unknown mechanism (Novick *et al.*, 1993). A few transcription factors act in global regulation of virulence factors. SarA (*staphylococcal* accessory regulator A) is a transcription factor up regulating production of several surface proteins and is also required for the expression of *agr* (Cheung *et al.*, 1992). Rot (repressor of toxins), another transcription factor, up regulates surface adhesin production and down regulates several secreted proteins (McNamara and Proctor, 2000). Additionally, an alternative sigma factor, Sigma B, is directly and indirectly, through SarA, involved in regulation of virulence genes (Kullik *et al.*, 1998).

2.3.20 MecA

Methicillin resistance is primarily caused by the *mecA* gene encoding a 78-kDa penicillin-binding protein 2A (PBP 2A or PBP 2’); (Hartman and Tomasz, 1984; Matsuhashi *et al.*, 1986; Ubukata *et al.*, 1985). Beta lactam antibiotics quickly acylate these enzymes because of their structural similarity with the natural substrates of the
PBP2s. The affinity of these antibiotics towards PBP2A is, however, much lower and this enzyme remains functional. Actually, a partially functional native PBP in addition to PBP2A is needed in the presence of beta lactam antibiotics (Pinho et al., 2001). mecA is preceded by the regulatory genes mecR1 and mecI, which are transcribed in the opposite direction as compared to mecA (Tesch et al., 1990). Mainly based on homology to the plasmid-encoded penicillinase regulators blaRI and blaI, which in addition to regulation of penicillinase production are also able to regulate PBP2A production, mecR1 is presumed to act as a signal transducer sensing the extracellular beta lactam antibiotic. This leads to activation of its cytoplasmic protease domain and specific cleavage of mecI, the transcriptional repressor of mecA. Intact regulator genes strongly repress PBP2A production and it is poorly inducible even in the presence of many beta lactam antibiotics, including methicillin.

2.3.21 SCCmec

Staphylococcal cassette chromosome (SCC) a unique family of mobile genetic elements found on the chromosomes of Staphylococcal spp. If SCC carries methicillin resistant gene (mecA), it is called SCC mec (Katayama et al., 2000). It is not present in methicillin sensitive Staphylococcus epidermidis (MSSE). Five types of SCCmec have been characterized so far (Ito et al., 1999; Ito et al., 2004). Each type contains a mec gene complex with the resistance encoding mecA gene and intact or partly deleted regulatory genes. The region also contains a cassette chromosome recombinase (ccr) gene complex encoding site-specific recombinases of the invertase/resolvase family, which are responsible for integration and excision of SCCmec, as well as additional type specific DNA. Type I, IV and V SCCmec elements carry no other resistance genes in addition to mecA. Type II and III SCCmec
elements carry resistance for spectinomycin and erythromycin encoded by Tn554 (Ito et al, 2001). SCC elements without the mecA gene have recently been found in both S. aureus and coagulase negative staphylococci. These elements are inserted in the chromosome at the same site as SCCmec and have either functional ccr genes identical or slightly different from the ones found in SCCmec or nonfunctional gene areas resembling the ccr genes (Luong et al, 2002). In contrast, the SCCmec types of oxacillin-resistant coagulase-negative staphylococci (MRCoNS) were of completely different composition. The other MRCoNS displayed 11 different, complex patterns suggesting frequent recombination between different SCCmec elements. With one ccr-negative exception, these strains amplified between one and three different ccr products, indicating either new varied complexes or multiple ccr loci. This suggests the presence of novel SCCmec types in MRCoNS and no extensive interspecies SCCmec transfer between MRSA and MRCoNS (Qi et al, 2005).

2.3.22 Other genes affecting methicillin resistance

Fem (factor essential for methicillin resistance) AB like gene that encodes an LPXTG protein of unknown function (Komatsu2awa et al, 2000) have also been identified in S. epidermidis (Alborn et al, 1996). Defective building blocks in fem mutants are not optimal substrates for PBP2A and lead to reduced resistance (Berger-Bäch, 1994; Chambers, 1997; de Lencastre et al, 1994).
2.3.23 Pathogenesis and host response against *Staphylococcal* infection

*Staphylococcal* pathogenesis results from various bacterial activities mediated by virulence factors and from the immunological response by the host. It is commonly thought that bacterial adherence to host tissue is a pre requisite for colonization and infection. This is achieved by the Microbial surface component recognized adhesive matrix (MSCRAMMs) (Patti *et al.*, 1994). Subsequent survival, growth, and establishment of infection depend on the ability of the bacterium to circumvent host defense. The primary host response is mediated by polymorphonuclear leucocytes (Verdrengh and Tarkowski, 1997), which are attracted by expression of adhesion molecules on endothelial cells. The cell wall components, peptidoglycan and teichoic acids trigger signaling pathways leading to their release of cytokines (Ellingsen *et al.*, 2002). Leucocytes and other host cellular factors can be destructed by locally acting bacterial toxins. Anti-inflammatory response is also achieved by the *staphylococcal* extracellular adherence protein, Eap that inhibits the recruitment of host leucocytes by direct interaction with the host adhesive proteins ICAM-1, fibrinogen, and vitronectin (Chavakis *et al.*, 2002). If not attenuated enough, however, the robust local inflammatory response may lead to the formation of an abscess. Inside an abscess, the bacteria gradually fall into a state of nutritional stress as the density of bacteria increases. At this point the auto induction of secreted virulence factors could enable the bacteria to break out and spread to new locations (Novick *et al.*, 1993). In invasive diseases, such as sepsis and endocarditis, *staphylococci* must interact with the endothelium. By using MSCRAMMs, the bacteria can adhere to damage areas of the endothelium or directly to the endothelial cell via the adhesin-receptor mechanism or via bridging ligands (Joh *et al.*, 1999). The bacteria may then be phagocytized into
endothelial cells (Hamill et al, 1986) and/or reach the underlining tissue (Lowy, 1998). Both endothelial phagocytosis and tissue invasion elicit an inflammatory response leading to the release of IL-1, IL-6, IL-8, tumor necrosis factor (TNF) and subsequently interferon gamma. Leucocytes adhere to endothelial cells and increase vascular permeability. Intracellular staphylococci often appear as small colony variants, which have mutations affecting electron transport (McNamara et al, 2000) and show slowly growing, non-pigmented colonies with reduced production of virulence factors (Von, 1997).

2.3.24 Pathogenicity of Staphylococcus epidermidis

2.3.24.1 Cellular invasion

Traditionally, species of Staphylococci has been considered as a strictly extracellular pathogen. In recent years, increasing evidence has emerged on its ability to invade eukaryotic cells. It has been suggested that cellular invasion is beneficial for the bacterium as it achieves protection from host defense and antibiotics and reaches a nutrient-rich environment (Lowy, 1998). Internalization of Staphylococcus into nonprofessional phagocytic cells (epithelial cells, endothelial cells, fibroblasts) has been shown to depend on Fn-mediated binding between the host cell and the Fn-binding proteins FnBPA or FnB PB on the surface of staphylococci (Dziewanowska et al, 1999; Fowler et al, 2000; Lammers et al, 1999). The specific molecule involved on the host cell surface is integrin α5β1 (Fowler et al, 2000). Additionally, heat shock protein 60 (Hsp60) has been shown to directly bind FnBP and mediate internalization into epithelial cells (Dziewanowska et al, 1999). In the host cells, internalization of staphylococci is an active cellular process dependent on actin polymerization (Menzies and Kourteva, 1998). Host cell tyrosine kinase activity is required
(Dziewanowska et al, 1999). Internalization of living *Staphylococcal* cells is known to induce apoptosis in human endothelial (Menzies and Kourteva, 1998) and pulmonary epithelial cells (Kahl *et al*, 2000). Bacteria, which are internalized in endosomes, can escape into the cytoplasm (Bayles *et al*, 1998; Menzies and Kourteva, 1998) or replicate inside the endosomes (Kahl *et al*, 2000). So, *Staphylococcus* seems to have an ability to invade various host cells. Whether this ability actually contributes to pathogenicity or rather is a part of host defense is still controversial. High frequency of recurrent infections after antibiotic treatment and ability to metastasize from a local infection site are in favour of a function beneficial for the bacteria (Sinha *et al*, 2000).

### 2.3.24.2 Bacteremia

*S. epidermidis* and other CoNS are the most frequently reported pathogens in nosocomial blood stream infections (Kloos and Bannerman, 1994). According to the Centers for Disease Control and Prevention's National Nosocomial infection surveillance system *S. epidermidis* is responsible for 33.5% of nosocomial blood stream infections (NNIS 2001). These bacteremias are largely due to intravascular catheter associated infection. Unfortunately, nosocomial bacteremia due to *S. epidermidis* is a rapidly increasingly problem (Rupp and Archer, 1994). Bacteremia originating from these sites can result from a compromise in both general and local defense mechanism in severely immunocompromised patients. *S. epidermidis* frequently contaminate blood cultures making their interpretation a major concern for clinicians and for microbiologists. Although, criteria such as sepsis symptoms, the number of positive blood cultures from separate veni puncture or access site and the similarity of their antibiotic resistance profiles are often considered. A large
proportion of nosocomial isolates of CoNS are resistant to multiple antibiotics, including penicillinase resistant penicillins (Archer et al, 1994). Localized infections with positive blood cultures are associated with higher mortality rate than localized infections without positive blood cultures (Souvenir et al, 1998).

2.3.24.3 Infections associated with medical devices

*S. epidermidis* is the most prominent cause of intravascular catheter associated infection (Rupp and Archer, 1994). In one study it was demonstrated that two clones of *S. epidermidis* were predominantly involved in colonization and subsequent infection in neutropenic hemato-oncologic patients in a setting with a high incidence of Catheter Related Infection (6.0/1000 catheter days) and a very high catheter removal rate for CRI 70%, despite prompt treatment with vancomycin. Migration of skin organisms at the insertion site into the cutaneous catheter tract with colonization of the catheter tip is the most common route of infection for peripherally inserted, short-term catheters. Some catheter materials have surface irregularities that enhance the microbial adherence. Additionally, certain catheter materials are more thrombogenic than others, a characteristic that also might predispose to catheter colonization and catheter related infection (Naomi et al, 2002). Rarely, catheter might become hematogenously seeding from another focus of infection (Salzman et al, 1993). It might be possible to eradicate methicillin resistant CoNS at the site of catheter implantation by using antiseptics and to prevent ingress of CoNS by a combination of occlusive dressings and careful handling of catheters and insertion sites with gloved hands (Frebourg et al, 1999).
2.3.24.4 Endocarditis

Coagulase negative *staphylococci*, usually *S. epidermidis* were previously a minor cause of native valve endocarditis. But now a day it is an important cause of prosthetic valve endocarditis and nosocomial infective endocarditis. Nosocomial native valve infections generally result from infected intravascular devices; the affected valve may or may not have been previously abnormal (Vander *et al.*, 1992). *S. epidermidis*, are the predominant cause of nosocomial prosthetic valve endocarditis. It can be acquired in the theatre or shortly there after at the time of the original valve replacement and presents within weeks or more often diagnosed within 60 days after surgery (early onset) and the cause of 3 to 8% of native valve endocarditis cases, usually in the setting of prior valve abnormalities (Karchmer, 1992). The vast majority of CoNS causing prosthetic valve endocarditis (PVE), when speciated were *S. epidermidis*. In contrast, when infection involves native valves, only 50% of isolates were *S. epidermidis* (Whitener *et al.*, 1993). Some cases of endocarditis following implantation of a prosthetic valve were recently shown to be attributable to polyclonal *S. epidermidis* populations (Van Wijngaerden *et al.*, 1997). Therefore, the detection in samples from the same patient of *S. epidermidis* strains with different antibiograms does not necessarily indicate contamination of the samples during collection. Complications, such as dehiscence of the valve or obstruction are relatively common. Blood cultures are usually positive and diagnosis is occasionally difficult. Antibiotic treatment of prosthetic valve is required in most cases (Heilmann and Peters, 2000).
2.3.24.5 Urinary tract infections

*S. epidermidis* is the predominant species cultured from the urine in significant numbers ($10^4$ cfu/ml). It accounts for 80-90% of non- *S. saprophyticus* CoNS isolates. It is cultured almost exclusively from the urine of hospitalized patients with complications of the urinary tract. About half of them have an indwelling urinary catheter. Both males and females are equally affected and most patients are 50 or more years of age. In at least half of the cases, the organisms are multiple drug resistant (Nicolle *et al*, 1983).

2.3.24.6 Eye Infections

*S. epidermidis* is commonly cultured from the conjunctive and lid margins of normal subjects (Bannerman *et al*, 1997). Because of its ubiquitous nature and relatively low virulence, *S. epidermidis* has received so far little attention for its role in ocular infections. However, in different studies *S. epidermidis* has been reported to play a significant role in several ocular external diseases such as chronic blepharitis and suppurative keratitis. The multiple resistances of *S. epidermidis* are a recognized problem. It might possibly represent a response to prolonged treatment (Mozayeni and Lam, 1996).

2.3.24.7 Pediatrics

Major advances in perinatal and neonatal care units have significantly improved for survival of very low birth weight infants. However late onset nosocomial neonatal septicemia, after more than 72 hours post delivery by CoNS, the most common organism accounting for more than 50% cases. They show multiple antibiotic resistances including resistance to methicillin (Stoll *et al*, 2002). CoNS are ubiquitous
and every human is colonized soon after birth: during invasive procedures these organism may then gain entry to the blood and result in sepsis (Dear, 1999). There is a clear co-relation between very low birth weight and the risk of a nosocomial infection with CoNS. The intensive use of antibiotics in an NICU setting with highly susceptible patients causes selection of multiresistant clones of CoNS, which subsequent becomes endemic (Hall, 1991). *S. epidermidis* distinct clones can become endemic in NICUs as long as a decade and nosocomial transmission plays an important role in *S.epidermidis* bacteremia (Huebner and Goldmannm, 1999).

2.4 Mechanism of resistance to commonly used antimicrobial agents

2.4.1 Penicillin

*Staphylococcal* resistance to penicillin is mediated by *blaZ*, the gene that encodes beta-lactamase. This predominantly extracellular enzyme, synthesized when *staphylococci* are exposed to beta lactum antibiotics, hydrolyzes the beta-lactum ring, rendering the beta lactam inactive. *blaZ* is under the control of to adjacent regulatory genes, the antirepressor *blaR1* and the repressor *bla1* (Kemodle, 2000). Recent studies have demonstrated that the signaling pathway responsible for beta-lactamase synthesis requires sequential cleavage of the regulatory protein *BlaR1 and Bla1*. Following exposure to beta lactams, *BlaR1*, a transmembrane sensor-transducer cleaves itself (Zhang and Stewart, 2000). Zhang and Stewart hypothesised that the cleaved protein functions as a proteases that cleaves the repressor *Bla1*, directly or indirectly (an additional protein, *BlaR2*, may be involved in this pathway) and allows *blaZ* to synthesize enzyme.
2.4.2 Methicillin

Resistance to β-lactam compounds that are not hydrolysed by β-lactamase such as methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin is termed "intrinsic" or "methicillin" resistance. Methicillin resistant coagulase negative staphylococci isolates are broadly resistant to penicillins and cephalosporins (Maranan et al., 1997). Methicillin resistance is most commonly mediated by the meca gene, which encodes for a single additional penicillin binding protein, PBP2a, with low affinity for all β-lactams (Spratt, 1994). The meca gene is widely distributed in both coagulase positive and coagulase negative staphylococci. It is carried on a transposon and appears to integrate into a single site in the staphylococcal chromosome along with an additional 30 kb of DNA, the mec locus (Hiramatsu, 1995). In some strains, this includes a regulatory locus, mecR1-mecI and may include an insertion element that is a potential integration site for unrelated resistance determinants. Expression of meca can be either constitutive or inducible. Other regulatory components that control the expression of the gene are the β-lactamase genes (blaI, blaRI, blaZ), which because of sequence similarities to the mecR1-mecI genes also can down regulate meca gene transcription. Expression of resistance also depends in part on other chromosomal genes where there are a series of five auxiliary genes that can modify expression of methicillin resistance. These are the fem (factor essential for the expression of methicillin resistance) A to E genes where they affect different steps in the synthesis of peptidoglycan; they are part of cellular peptidoglycan metabolism and can regulate the degree of resistance without altering levels of PBP2a (Murakami and Tomasz, 1989). The phenotypic expression of methicillin resistance shows great variability (Tomasz et al., 1991). In a heterogeneous bacterial population, all cells carry the genetic markers of methicillin resistance but the resistant phenotype occurs only in a small
fraction of the population. The proportion of the population that is resistant to higher concentrations of methicillin is a strain specific characteristic under genetic control and varies from $10^{-2}$ to $10^{-8}$ (Hartman and Tomasz, 1984). The least frequent phenotype is homogeneous resistance, with a single population of cells that is inhibited by high levels of antibiotic concentration (de Lencastre et al, 1994).

### 2.4.3 Quinolones

Methicillin resistant *staphylococci* are associated with resistance to several commonly used antimicrobial agents. Fluoroquinolones exert their bactericidal activity by interacting with two types II topoisomerases, DNA gyrase (subunits encoded by *gyrA* and *gyrB*) and topoisomerase IV (subunits encoded by *grlA* and *grlB* for *S. aureus* and termed *parC* and *parE* for other organisms) (Hooper, 2001). Resistance to quinolones in *Staphylococci* arises primarily from mutations in the quinolone resistance-determining region (QRDR) of topoisomerase IV and/or DNA gyrase (Diskotto et al, 2001). However, the multidrug efflux pump *NorA* has also been shown to contribute to quinolone resistance (Kaatz et al, 1993).

### 2.4.4 Erythromycin

Erythromycin resistant *staphylococci* can be divided into two phenotypic classes based on their pattern of cross-resistance to other macrolides (MLS), lincosamides and type B streptogramins. Strains inducibly or constitutively resistant to all MLS antibiotics possess erythromycin ribosomal methylase (*erm*) genes, whereas strains inducibly resistant to only 14 and 15 membered ring macrolides and type B streptogramins harbour *msrA*, which encodes an ATP dependent efflux pump. Resistance to macrolides (e.g. erythromycin) can occur by two different mechanisms:
efflux due to macrolide streptogramin resistance (*msrA* gene) and ribosome alteration due to erythromycin ribosome methylase (*erm* gene) (Weisblum, 1999). Macrolide resistance due to efflux encoded by *msrA* has been more prevalent in CoNS than in *S. aureus* (Eady *et al*., 1993).

### 2.4.5 Aminoglycosides

Aminoglycosides are antibiotics, which are often used along with a β-lactamase stable penicillin (Isaacs, 2000). The anti-*staphylococcal* activity of aminoglycosides is considered important (Isaacs, 2003) as methicillin resistance is frequently observed among CoNS blood isolates (De Giusti *et al*., 1999). The main mechanism of aminoglycoside resistance is drug inactivation by aminoglycoside modifying enzymes (AMEs) encoded within mobile genetic elements (Ida *et al*., 2001). The following three AMEs are of particular significance among *staphylococci* since they modify and thereby inactivate the traditional aminoglycosides of therapeutic importance aminoglycoside-6'-N-acetyltransferase/2''-O-phosphoryltransferase [AAC (6')/APH (2'')], aminoglycoside-4'-O-nucleotidytransferase I [ANT (4')-I] and aminoglycoside-3'-O-phosphoryltransferase III [APH (3')-III (Schmitz *et al*., 1999). Variations in AME substrate specificity explain differences in antibacterial activity among the aminoglycosides (Livermore *et al*., 2001). The bifunctional enzyme AAC (6')/APH (2'') is the most frequently encountered AME in *staphylococcal* isolates and modifies to a different degree essentially all clinically available aminoglycosides, except streptomycin (Udo and Dashti, 2000).
2.4.6 Tetracycline

Two mechanisms of tetracycline resistance have been identified in *Staphylococcus* species: (i) active efflux resulting from the acquisition of the *tetK* and *tetL* genes located on a plasmid and (ii) ribosomal protection mediated by *tetM* or *tetO* determinants located on either a transposon or the chromosome (Warsa *et al.*, 1996).

2.4.7 Fusidic acid

Fusidic acid was first isolated from the fungus *Fusidium coccineum* (Godtfredsen and Jahnsen, 1962) and released on to the market in 1963 where is was used often in combination therapy together with methicillin and penicillin G (Alexander and Hutchinson, 1963). Resistance to fusidic acid is determined by a number of mechanisms. The best described are alterations in elongation factor G, which appear in natural mutants that are harbored at low rates in normal populations of *staphylococci* ($10^6$ to $10^8$). Altered drug permeability has also been described and appears to be plasmid-borne. Binding by chloramphenicol acetyltransferase type efflux and I are other described mechanisms of resistance whose prevalence is unclear (John and Peter, 1999).

2.4.8 Rifampicin

Rifampicin is a valuable antibiotic in combination therapy especially for deep-seated *staphylococcal* infections, owing to its excellent pharmacokinetic properties and bacteriocidal activity (Yao and Moellering, 1999). Resistance of rifampicin is due to chromosomal mutation in *ropB* gene which encoding the β subunit of RNA polymerase (Wichelhaus *et al.*, 1999) resulting in effective binding of the drug (Franklin, 1998).
2.4.9 Vancomycin

According to NCCLS for *Staphylococcus epidermidis* which has a MIC of \(\leq 4 \, \mu g/ml\) is considered vancomycin-susceptible, while that the MIC is 8 to 16 \(\mu g/ml\) are vancomycin intermediate (VIS) and those for which the MIC is \(\geq 32 \, \mu g/ml\) are vancomycin resistant (VRS). The reduced susceptibility to vancomycin appears to result from changes in peptidoglycan biosynthesis. The VIS strains are notable for the additional quantities of synthesized peptidoglycan that result in irregular shaped, thickened cell wall. There is also decreased cross-linking of peptidoglycan strands, which leads to the exposure of D-Ala–Ala residues (Hanaki *et al.*, 1998). The altered cross-linking results from reduced amount of L glutamine that are available for amidation of D-glutamine in the penta peptide bridge (Walsh and Howe, 2002). As a result there are more D-Ala-D-Ala residues available to bind and trap vancomycin. The bound vancomycin then acts as a further impediment to drug molecules reaching their target on the cytoplasmic membrane.

The second form of vancomycin resistance has resulted from the probable conjugal transfer of *vanA* operon from a vancomycin resistant *E. faecalis* (Showsh and Clewell, 2001). These VRS isolates demonstrate complete vancomycin resistance, with MIC of 128 \(\mu g/ml\). Resistance in these isolates is caused by alteration of the terminal peptide to D-Ala-D-Lac instead of D-Ala-D-Ala. Synthesis of D-Ala-D-Lac occurs only with exposure to low concentration of vancomycin. As a result, the additional biosynthetic demands are limited and the VRS strain is ecologically fit (Gonzalez and Courvalin, 2003).
2.4.10 Currently available agents

Quinupristin / dalfopristin, a combination of two streptogramins are currently available newer antimicrobial agents. It has been shown to have excellent activity in vitro against a broad range of *staphylococci* and *streptococci* including multi drug resistant strains (Jones *et al*, 1998). Resistance to streptogramin can develop through one of several mechanism- alteration of the target site (the most common mechanism), active transport or efflux mediated by an ATP binding protein and enzyme modification (Thal *et al*, 1999). Oxazolidinones are a new class of compounds with activity against resistant Gram positive organisms (Tsiodras *et al*, 2001). Resistance of linezolid is due to a mutation in the 23S rRNA at position 2576 in the peptidyl transferase centre of domain V (Wilson *et al*, 2003).

2.5 Laboratory diagnosis of methicillin resistant *Staphylococcus epidermidis*

Methicillin resistant *Staphylococcus* (MRS) requires timely detection in order to prevent infection and nosocomial transmission (Mellman *et al*, 2006). Health care workers and infection control personnel depend on the laboratory for the reliable detection of MRS in clinical specimens (Duckworth, 1993). Classical microbiologists routinely use culture based enrichment assays as the basis for detection and subsequent identification. The inclusion of an elevated salt concentration and specific antibiotics in the growth medium allows for highly specific detection (Wertheim *et al*, 2001). Molecular assays may offer benefits over more traditional culture-based assays, such as reduced time to identification and better specificity and sensitivity. The commercial diagnostics industry has introduced a range of molecular assays in recent years. However, there is little information available on their performance and
some studies suggest that they may suffer from reduced sensitivity as a result of sample inhibition (Donoso et al, 2007). Specificity may also be an issue as the presence of methicillin-resistant coagulase negative *staphylococci*, including methicillin-resistant *Staphylococcus epidermidis* (MRSE), which may cause false positive results. (www.qcmd.org).

The literature on methicillin susceptibility testing is extensive and often conflicting in recommendations regarding the most reliable method for routine use. This is partly because the various studies of phenotypic methods have included different strains, which may differ significantly in heterogeneity (Tomasz et al, 1991). Optimal conditions for detection of resistance vary among strains. No single set of test conditions is suitable for detection of all resistant strains. In assessing the performance of susceptibility testing methods the MIC determined by a dilution method has traditionally been the reference method; but methicillin MICs are affected by test conditions and some reports of erroneous results in studies of MRS detection methods may actually be due to failure to detect resistance with the reference MIC tests. MIC methods have now been replaced as the reference method by molecular methods, which detect the meca gene. Disk diffusion methods remain the most widely used in routine clinical laboratories, although some commercial systems for detection of methicillin resistance are available and automated methods are increasingly used (Brown and Walpole, 2001).

### 2.5.1 Disk diffusion test

Many different combinations of conditions have been recommended for disk diffusion. Standardized methods have however, been defined by the British Society for Antimicrobial Chemotherapy (BSAC) and the NCCLS (NCCLS, 2004). The
recent development of cefoxitin disk diffusion tests is likely to alter the recommendations for these methods as studies all suggest that tests with cefoxitin are more reliable than those with oxacillin (Felten et al., 2002). It is suggested that no special medium or incubation temperature is required with cefoxitin (Mougeot et al., 2001). Although some effect of temperature has been reported, there may be medium effects and the effects of inoculum have not been reported.

Oxacillin disk susceptibility testing was performed according to Clinical and Laboratory Standard Institute. Briefly a bacterial suspension adjusted to 0.5 MacFarland standard was inoculated into Muller Hinton agar media. A filter paper disk containing 1 µg oxacillin was placed on the inoculated Muller Hinton agar media. All plates were incubated in 35°C for 24 – 48 hrs. The diameter of zone of inhibition was measured and interpreted as follows

The strains of Staphylococcus epidermidis that showed zone of inhibition ≤17 mm around 1 micro gram Oxacillin disk was designated as MRSE (CLSI, 2007).

2.5.2 Oxacillin agar screening method

This method has been recommended for screening colonies isolated on routine media and for confirmation of suspected resistance seen in disk diffusion tests. The method recommended by the NCCLS (NCCLS, 2003) requires suspending the test organism to the density of a 0.5 McFarland standard and inoculating MH agar containing 4% NaCl and 6 mg/L oxacillin with a spot or a streak of the organism. Plates are incubated at 35°C or less for 24 h and any growth other than a single colony is indicative of resistance. This method is 95% sensitive for detection of coagulase negative Staphylococci (Mindler and Warner, 1987).
2.5.3 Broth microdilution

The NCCLS method, which requires the use of MH broth with 2% NaCl, an inoculum of $5 \times 10^5$ cfu/mL and incubation at 33–35°C for 24 hrs is the only defined method in general use (NCCLS, 2003).

2.5.4 E-test method

E-test (AB Biodisk) was used as a quantitative technique for determination of antimicrobial susceptibility and minimal inhibitory concentration (MIC). Mueller-Hinton medium supplemented with 2% NaCl was used for inoculation. Incubation time with E-test strips was 48 hrs for CoNS (Bolstrom et al., 1988). Evaluations comparing the E test with dilution MIC and molecular methods have generally found good essential agreement (Weller et al., 1997). The E test has an advantage over other MIC methods in that it is as easy to set up as a disk diffusion test (Huang et al., 1993).

2.5.5 Mannitol salt agar – cefoxitin screening medium

It is one of the recently developed screening media. In this media mannitol salt agar contain 4-mg/L cefoxitin and 3% NaCl salt concentration. The test is performed by direct $10^2$ CFU/ml colony-forming suspension inoculated on the agar plate and incubated for 24-48 hr at 35°C aerobically. Any growth on the plate containing cefoxitin considered as resistant to methicillin. The sensitivity and specificity of this method 100% over oxacillin agar screening method (Smyth and Gunnar, 2005).
2.5.6 Automated methods

Automated systems are widely used in clinical laboratories but they may lack accuracy for the detection of heterogeneously resistant isolates (Mackenzie et al, 1995). However, in the past few years, several reports have emphasized the performance characteristics of different rapid methods, such as the Rapid MicroScan panel (Baxter Microscan, West Sacramento, Calif.) (Woods et al, 1994) Phoenix (Becton Dickinson) and the Vitek system (bioMérieux Vitek, Inc., Hazelwood, Mo.) (Knapp et al, 1994). In particular, Knapp et al, showed the usefulness of the Vitek system for the detection of low-level-expression class isolates of Staphylococcus aureus and Staphylococcus epidermidis. However, these authors raised concerns over the accuracy of the Vitek system for detecting borderline susceptible isolate that lack mecA (Knapp et al, 1996). Moreover, the Vitek system may miss a significant number of coagulase-negative staphylococci that have the mecA gene and for which the oxacillin MICs are in the susceptible range (1 to 2 µg/ml) (Ramotar et al, 1996).

2.5.7 Quenching fluorescence method

With the Crystal MRSA method (Becton Dickinson) inhibition of growth of an isolate by oxacillin is indicated by the quenching of fluorescence of an oxygen sensitive fluorescent indicator by oxygen remaining in the broth. The method is reasonably reliable but requires several hours of incubation (Jureen et al, 2001). The sensitivity and specificity of the test were 54% and 100% for vials containing coagulase negative staphylococci (Kubina et al, 1999).
2.5.8 Molecular methods

The fact that high-level resistance to penicillinase resistant penicillins is generally related to the presence of the *mecA* gene means that a genotypic method for the detection of *mecA* allows rapid and unambiguous characterization of this resistance mechanism. The earliest molecular methods for the detection of *mecA* relied on either radiolabelled or digoxigenin (DIG)-labelled DNA probes (Archer and Pennell, 1990). The non-radioactive DIG-labelled probe performed as well as the radioactive label, enabling the safer utilization of the test system in a diagnostic laboratory, but even when used in either a dot blot or colony blot format, DNA probing involves a number of time-consuming manipulations resulting in delayed reporting (Ligozzi *et al.*, 1991). The “gold standard” for the detection of methicillin resistance is PCR or DNA hybridization of the *mecA* gene (Chambers, 1997). From detection of *mecA* by PCR, a single bacterial colony is obtained from a fresh subculture and is resuspended in 100 µl of sterile water. One micro liter of the suspension is added to each PCR mixture supplied by the manufacturer. The PCR program consists of a bacterial lysis and DNA denaturation step of 5 min at 95º C; 30 cycles with a 30-s annealing step at 42º C; and a 30-s extension at 72º C. The primer pair used (5’-CTC AGG TAC TGC TAT CCA CC-3’ and 5’ CAC TTG GTA TAT CTT CAC C-3’; Life Tecnologies, Rockville, Md), as described by Ryffel *et al.*, (1990) yielded a 533 –bp DNA fragment that was detected by 1% agarose gel electrophoresis with ethidium bromide staining under UV light.

2.6 Detection of biofilm

A number of tests are available in clinical laboratories to detect biofilm production by *Staphylococcus epidermidis*. Methods include Modified mirotiter plate test, (Srdjan *et al*.
al., 2000), Tissue culture plate method (Christensen et al., 1985), Tube method (Christensen et al., 1982) and Congo red agar test (Freeman et al., 1989). These methods are often subject to severe analytical limitations and are unable to detect bacterial adherence accurately (Raad et al., 1995).

### 2.6.1 Modified mirotiter plate method

The modified mirotiter plate test is the most frequently used technique for quantifying biofilm formation, which is an important indicator for the pathogenesis of *Staphylococcus epidermidis*. In this technique isolates from fresh agar plates were inoculated in Tryptic soy broth and incubated for 24 hour at 37°C in stationary condition. In brief three wells of a sterile 96-well flat-bottomed plastic tissue culture plates with a lid were filled with 200 µl of *Staphylococcus epidermidis* fresh culture suspension each. Record was kept on the sample inoculated each well. *S. aureus* ATCC 25923 was used as positive control. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 hrs at 37°C. Then the content of each well was aspirated and each well was washed three times with 250 µl of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200 µl of 99% methanol per well and after 15 minutes plates were emptied and left to dry. Then plates were stained for 5 minutes with 0.2 ml of 2% crystal violet used for Gram staining per well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 160 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm by using an enzyme immunosorbent assay reader. All strains were classified into the following categories: non adherent (0), weakly (+),
moderately (+++) or strongly (++++) adherent, based upon the OD of bacterial films. This test is superior to other tests in terms of objectivity and accuracy because it enables indirect measuring of bacteria attached both to the bottoms and walls of the wells, while in other tests only the dye bound to the bacteria attached to the bottom of the wells is spectrophotometrically registered (Srdjan et al., 2000).

2.6.2 Tissue culture plate test (TCP)

The Tissue culture plate (TCP) assay is the most widely used technique and is considered as standard test for detection of biofilm formation. Isolates from fresh agar plates were inoculated in respective media (trypsinase soy broth, trypsinase soy broth with 1% glucose and brain heart infusion broth with 2% sucrose) and incubated for 18 hour at 37°C in stationary condition and diluted 1 in 100 with fresh medium. Individual wells of sterile, polystyrene, 96 well flat bottom tissue culture plates wells were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. After incubation content of each well was gently removed by tapping the plates. The wells were washed with 0.2 ml of phosphate buffer saline (PBS pH 7.2) and fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by deionized water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 570 nm (OD_{570}). The TCP method was found to be the most sensitive, accurate and reproducible screening method for detection of biofilm formation by Staphylococci and has the advantage of being a quantitative model to study the adherence of staphylococci on biomedical devices (Christensen et al., 1985).
2.6.3 Tube method (TM)

By Tube method, qualitative assessment of biofilm formation is determined. In this test TSBglu (Tryptica soya broth with glucose) is inoculated with loopful of microorganism from overnight culture plates and 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 tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Biofilm detection by the tube method is easy to perform but it cannot discriminate between moderate and weakly biofilm producing isolates (Christensen et al, 1982).

2.6.4 Congo red agar method (CRA)

This is an alternative method for screening of biofilm formation by Staphylococcus epidermidis. This method requires the use of a specially prepared solid medium brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result. Most laboratories avoid this test because results of Congo red agar method do not correlate with other standard methods (Freeman et al, 1989).

2.7 Detection of hemolysin

Detection of hemolysin of S. epidermidis is done by following methods
2.7.1 The production of hemolysins is determined on plates containing blood agar base with 10% sheep blood incubated at 37°C for 24 hrs. A positive result is indicated by the formation of hemolysis zones around the isolated colonies. Formation of no hemolysis zones around the isolated colonies indicates negative result (Cheesbrough, 2000).

2.7.2 Hemolysins production is also determined on plates containing Tryptica soya agar supplemented with 5% sheep blood incubated at 37°C for 48 hrs. Positive result indicates formation of hemolysis zones around the isolated colonies. Formation of no hemolysis zones around the isolated colonies indicates negative result (Michelim et al, 2005).

2.8 Treatment of *Staphylococcus epidermidis* infection

Over the last decades, there has been an enormous increase and emergence of CoNS strains particularly *S.epidermidis* that are resistant to the antibiotic methicillin, especially in nosocomial settings. Methicillin resistance/multiple drug resistance has been documented more often in disease causing strains of *S. epidermidis* than in skin colonizing strains (Archer *et al*, 1994). Methicillin sensitive *S. epidermidis* strains should be treated with oxacillin or cefazolin or clindamicin. Methicillin resistance is equivalent to resistance to oxacillin, which is commonly used and extremely effective anti-staph drug. For treatment of MRSE infection, antibiotic susceptibility test in all potential cases is essential to determine the appropriate therapy. Before the results of susceptibility tests are available, decision of empirical therapy need to be made, based on the local resistance pattern, the patient’s response to any previous antibiotic therapy, the site of infection and the patient’s underlying clinical conditions. In
addition to initiating appropriate antibiotic therapy and/or drainage of purulent fluid/dischARGE are essential element of treatment. Though MRSE strains are frequently resistant to other antibiotic agents in clinical use, including other β-lacams, fluoroquinolones, aminoglycosides, rifampicin and mupirocin resistance rates to these antibiotics vary considerably between different centers and countries (Morgan et al., 1999). Thus, in centers where MRSE have become multidrug resistant, therapeutic options are limited. Often the glycopeptides (Vancomycin and Teicoplanin) are the only effective antibiotics remaining. This can present therapeutic difficulties when the infection is less accessible to drug penetration (e.g. Infection of CoNS in bone) or involves adherent bacteria (e.g. Endocarditis or infections developing on prosthetic materials). In the later situation, organisms that may be highly susceptible to the glycopeptides under standard in laboratory conditions are in fact tolerant in the biofilm environment, possibly due to a reduction in metabolic rate (Raad et al., 1998). As a result of poor extravascular penetration of the glycopeptides and the emergence of resistance to both vancomycin and teicoplanin in MRSE, there is an urgent need for effective alternative antimicrobials for the treatment of infections caused by this pathogen. A number of antibiotics currently under clinical development may be suitable; these include streptogramins, third generation fluoroquinolones (moxifloxacin, trovafloxacin), glyceryclines (GAR 936), oxazolidinones (linezolid) and carbapenems (Urban et al., 1996). Amoxycillin/clavulanic acid may also be of utility in MRSE infection; although at present only experimental data in an endocarditis model are available (Moreillion and Que, 2004).

MATERIALS AND METHODS
3.1 Place and period

The study was done in the Department of Microbiology, Mymensingh Medical College, during the period from July 2007 to June 2008. Cases were selected from in patient department of Mymensingh Medical College Hospital.

3.2 Type of the study

The study was designed as cross sectional study.

3.3 Study population

200 cases and 30 healthy controls were included in this study.

3.3.a Cases

A total of 200 patients (clinical isolates) irrespective of age and sex were selected from in patient department of Mymensingh Medical College Hospital on the basis of following criteria:

1. Surgical wound showing clinically no response to multiple courses of antibiotics.
2. Patient with infected skin lesion (Boil, Abscess, Cellulites).
3. Patient with stitch infection.
4. Patient with diabetic ulcer.
5. Patient with burn ulcer.
6. Patient with middle ear infection (Chronic suppurative otitis media).

3.3.b Controls

In control group swabs were taken from the forearms of 30 healthy volunteers irrespective of age and sex from Microbiology Department of Mymensingh Medical
College and Surgery Department of Mymensingh Medical College Hospital, having no history of disease within past 6 months. *Staphylococcus epidermidis* isolates obtained from control group represented as normal flora.

### 3.4 Data collection and analysis

Relevant history, clinical findings and laboratory records of every subject were recorded in a pre-designed data sheet (appendix I). Subsequently, statistical analysis of data was done by computer programme SPSS version 12.0.

### 3.5 Collection of specimens

Specimens comprising of wound swab, pus, exudates from diabetic ulcer, exudates from burn ulcer, aural swab and swab from stitch infection were collected with proper aseptic precaution. Before taking swab, the wounds were cleaned by normal saline and debris was removed. Swabs were collected by sterile swab sticks applying gentle rubbing and pus were also collected by immersing the swab stick into free flowing pus. Before taking aural swab there should be discontinuation of aural antibiotics or other aural chemotherapeutic agents for at least three hours. Then the swab stick was placed in the outer ear and rotated slowly and gently, avoiding trauma to the ear. Immediately after collection the swab stick was put into sterile test tube with proper labeling and carried to the laboratory for inoculation into culture media within two hours after collection.

### 3.6 Microbiological methods
3.6.1 Inoculation of specimens
All specimens were inoculated into nutrient agar, blood agar and mannitol salt agar media within two hours after collection. Inoculated media were incubated at 37°C for 24 hours aerobically.

3.6.2 Isolation and identification of organism
All the suspected colonies were subjected to gram staining for initial identification of organism according to their gram reaction and morphology. The isolates were identified as *S. epidermidis* on the basis of colony morphology on nutrient agar media, blood agar media and mannitol salt agar media and also by different biochemical tests.

3.6.2.1 Colony morphology
After 24 hours incubation, colony morphology was noted in different culture media.

3.6.2.1.a In nutrient agar the colonies those appeared white coloured, circular, having 2-4 mm in diameter, convex with shining surface were designated as *Staphylococcus epidermidis*.

3.6.2.1.b In blood agar gamma hemolysins (no hemolysis) have been detected in isolates of *S. epidermidis*.

3.6.2.1.c In mannitol salt agar *S. epidermidis* isolates failed to ferment mannitol and showed red or purple zone of colonies (Dugid, 1996).

3.6.2.2 Method of Gram’s staining
Gram’s staining was done for morphological identification of *Staphylococcus epidermidis*. A drop of distilled water was taken on the middle of a clear glass slide. Then a loopfull of bacterial suspension (young culture) was transferred with a sterilized inoculating wire loop on the drop of water. Then a very thin smear was prepared on the slide by spreading specimen uniformly. The smear was fixed by passing it over the flame for two or three times. The smear was flooded with crystal violet solution and allowed to stand for two minutes and then washed thoroughly with gentle stream of tap water. Then the slide was immersed in Gram iodine solution for 1 minute and washed with tap water. Then the smear was decolorized with 95% alcohol for 10 seconds. Alcohol was drained off and washed thoroughly with gentle stream of tap water. The slide was then counter stained with diluted carbol fuchsins (1:10) for 1 minute. After washing with tap water the slide was dried and examined under microscope (Cheesbrough, 2000).

### 3.6.2.3 Biochemical tests

Following biochemical tests were performed for suspected growth of *Staphylococcus epidermidis*.

#### 3.6.2.3.1 Catalase test

A small amount of culture was picked with a sterile toothpick and placed into a small, clean test tube containing 3% hydrogen peroxide solution. The production of gas bubbles on the surface of hydrogen peroxide was indicated as positive reaction. Known strain of *S. aureus* was used as positive control and *Streptococcus pyogenes* as negative control (Cheesbrough, 2000).

#### 3.6.2.3.2 Coagulase test
Human plasma was collected from transfusion bag and preserved in 15 ml screw capped test tube. Both slide and tube coagulase tests were done (Cheesbrough, 2000).

**3.6.3.2.a Slide Coagulate test**

Slide coagulation test was used to differentiate coagulate producing *Staphylococcus aureus* from coagulate negative. Coagulate causes plasma to clot by converting fibrinogen to fibrin. In this method, a drop of normal saline was placed in a slide. With minimum of spreading two colonies of test organism were mixed with it to form a smooth milky suspension. Then a drop of undiluted human plasma was added to that suspension. Coagulate positive strains were shown coarse visible clumping within 10 seconds. Known strain of Coagulate positive *S. aureus* was used as positive control.

**3.6.3.2.b Tube coagulate test**

Plasma was diluted as 1:10 with normal saline and 0.2 ml of diluted plasma was added to 0.8 ml of broth culture done in Brain Heart Infusion broth (Appendix-II) of the strain to be tested. The tubes were incubated at 37°C and observed for coagulation after 1, 3 and 6 hours. The conversion of plasma into a soft or stiff gel, best seen on tilting the tube to horizontal position was considered as positive. Both Coagulate positive and Coagulate negative strains were tested simultaneously as control.

**3.6.3.3 Oxidase test**

Objective

To distinguish *Staphylococcus* from *Micrococcus* species.

In this test, Oxidase discs from Himedia (DD018) were used to test the presence of the enzyme cytochrome oxidase. A loopful of bacterial cell from solid media was
brought in contact with the oxidase disc and the change in colour was observed within 60 seconds.

A positive reaction is characterized by a dark purple/blue colour and developed within one minute. No change of colour indicated negative reaction. Strains of *Staphylococcus epidermidis* did not change colour and were oxidase negative. Both *Pseudomonas spp* and *E. coli* were used as positive and negative control respectively (Tammey *et al*, 2007).

### 3.6.3.4 Mannitol fermentation test

Mannitol salt agar media was used to distinguish mannitol fermenter bacteria (*S. aureus*) from other bacteria. It is both selective and indicator media for *S. aureus*. *Staphylococcus epidermidis* failed to ferment mannitol and formed colonies with red or purple zone (Dugid, 1996).

### 3.6.3.5 Novobiocin susceptibility test

**Objective**

To distinguish *Staphylococcus epidermidis* from *Staphylococcus saprophyticus*.

Novobiocin susceptibility test was done in Muller Hinton agar medium. 5µg novobiocin disk in the usual disk diffusion test was done for antibiotic sensitivities. With an inoculum suspension equivalent in turbidity to a 0.5 McFarland opacity standard and incubated at 37°C for 24 hrs aerobically. *S. epidermidis* showed a large zone of inhibition of growth, e.g. over 15 mm in diameter around a 7 mm disk, whilst *S.saprophyticus* showed a much smaller zone or growth right up to the disk (Dugid, 1996).
3.6.3.6 Bacitracin susceptibility test

Objective

To distinguish *Staphylococcus* from *Micrococcus* species.

Bacitracin susceptibility test was done in Muller Hinton agar medium. Bacitracin disk in the usual disk diffusion test was done for antibiotic sensitivities. With an inoculum suspension equivalent in turbidity to a 0.5 McFarland opacity standard and incubated at 37°C for 24 hrs aerobically. A zone of inhibition ≥14 mm indicates susceptibility to bacitracin and a zone of inhibition <14 mm indicates resistant to bacitracin. *S. epidermidis* showed zone of inhibition of growth <14 mm in diameter. *Streptococcus pyogenes* ATCC 19615 and *Streptococcus aureus* ATCC 25923 were used as positive and negative control respectively (Tammey *et al*, 2007).

Bacterial growth which showed colony character and biochemical tests compatible with *Staphylococcus epidermidis* were considered for further procedure.

3.7 Maintenance and preservation of culture strains

Organisms grown in appropriate medium for 24 hours were preserved in a nutrient agar slant at 2-8°C in a refrigerator and this culture was used for two weeks for routine laboratory works. For long-term preservation selected and identified strains were stored in brain heart infusion broth with 16% glycerol and stored frozen without significant loss of viability at −20°C until further study (Cheesbrough, 2000).

3.8 Antimicrobial susceptibility test
Antimicrobial susceptibility test of the isolated organisms was done by disk diffusion method using the Kirby-Bauer technique (Bauer et al, 1966) and as per recommendation of CLSI, 2007.

### 3.8.1 Antimicrobial susceptibility test by disk diffusion method

All the isolated *Staphylococcus epidermidis* were put into antibiotic susceptibility test by Kirby-Bauer disk diffusion technique as per recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2007). Panel of antibiotics were used - namely penicillin, amoxycillin, cefuroxime, oxacillin, cloxacillin, cephradine, gentamicin, ceftriaxone, erythromycin, ciprofloxacin, rifampicin, fusidic acid and vancomycin. All tests were performed on Muller-Hinton agar (Appendix II) media. The surface was lightly and uniformly inoculated by sterile cotton swab stick. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standard (Appendix IV). The swab stick then took out and squeezed on the wall of the test tube to discard extra suspension. Inoculated plates were incubated at 35 to 37°C for 24 hours. On the next day, plates were read by taking measurement of zone of inhibition. Inhibition zones were measured in millimeter (mm) by using a ruler over the surface of the plate with the lid open. The plates were hold a few inches above a black, non-reflecting background and illuminated with reflected light. Results were recorded and graded as Resistant (R) and Sensitive (S) according to the reference zone of inhibition for particular antibiotic (CLSI, 2007).

### 3.8.1.2 Standardization of the disk
In order to standardize the disk potency, a representative disk was tested against the reference strains of *S. aureus* ATCC 25923. The zone of inhibition was compared with standard value as recommended by CLSI (2007).

### 3.8.1.3 Designation of MRSE

The strain of *Staphylococcus epidermidis* that showed zone of inhibition $\leq 17$ mm around 1 micro gram Oxacillin disk was designated as MRSE (CLSI, 2007). Oxacillin resistance was verified on Muller-Hinton agar media. The medium was prepared according to the manufacturer’s instruction. After autoclaving the medium, the medium was mixed well and poured into the sterile petri dishes. All plates were dried and cooled to solidify. Inoculums of bacteria was prepared as follows:

Three to five bacterial colonies were given in 3-4 ml of sterile normal saline, mixed well and adjusted with 0.5 McFarland standard. A sterile swab stick was soaked in this suspension, pressed gently against the wall of the test tube to squeeze extra suspension. Then the surface of the medium was uniformly inoculated by gentle swabbing and rotating the plate 60 degree for three times. Then Oxacillin 1 micro gram disk was applied to the agar surface by sterile disk holder and ensure that there was close contact between the disk and the surface of the medium. Then the plates were incubated at 35° C for 24 hours. On the following day the reading of zone of inhibitions were taken by measuring scale against good light. The result was interpretated as follows (CLSI, 2007)

- Zone of inhibition $\geq 18$ mm was considered as sensitive (S).
- Zone of inhibition $\leq 17$ mm was considered as resistant (R).

### 3.9 Detection of biofilm formation by Modified microtiter-plate test
Modified micotiter-plate test was done to detect the ability of *Staphylococcus epidermidis* to form biofilm according to the method discussed by Srdjan *et al*, 2000. In this technique isolates from fresh agar plates were inoculated in tryptic soy broth (TSB) and incubated for 24 hour at 37°C in stationary condition. In brief three wells of a sterile 96 well flat-bottomed plastic tissue culture plate with a lid were filled with 200 µl of *Staphylococcus epidermidis* fresh culture suspension each. Record was kept on the sample inoculated each well. *S. aureus* ATCC 25923 was used as positive control. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 hrs at 37°C. Then, the content of each well was aspirated and each well was washed three times with 250 µl of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200 µl of 99% methanol per well and after 15 minutes plates were emptied and left to dry. Then, plates were stained for 5 minutes with 0.2 ml of 2% crystal violet used for Gram staining per well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 160 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm by using an enzyme immunosorbent assay reader. All strains were classified into the following categories: non adherent (0), weakly (+), moderately (+++) or strongly (+++) adherent, based upon the OD of bacterial films. The cut-off OD (OD<sub>K</sub>) for the modified microtiter plate test was three standard deviations above the mean OD of the negative control (OD<sub>C</sub>). Any OD above the cut-off value was considered as positive for biofilm formation as follows

**Calculation formula**
<table>
<thead>
<tr>
<th>Formula</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD ≤ ODₖ</td>
<td>Non adherent</td>
</tr>
<tr>
<td>ODₖ &lt; OD ≤ 2ODₖ</td>
<td>Weakly adherent</td>
</tr>
<tr>
<td>2ODₖ &lt; OD ≤ 4ODₖ</td>
<td>Moderately adherent</td>
</tr>
<tr>
<td>4ODₖ &lt; OD</td>
<td>Strongly adherent</td>
</tr>
</tbody>
</table>

Here, $ODₖ = \text{Mean } OD_C + 3SD \text{ of } OD_C$

All tests were carried out three times and results were averaged.

### 3.10 Determination of hemolysin production

The production of hemolysins was determined on plates containing blood agar base with 10% sheep blood incubated at 37°C for 24 hrs. A positive result was indicated by the formation of hemolysis zones around the isolated colonies. Formation of no hemolysis zones around the isolated colonies was indicated as negative result. *Staphylococcus epidermidis* formed no hemolysis zone around the isolated colony (Cheesbrough 2000).
RESULTS

A total of 62 *Staphylococcus epidermidis* were isolated from 230 specimens. Among them 32 *Staphylococcus epidermidis* were isolated from cases and 30 were from healthy controls. Different findings and observations were shown in this section.

**Table no 1.** Shows culture positivity of different isolates from clinical specimens and healthy controls. Clinical specimens comprising of 140 (70%) culture positive isolates and 60 (30%) culture negative strains. All 30 (100%) isolates from control group were culture positive for *Staphylococcus epidermidis*.

**Figure no 1.** Shows culture positivity of different isolates from clinical specimens and healthy controls.

**Table no 2.** Shows distribution of culture positive bacterial isolates from case group. Total 140 isolates were culture positive. Among them 32 (23%) were *Staphylococcus epidermidis*, 68 (49%) were *Staphylococcus aureus*, 27 (19%) were *Pseudomonas spp* and 13 (9%) were *E. coli*. In healthy control group comprising of 30 (100%) culture positive isolates which were *Staphylococcus epidermidis*.

**Figure no 2.** Shows distribution of culture positive bacterial isolates from case group.

**Table no 3.** Shows distribution of culture positive bacterial isolates from cases as per specimens. Total isolates of *S. epidermidis* were 32 (23%) from cases. Of which 11 (15%) were from surgical wound swab, 12 (20%) from pus of skin infection, 06
(17%) were from stitch infection swab, 01 (08%) were from aural swab, 01 (09%) were from burn ulcer exudates and 01 (14%) from diabetic ulcer exudates.

Isolates of *S. aureus* were 68 (49%) from cases. Of which 27 (36%) were from surgical wound swab, 24 (40%) from pus of skin infection, 08 (23%) were from stitch infection swab, 05 (42%) were from aural swab, 03 (27%) were from burn ulcer exudates and 01 (14%) from diabetic ulcer exudates.

Isolates of *Pseudomonas spp* were 27 (19%) from cases. Of which 10 (13%) were from surgical wound swab, 13 (22%) from pus of skin infection, 03 (27%) were from burn ulcer exudates and 01 (14%) from diabetic ulcer exudates.

Isolates of *Escherichia coli* were 13 (9%) from cases. Of which 09 (12%) were from surgical wound swab, 03 (05%) from pus of skin infection, 01 (09%) were from burn ulcer exudates.

**Figure no 3.** Shows distribution of *Staphylococcus epidermidis* as per clinical specimens.

**Table no 4.** Shows MRSE detected by disk diffusion method. Out of 32 *Staphylococcus epidermidis* from different clinical specimens, 18 (56.25%) isolates were detected as MRSE by disk diffusion method.

**Figure no 4.** Shows the detection rate of MRSE and MSSE by disk diffusion method.
Table 1. Culture positivity of different isolates from clinical specimens and healthy controls

<table>
<thead>
<tr>
<th>Source of specimens</th>
<th>Culture positive</th>
<th>Culture negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimens from cases</td>
<td>140 (70)</td>
<td>60 (30)</td>
</tr>
<tr>
<td>(n=200)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimens from controls</td>
<td>30 (100)</td>
<td>00 (100)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total n=230</td>
<td>170 (74)</td>
<td>60 (26)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 1. Culture positivity of different isolates from clinical specimens and healthy controls

Table 2. Distribution of culture positive bacterial isolates from case group
<table>
<thead>
<tr>
<th>Name of culture positive isolates</th>
<th>No. of culture positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>32 (23)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>68 (49)</td>
</tr>
<tr>
<td><em>Pseudomonas spp.</em></td>
<td>27 (19)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>13 (9)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages

**Figure 2. Distribution of culture positive bacterial isolates from case group**

**Table 3. Distribution of culture positive bacterial isolates from cases as per specimens (n=140)**
<table>
<thead>
<tr>
<th>Clinical Specimen</th>
<th>Count</th>
<th>Percentage</th>
<th>Count</th>
<th>Percentage</th>
<th>Count</th>
<th>Percentage</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical wound swab (n=75)</td>
<td>11 (15)</td>
<td>27 (36)</td>
<td>10 (13)</td>
<td>9 (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pus from skin infection (n=60)</td>
<td>12 (20)</td>
<td>24 (40)</td>
<td>13 (22)</td>
<td>3 (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swab from stitch infections (n=35)</td>
<td>6 (17)</td>
<td>8 (23)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aural swab (n=12)</td>
<td>1 (8)</td>
<td>5 (42)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exudates from burn ulcer (n=11)</td>
<td>1 (9)</td>
<td>3 (27)</td>
<td>3 (27)</td>
<td>1 (9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exudates from diabetic ulcer (n=7)</td>
<td>1 (14)</td>
<td>1 (14)</td>
<td>1 (14)</td>
<td>0 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (n=140)</td>
<td>32 (23)</td>
<td>68 (49)</td>
<td>27 (19)</td>
<td>13 (9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages

Figure 3. Distribution of *Staphylococcus epidermidis* as per clinical specimens
Table 4. Detection of MRSE by disk diffusion method

<table>
<thead>
<tr>
<th>Total number of <em>S. epidermidis</em> (isolated from clinical specimens)</th>
<th>MRSE detected by disk diffusion method (DDM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>

DDM= Disk diffusion method.

Figures in the parentheses indicate percentages.
Figure 4. Detection rate of MRSE and MSSE by DDM

Table no 5. Shows specimen wise proportion of *Staphylococcus epidermidis* and MRSE by disk diffusion method. Majority 07 (64%) of MRSE detected by disk diffusion method were isolated from surgical wound swab, followed by pus of skin infection 06 (50%), 04 (67%) were from stitch infection swab and 1 (100%) from diabetic ulcer exudates.

Figure no 5. Shows specimen wise proportion of MRSE by disk diffusion method.

Table no 6. Shows Antimicrobial susceptibility pattern of 32 *Staphylococcus epidermidis* isolated from different clinical specimens. Highest numbers (94%) of isolates were resistant to penicillin, followed by oxacillin (56%), cloxacillin (56%), gentamicin (44%), erythromycin (41%), Doxycycline (37%), cephradine (34%), ciprofloxacin (28%), ceftriaxone (28%), fusidic acid (22%) and cefuroxime (19%). All isolates of *Staphylococcus epidermidis* from cases were susceptible to rifampicin and vancomycin.
**Figure no 6.** Shows Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from different clinical specimens.

**Table 5. Specimen wise proportion of *Staphylococcus epidermidis* and MRSE by disk diffusion method**

<table>
<thead>
<tr>
<th>Type of specimens</th>
<th><em>S. epidermidis</em></th>
<th>MRSE by DDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical wound swab</td>
<td>11</td>
<td>07 (64)</td>
</tr>
<tr>
<td>Pus from skin infection</td>
<td>12</td>
<td>06 (50)</td>
</tr>
<tr>
<td>Swab from stitch infection</td>
<td>06</td>
<td>04 (67)</td>
</tr>
<tr>
<td>Aural swab</td>
<td>01</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Exudates from burn ulcer</td>
<td>01</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Exudates from diabetic ulcer</td>
<td>01</td>
<td>01 (100)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>32</strong></td>
<td><strong>18 (56.25)</strong></td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 5. Specimen wise proportion of MRSE by DDM

Table 6. Antimicrobial susceptibility pattern of *S. epidermidis* isolated from different clinical specimens (n= 32).

<table>
<thead>
<tr>
<th>Name of Antibiotics</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>2 (6)</td>
<td>30 (94)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>14 (44)</td>
<td>18 (56)</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>14 (44)</td>
<td>18 (56)</td>
</tr>
<tr>
<td>Cephradine</td>
<td>21 (66)</td>
<td>11 (34)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>18 (56)</td>
<td>14 (44)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>23 (72)</td>
<td>09 (28)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>20 (63)</td>
<td>12 (37)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>23 (72)</td>
<td>09 (28)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>19 (59)</td>
<td>13 (41)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>26 (81)</td>
<td>6 (19)</td>
</tr>
<tr>
<td>Medication</td>
<td>Susceptibility</td>
<td>Resistance</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>------------</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>32 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>32 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>25 (78)</td>
<td>7 (22)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages

**Figure 6.** Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from different clinical specimens.

**Table no 7.** Shows Antimicrobial susceptibility pattern of 30 *Staphylococcus epidermidis* isolated from control group. All isolates of *Staphylococcus epidermidis* from control group were susceptible to all antibiotics except penicillin, which was only 10% resistant.

**Figure no 7.** Shows Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from control group.

**Table no 8.** Shows pattern of Antimicrobial resistance among MRSE. 100% isolates of MRSE were resistant to penicillin, amoxycillin, oxacillin, cloxacillin, followed by
gentamicin (56%), erythromycin (50%), Doxycycline (44%), cephradine (44%), ciprofloxacin (39%), fusidic acid (33%), cefuroxime (33%) and ceftriaxone (28%). All isolates of MRSE were susceptible to rifampicin and vancomycin.

**Figure no 8.** Shows pattern of Antimicrobial resistance among MRSE isolates.

**Table no 9.** Shows prevalence of biofilm production of *Staphylococcus epidermidis*.

Among 32 clinical isolates of *Staphylococcus epidermidis*, 13 (40.62%) produced biofilm. Of which 09 (28.12%) were weak adherence and 04 (12.5%) were moderate adherence in nature. None of the isolate was found positive for strong adherence. In 30 healthy controls, 5 (16.66%) *Staphylococcus epidermidis* produced biofilm, which were weak adherence in nature.

**Figure no 9.** Shows prevalence of biofilm production of *Staphylococcus epidermidis* in case and control groups.

**Table 7. Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from healthy controls**

<table>
<thead>
<tr>
<th>Name of Antibiotic</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicilllin</td>
<td>27 (90)</td>
<td>03 (10)</td>
</tr>
<tr>
<td>Oxacilllin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cloxacilllin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cephradine</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Drug</td>
<td>Susceptibility</td>
<td>Resistance</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>------------</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages

Figure 7. Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from healthy controls
Table 8. Pattern of Antimicrobial resistance among MRSE

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Antibiotics</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>18 (100)</td>
<td>Doxycycline</td>
<td>08 (44)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>18 (100)</td>
<td>Ciprofloxacin</td>
<td>07 (39)</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>18 (100)</td>
<td>Erythromycin</td>
<td>09 (50)</td>
</tr>
<tr>
<td>Cephradine</td>
<td>08 (44)</td>
<td>Cefuroxime</td>
<td>06 (33)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 (56)</td>
<td>Vancomycin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>18 (100)</td>
<td>Rifampicin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>05 (28)</td>
<td>Fusidic acid</td>
<td>06 (33)</td>
</tr>
</tbody>
</table>
Figures in the parentheses indicate percentages

**Figure 8. Pattern of Antimicrobial resistance among MRSE.**

**Table 9. Prevalence of biofilm production of *Staphylococcus epidermidis* isolated from case and control groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>No adherence (0)</th>
<th>Weak adherence (+)</th>
<th>Moderate adherence (++)</th>
<th>Strong adherence (+++)</th>
<th>Prevalence of biofilm production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case (n=32)</td>
<td>19 (59.37)</td>
<td>09 (28.12)</td>
<td>04 (12.5)</td>
<td>00 (00)</td>
<td>13 (40.62)</td>
</tr>
<tr>
<td>Control (n=30)</td>
<td>25 (83.33)</td>
<td>05 (16.66)</td>
<td>00 (00)</td>
<td>00 (00)</td>
<td>05 (16.66)</td>
</tr>
<tr>
<td>Total (n = 62)</td>
<td>44 (70.96)</td>
<td>14 (22.58)</td>
<td>04 (6.45)</td>
<td>00 (00)</td>
<td>18 (29.03)</td>
</tr>
</tbody>
</table>
Figures in the parentheses indicate percentages

Figure 9. Prevalence of biofilm production of *Staphylococcus epidermidis* isolated from case and control groups

**Table no 10.** Overall biofilm production of *Staphylococcus epidermidis* isolated from case and control groups. In case group, 13 (40.62%) *Staphylococcus epidermidis* produced biofilm. In healthy control group, 5 (16.66%) *Staphylococcus epidermidis* produced biofilm.
**Figure no 10.** Overall biofilm production of *Staphylococcus epidermidis* isolated from case and control groups.

**Table no 11.** Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by Modified microtiter plate test. In this test sensitivity, specificity, positive predictive value and negative predictive value were 40.62%, 83.33%, 72.22% and 56.81% respectively.

**Figure no 11.** Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by Modified microtiter plate test.

**Table no 12.** Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from case and control groups. Among 32 clinical isolates and 30 isolates from control group, no hemolysin production was detected.

**Figure no 12.** Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from case group.

**Figure no 13.** Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from control group.
Table 10. Overall biofilm production of *Staphylococcus epidermidis* isolated from case and control groups

<table>
<thead>
<tr>
<th>Test Results</th>
<th>Case</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive biofilm production</td>
<td>13 (40.62)</td>
<td>05 (16.66)</td>
<td>18 (29.03)</td>
</tr>
<tr>
<td>Negative biofilm production</td>
<td>19 (59.37)</td>
<td>25 (83.33)</td>
<td>44 (70.96)</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>30</td>
<td>62</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentages.

Detection of biofilm production of *S. epidermidis* isolated from case group is significantly different from that of control group.

Chi – squire value = 4.31, p value = <0.05, degree of freedom (df) = 1
Table 11. Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by modified microtiter-plate test

<table>
<thead>
<tr>
<th>Test Results</th>
<th>Case</th>
<th>Control</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive biofilm production</td>
<td>13 (a)</td>
<td>05 (b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative biofilm production</td>
<td>19 (c)</td>
<td>25 (d)</td>
<td>40.62</td>
<td>83.33</td>
<td>72.22</td>
<td>56.81</td>
</tr>
<tr>
<td>Total</td>
<td>32 (a+c)</td>
<td>30 (b+d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

65
N.B.: Sensitivity, Specificity, Positive predictive value and negative predictive value were calculated by the following formula

\[ a = \text{True positive}, \ b = \text{False positive}, \ c = \text{False negative} \text{ and } d = \text{True negative}. \]

Sensitivity = \( \frac{a}{a+c} \times 100 = 40.62\% \)

Specificity = \( \frac{d}{b+d} \times 100 = 83.33\% \)

Positive predictive value = \( \frac{a}{a+b} \times 100 = 72.22\% \)

Negative predictive value = \( \frac{d}{c+d} \times 100 = 56.81\% \)
Figure 11. Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by modified microtiter-plate test

Table 12. Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from case and control groups
<table>
<thead>
<tr>
<th>Source of specimens</th>
<th>No hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em> isolated from cases</td>
<td>32 (100)</td>
</tr>
<tr>
<td>(n=32)</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em> isolated from controls</td>
<td>30 (100)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
</tr>
<tr>
<td>Total (n=62)</td>
<td>62 (100)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages.
Figure 12. Hemolytic property of *Staphylococcus epidermidis* isolated from case group
Figure 13. Hemolytic property of *Staphylococcus epidermidis* isolated from control group
MATERIALS AND METHODS

3.1 Place and period

The study was done in the Department of Microbiology, Mymensingh Medical College, during the period from July 2007 to June 2008. Cases were selected from in patient department of Mymensingh Medical College Hospital.

3.2 Type of the study

The study was designed as cross sectional study.

3.3 Study population

200 cases and 30 healthy controls were included in this study.

3.3.a Cases

A total of 200 patients (clinical isolates) irrespective of age and sex were selected from in patient department of Mymensingh Medical College Hospital on the basis of following criteria:

1. Surgical wound showing clinically no response to multiple courses of antibiotics.
2. Patient with infected skin lesion (Boil, Abscess, Cellulites).
3. Patient with stitch infection.
4. Patient with diabetic ulcer.
5. Patient with burn ulcer.
6. Patient with middle ear infection (Chronic suppurative otitis media).
3.3.b Controls

In control group swabs were taken from the forearms of 30 healthy volunteers irrespective of age and sex from Microbiology Department of Mymensingh Medical College and Surgery Department of Mymensingh Medical College Hospital, having no history of disease within past 6 months. *Staphylococcus epidermidis* isolates obtained from control group represented as normal flora.

3.4 Data collection and analysis

Relevant history, clinical findings and laboratory records of every subject were recorded in a pre-designed data sheet (appendix I). Subsequently, statistical analysis of data was done by computer programme SPSS version 12.0.

3.5 Collection of specimens

Specimens comprising of wound swab, pus, exudates from diabetic ulcer, exudates from burn ulcer, aural swab and swab from stitch infection were collected with proper aseptic precaution. Before taking swab, the wounds were cleaned by normal saline and debris was removed. Swabs were collected by sterile swab sticks applying gentle rubbing and pus were also collected by immersing the swab stick into free flowing pus. Before taking aural swab there should be discontinuation of aural antibiotics or other aural chemotherapeutic agents for at least three hours. Then the swab stick was placed in the outer ear and rotated slowly and gently, avoiding trauma to the ear. Immediately after collection the swab stick was put into sterile test tube with proper labeling and carried to the laboratory for inoculation into culture media within two hours after collection.
3.6 Microbiological methods

3.6.1 Inoculation of specimens

All specimens were inoculated into nutrient agar, blood agar and mannitol salt agar media within two hours after collection. Inoculated media were incubated at 37°C for 24 hours aerobically.

3.6.2 Isolation and identification of organism

All the suspected colonies were subjected to gram staining for initial identification of organism according to their gram reaction and morphology. The isolates were identified as *S. epidermidis* on the basis of colony morphology on nutrient agar media, blood agar media and mannitol salt agar media and also by different biochemical tests.

3.6.2.1 Colony morphology

After 24 hours incubation, colony morphology was noted in different culture media.

3.6.2.1.a In nutrient agar the colonies those appeared white coloured, circular, having 2-4 mm in diameter, convex with shining surface were designated as *Staphylococcus epidermidis*.

3.6.1.b In blood agar gamma hemolysins (no hemolysis) have been detected in isolates of *S. epidermidis*.

3.6.1.c In mannitol salt agar *S. epidermidis* isolates failed to ferment mannitol and showed red or purple zone of colonies (Dugid, 1996).
3.6.2.2 Method of Gram’s staining

Gram’s staining was done for morphological identification of *Staphylococcus epidermidis*. A drop of distilled water was taken on the middle of a clear glass slide. Then a loopfull of bacterial suspension (young culture) was transferred with a sterilized inoculating wire loop on the drop of water. Then a very thin smear was prepared on the slide by spreading specimen uniformly. The smear was fixed by passing it over the flame for two or three times. The smear was flooded with crystal violet solution and allowed to stand for two minutes and then washed thoroughly with gentle stream of tap water. Then the slide was immersed in Gram iodine solution for 1 minute and washed with tap water. Then the smear was decolorized with 95% alcohol for 10 seconds. Alcohol was drained off and washed thoroughly with gentle stream of tap water. The slide was then counter stained with diluted carbol fuchsin (1:10) for 1 minute. After washing with tap water the slide was dried and examined under microscope (Cheesbrough, 2000).

3.6.2.3 Biochemical tests

Following biochemical tests were performed for suspected growth of *Staphylococcus epidermidis*.

3.6.2.3.1 Catalase test

A small amount of culture was picked with a sterile tooth pick and placed into a small, clean test tube containing 3% hydrogen per oxide solution. The production of gas bubbles on the surface of hydrogen per oxide was indicated as positive reaction.
Known strain of *S. aureus* was used as positive control and *Streptococcus pyogenes* as negative control (Cheesbrough, 2000).

### 3.6.2.3.2 Coagulase test

Human plasma was collected from transfusion bag and preserved in 15 ml screw capped test tube. Both slide and tube coagulase tests were done (Cheesbrough, 2000).

#### 3.6.3.2.a Slide Coagulase test

Slide coagulation test was used to differentiate coagulase producing *Staphylococcus aureus* from coagulase negative. Coagulase causes plasma to clot by converting fibrinogen to fibrin. In this method, a drop of normal saline was placed in a slide. With minimum of spreading two colonies of test organism were mixed with it to form a smooth milky suspension. Then a drop of undiluted human plasma was added to that suspension. Coagulase positive strains were shown coarse visible clumping within 10 seconds. Known strain of Coagulase positive *S. aureus* was used as positive control.

#### 3.6.3.2.b Tube coagulase test

Plasma was diluted as 1:10 with normal saline and 0.2 ml of diluted plasma was added to 0.8 ml of broth culture done in Brain Heart Infusion broth (Appendix-II) of the strain to be tested. The tubes were incubated at 37°C and observed for coagulation after 1, 3 and 6 hours. The conversion of plasma into a soft or stiff gel, best seen on tilting the tube to horizontal position was considered as positive. Both Coagulase positive and Coagulase negative strains were tested simultaneously as control.

### 3.6.3.3 Oxidase test

Objective
To distinguish *Staphylococcus* from *Micrococcus* species.

In this test, Oxidase discs from Himedia (DD018) were used to test the presence of the enzyme cytochrome oxidase. A loopful of bacterial cell from solid media was brought in contact with the oxidase disc and the change in colour was observed within 60 seconds.

A positive reaction is characterized by a dark purple/blue colour and developed within one minute. No change of colour indicated negative reaction. Strains of *Staphylococcus epidermidis* did not change colour and were oxidase negative. Both *Pseudomonas spp* and *E. coli* were used as positive and negative control respectively (Tammey *et al.*, 2007).

### 3.6.3.4 Mannitol fermentation test

Mannitol salt agar media was used to distinguish mannitol fermenter bacteria (*S. aureus*) from other bacteria. It is both selective and indicator media for *S. aureus*. *Staphylococcus epidermidis* failed to ferment mannitol and formed colonies with red or purple zone (Dugid, 1996).

### 3.6.3.5 Novobiocin susceptibility test

**Objective**

To distinguish *Staphylococcus epidermidis* from *Staphylococcus saprophyticus*.

Novobiocin susceptibility test was done in Muller Hinton agar medium. 5µg novobiocin disk in the usual disk diffusion test was done for antibiotic sensitivities. With an inoculum suspension equivalent in turbidity to a 0.5 McFarland opacity standard and incubated at 37°C for 24 hrs aerobically. *S. epidermidis* showed a large zone of inhibition of growth, e.g. over 15 mm in diameter around a 7 mm disk, whilst
S. saprophyticus showed a much smaller zone or growth right up to the disk (Dugid, 1996).

### 3.6.3.6 Bacitracin susceptibility test

**Objective**

To distinguish *Staphylococcus* from *Micrococcus* species.

Bacitracin susceptibility test was done in Muller Hinton agar medium. Bacitracin disk in the usual disk diffusion test was done for antibiotic sensitivities. With an inoculum suspension equivalent in turbidity to a 0.5 McFarland opacity standard and incubated at 37°C for 24 hrs aerobically. A zone of inhibition ≥14 mm indicates susceptibility to bacitracin and a zone of inhibition <14 mm indicates resistant to bacitracin. *S. epidermidis* showed zone of inhibition of growth <14 mm in diameter. *Streptococcus pyogenes* ATCC 19615 and *Streptococcus aureus* ATCC 25923 were used as positive and negative control respectively (Tammey et al, 2007).

Bacterial growth which showed colony character and biochemical tests compatible with *Staphylococcus epidermidis* were considered for further procedure.

### 3.7 Maintenance and preservation of culture strains

Organisms grown in appropriate medium for 24 hours were preserved in a nutrient agar slant at 2-8°C in a refrigerator and this culture was used for two weeks for routine laboratory works. For long-term preservation selected and identified strains were stored in brain heart infusion broth with 16% glycerol and stored frozen without significant loss of viability at –20 °C until further study (Cheesbrough, 2000).
3.8 Antimicrobial susceptibility test

Antimicrobial susceptibility test of the isolated organisms was done by disk diffusion method using the Kirby-Bauer technique (Bauer et al., 1966) and as per recommendation of CLSI, 2007.

3.8.1 Antimicrobial susceptibility test by disk diffusion method

All the isolated *Staphylococcus epidermidis* were put into antibiotic susceptibility test by Kirby-Bauer disk diffusion technique as per recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2007). Panel of antibiotics were used - namely penicillin, amoxycillin, cefuroxime, oxacillin, cloxacillin, cephradine, gentamicin, ceftriaxone, erythromycin, ciprofloxacin, rifampicin, fusidic acid and vancomycin. All tests were performed on Muller-Hinton agar (Appendix II) media. The surface was lightly and uniformly inoculated by sterile cotton swab stick. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standard (Appendix IV). The swab stick then took out and squeezed on the wall of the test tube to discard extra suspension. Inoculated plates were incubated at 35 to 37°C for 24 hours. On the next day, plates were read by taking measurement of zone of inhibition. Inhibition zones were measured in millimeter (mm) by using a ruler over the surface of the plate with the lid open. The plates were hold a few inches above a black, non-reflecting background and illuminated with reflected light. Results were recorded and graded as Resistant (R) and Sensitive (S) according to the reference zone of inhibition for particular antibiotic (CLSI, 2007).
3.8.1.2 Standardization of the disk

In order to standardize the disk potency, a representative disk was tested against the reference strains of *S. aureus* ATCC 25923. The zone of inhibition was compared with standard value as recommended by CLSI (2007).

3.8.1.3 Designation of MRSE

The strain of *Staphylococcus epidermidis* that showed zone of inhibition ≤ 17 mm around 1 micro gram Oxacillin disk was designated as MRSE (CLSI, 2007). Oxacillin resistance was verified on Muller-Hinton agar media. The medium was prepared according to the manufacturer’s instruction. After autoclaving the medium, the medium was mixed well and poured into the sterile petri dishes. All plates were dried and cooled to solidify. Inoculum of bacteria was prepared as follows:

Three to five bacterial colonies were given in 3-4 ml of sterile normal saline, mixed well and adjusted with 0.5 McFarland standard. A sterile swab stick was soaked in this suspension, pressed gently against the wall of the test tube to squeeze extra suspension. Then the surface of the medium was uniformly inoculated by gentle swabbing and rotating the plate 60 degree for three times. Then Oxacillin 1 micro gram disk was applied to the agar surface by sterile disk holder and ensure that there was close contact between the disk and the surface of the medium. Then the plates were incubated at 35° C for 24 hours. On the following day the reading of zone of inhibitions were taken by measuring scale against good light. The result was interpreted as follows (CLSI, 2007)

Zone of inhibition ≥ 18 mm was considered as sensitive (S).

Zone of inhibition ≤ 17 mm was considered as resistant (R).
3.9 Detection of biofilm formation by Modified microtiter-plate test

Modified micotiter-plate test was done to detect the ability of *Staphylococcus epidermidis* to form biofilm according to the method discussed by Srdjan *et al*, 2000. In this technique isolates from fresh agar plates were inoculated in tryptic soy broth (TSB) and incubated for 24 hour at 37°C in stationary condition. In brief three wells of a sterile 96 well flat-bottomed plastic tissue culture plate with a lid were filled with 200 µl of *Staphylococcus epidermidis* fresh culture suspension each. Record was kept on the sample inoculated each well. *S. aureus* ATCC 25923 was used as positive control. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 hrs at 37°C. Then, the content of each well was aspirated and each well was washed three times with 250 µl of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200 µl of 99% methanol per well and after 15 minutes plates were emptied and left to dry. Then, plates were stained for 5 minutes with 0.2 ml of 2% crystal violet used for Gram staining per well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 160 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm by using an enzyme immunosorbent assay reader. All strains were classified into the following categories: non adherent (0), weakly (+), moderately (++) or strongly (+++) adherent, based upon the OD of bacterial films. The cut-off OD (ODₖ) for the modified microtiter plate test was three standard deviations above the mean OD of the negative control (ODₐ). Any OD above the cut-off value was considered as positive for biofilm formation as follows
Calculation formula

<table>
<thead>
<tr>
<th>Formula</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( OD \leq OD_K )</td>
<td>Non adherent</td>
</tr>
<tr>
<td>( OD_K &lt; OD \leq 2OD_K )</td>
<td>Weakly adherent</td>
</tr>
<tr>
<td>( 2OD_K &lt; OD \leq 4OD_K )</td>
<td>Moderately adherent</td>
</tr>
<tr>
<td>( 4OD_K &lt; OD )</td>
<td>Strongly adherent</td>
</tr>
</tbody>
</table>

Here, \( OD_K = \text{Mean } OD_C + 3\text{SD of } OD_C \)

All tests were carried out three times and results were averaged.

### 3.10 Determination of hemolysin production

The production of hemolysins was determined on plates containing blood agar base with 10% sheep blood incubated at 37°C for 24 hrs. A positive result was indicated by the formation of hemolysis zones around the isolated colonies. Formation of no hemolysis zones around the isolated colonies was indicated as negative result. *Staphylococcus epidermidis* formed no hemolysis zone around the isolated colony (Cheesbrough 2000).
RESULTS

A total of 62 *Staphylococcus epidermidis* were isolated from 230 specimens. Among them 32 *Staphylococcus epidermidis* were isolated from cases and 30 were from healthy controls. Different findings and observations were shown in this section.

**Table no 1.** Shows culture positivity of different isolates from clinical specimens and healthy controls. Clinical specimens comprising of 140 (70%) culture positive isolates and 60 (30%) culture negative strains. All 30 (100%) isolates from control group were culture positive for *Staphylococcus epidermidis*.

**Figure no 1.** Shows culture positivity of different isolates from clinical specimens and healthy controls.

**Table no 2.** Shows distribution of culture positive bacterial isolates from case group. Total 140 isolates were culture positive. Among them 32 (23%) were *Staphylococcus epidermidis*, 68 (49%) were *Staphylococcus aureus*, 27 (19%) were *Pseudomonas spp* and 13 (9%) were *E. coli*. In healthy control group comprising of 30 (100%) culture positive isolates which were *Staphylococcus epidermidis*.

**Figure no 2.** Shows distribution of culture positive bacterial isolates from case group.

**Table no 3.** Shows distribution of culture positive bacterial isolates from cases as per specimens. Total isolates of *S. epidermidis* were 32 (23%) from cases. Of which 11 (15%) were from surgical wound swab, 12 (20%) from pus of skin infection, 06
(17%) were from stitch infection swab, 01 (08%) were from aural swab, 01 (09%) were from burn ulcer exudates and 01 (14%) from diabetic ulcer exudates.

Isolates of *S. aureus* were 68 (49%) from cases. Of which 27 (36%) were from surgical wound swab, 24 (40%) from pus of skin infection, 08 (23%) were from stitch infection swab, 05 (42%) were from aural swab, 03 (27%) were from burn ulcer exudates and 01 (14%) from diabetic ulcer exudates.

Isolates of *Pseudomonas spp* were 27 (19%) from cases. Of which 10 (13%) were from surgical wound swab, 13 (22%) from pus of skin infection, 03 (27%) were from burn ulcer exudates and 01 (14%) from diabetic ulcer exudates.

Isolates of *Escherichia coli* were 13 (9%) from cases. Of which 09 (12%) were from surgical wound swab, 03 (05%) from pus of skin infection, 01 (09%) were from burn ulcer exudates.

**Figure no 3.** Shows distribution of *Staphylococcus epidermidis* as per clinical specimens.

**Table no 4.** Shows MRSE detected by disk diffusion method. Out of 32 *Staphylococcus epidermidis* from different clinical specimens, 18 (56.25%) isolates were detected as MRSE by disk diffusion method.

**Figure no 4.** Shows the detection rate of MRSE and MSSE by disk diffusion method.
Table 1. Culture positivity of different isolates from clinical specimens and healthy controls

<table>
<thead>
<tr>
<th>Source of specimens</th>
<th>Culture positive</th>
<th>Culture negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimens from cases</td>
<td>140 (70)</td>
<td>60 (30)</td>
</tr>
<tr>
<td>(n=200)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimens from controls</td>
<td>30 (100)</td>
<td>00 (100)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total n=230</td>
<td>170 (74)</td>
<td>60 (26)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 1. Culture positivity of different isolates from clinical specimens and healthy controls
Table 2. Distribution of culture positive bacterial isolates from case group

<table>
<thead>
<tr>
<th>Name of culture positive isolates</th>
<th>No. of culture positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>32 (23)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>68 (49)</td>
</tr>
<tr>
<td><em>Pseudomonas spp.</em></td>
<td>27 (19)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>13 (9)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 2. Distribution of culture positive bacterial isolates from case group
Table 3. Distribution of culture positive bacterial isolates from cases as per specimens (n=140)

<table>
<thead>
<tr>
<th>Type of specimens</th>
<th>S. epidermidis</th>
<th>S. aureus</th>
<th>Pseudomonas spp</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical wound swab (n=75)</td>
<td>11 (15)</td>
<td>27 (36)</td>
<td>10 (13)</td>
<td>9 (12)</td>
</tr>
<tr>
<td>Pus from skin infection (n=60)</td>
<td>12 (20)</td>
<td>24 (40)</td>
<td>13 (22)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Swab from stitch infections (n=35)</td>
<td>6 (17)</td>
<td>8 (23)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Aural swab (n=12)</td>
<td>1 (8)</td>
<td>5 (42)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Exudates from burn ulcer (n=11)</td>
<td>1 (9)</td>
<td>3 (27)</td>
<td>3 (27)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Exudates from diabetic ulcer (n=7)</td>
<td>1 (14)</td>
<td>1 (14)</td>
<td>1 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total (n=140)</td>
<td>32 (23)</td>
<td>68 (49)</td>
<td>27 (19)</td>
<td>13 (9)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 3. Distribution of *Staphylococcus epidermidis* as per clinical specimens
Table 4. Detection of MRSE by disk diffusion method

<table>
<thead>
<tr>
<th>Total number of <em>S. epidermidis</em> (isolated from clinical specimens)</th>
<th>MRSE detected by disk diffusion method (DDM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Percentage</td>
</tr>
<tr>
<td>32</td>
<td>18</td>
</tr>
</tbody>
</table>

DDM= Disk diffusion method.

Figures in the parentheses indicate percentages.
Figure 4. Detection rate of MRSE and MSSE by DDM
Table no 5. Shows specimen wise proportion of *Staphylococcus epidermidis* and MRSE by disk diffusion method. Majority 07 (64%) of MRSE detected by disk diffusion method were isolated from surgical wound swab, followed by pus of skin infection 06 (50%), 04 (67%) were from stitch infection swab and 1 (100%) from diabetic ulcer exudates.

Figure no 5. Shows specimen wise proportion of MRSE by disk diffusion method.

Table no 6. Shows Antimicrobial susceptibility pattern of 32 *Staphylococcus epidermidis* isolated from different clinical specimens. Highest numbers (94%) of isolates were resistant to penicillin, followed by oxacillin (56%), cloxacillin (56%), gentamicin (44%), erythromycin (41%), Doxycycline (37%), cephradine (34%), ciprofloxacin (28%), ceftriaxone (28%), fusidic acid (22%) and cefuroxime (19%). All isolates of *Staphylococcus epidermidis* from cases were susceptible to rifampicin and vancomycin.

Figure no 6. Shows Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from different clinical specimens.
Table 5. Specimen wise proportion of *Staphylococcus epidermidis* and MRSE by disk diffusion method

<table>
<thead>
<tr>
<th>Type of specimens</th>
<th>S. epidermidis</th>
<th>MRSE by DDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical wound swab</td>
<td>11</td>
<td>07 (64)</td>
</tr>
<tr>
<td>Pus from skin infection</td>
<td>12</td>
<td>06 (50)</td>
</tr>
<tr>
<td>Swab from stitch infection</td>
<td>06</td>
<td>04 (67)</td>
</tr>
<tr>
<td>Aural swab</td>
<td>01</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Exudates from burn ulcer</td>
<td>01</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Exudates from diabetic ulcer</td>
<td>01</td>
<td>01 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>18 (56.25)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 5. Specimen wise proportion of MRSE by DDM
Table 6. Antimicrobial susceptibility pattern of *S. epidermidis*
isolated from different clinical specimens (n= 32).

<table>
<thead>
<tr>
<th>Name of Antibiotics</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>2 (6)</td>
<td>30 (94)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>14 (44)</td>
<td>18 (56)</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>14 (44)</td>
<td>18 (56)</td>
</tr>
<tr>
<td>Cephradine</td>
<td>21 (66)</td>
<td>11 (34)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>18 (56)</td>
<td>14 (44)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>23 (72)</td>
<td>09 (28)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>20 (63)</td>
<td>12 (37)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>23 (72)</td>
<td>09 (28)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>19 (59)</td>
<td>13 (41)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>26 (81)</td>
<td>6 (19)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>32 (100)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>32 (100)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>25 (78)</td>
<td>7 (22)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 6. Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from different clinical specimens.
Table no 7. Shows Antimicrobial susceptibility pattern of 30 *Staphylococcus epidermidis* isolated from control group. All isolates of *Staphylococcus epidermidis* from control group were susceptible to all antibiotics except penicillin, which was only 10% resistant.

Figure no 7. Shows Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from control group.

Table no 8. Shows pattern of Antimicrobial resistance among MRSE. 100% isolates of MRSE were resistant to penicillin, amoxycillin, oxacillin, cloxacillin, followed by gentamicin (56%), erythromycin (50%), Doxycycline (44%), cephradine (44%), ciprofloxacin (39%), fusidic acid (33%), cefuroxime (33%) and ceftriaxone (28%). All isolates of MRSE were susceptible to rifampicin and vancomycin.

Figure no 8. Shows pattern of Antimicrobial resistance among MRSE isolates.

Table no 9. Shows prevalence of biofilm production of *Staphylococcus epidermidis*. Among 32 clinical isolates of *Staphylococcus epidermidis*, 13 (40.62%) produced biofilm. Of which 09 (28.12%) were weak adherence and 04 (12.5%) were moderate adherence in nature. None of the isolate was found positive for strong adherence. In 30 healthy controls, 5 (16.66%) *Staphylococcus epidermidis* produced biofilm, which were weak adherence in nature.

Figure no 9. Shows prevalence of biofilm production of *Staphylococcus epidermidis* in case and control groups.
Table 7. Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from healthy controls

<table>
<thead>
<tr>
<th>Name of Antibiotic</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>27 (90)</td>
<td>03 (10)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cephradine</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30 (100)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>30 (100)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 7. Antimicrobial susceptibility pattern of *Staphylococcus epidermidis* isolated from healthy controls
Table 8. Pattern of Antimicrobial resistance among MRSE

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Antibiotics</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>18 (100)</td>
<td>Doxycycline</td>
<td>08 (44)</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>18 (100)</td>
<td>Ciprofloxacin</td>
<td>07 (39)</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>18 (100)</td>
<td>Erythromycin</td>
<td>09 (50)</td>
</tr>
<tr>
<td>Cephradine</td>
<td>08 (44)</td>
<td>Cefuroxime</td>
<td>06 (33)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 (56)</td>
<td>Vancomycin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>18 (100)</td>
<td>Rifampicin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>05 (28)</td>
<td>Fusidic acid</td>
<td>06 (33)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 8. Pattern of Antimicrobial resistance among MRSE.
Table 9. Prevalence of biofilm production of *Staphylococcus epidermidis* isolated from case and control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No adherence (0)</th>
<th>Weak adherence (+)</th>
<th>Moderate adherence (++)</th>
<th>Strong adherence (+++)</th>
<th>Prevalence of biofilm production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case (n= 32)</td>
<td>19 (59.37)</td>
<td>09 (28.12)</td>
<td>04 (12.5)</td>
<td>00 (00)</td>
<td>13 (40.62)</td>
</tr>
<tr>
<td>Control (n=30)</td>
<td>25 (83.33)</td>
<td>05 (16.66)</td>
<td>00 (00)</td>
<td>00 (00)</td>
<td>05 (16.66)</td>
</tr>
<tr>
<td>Total (n = 62)</td>
<td>44 (70.96)</td>
<td>14 (22.58)</td>
<td>04 (6.45)</td>
<td>00 (00)</td>
<td>18 (29.03)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages
Figure 9. Prevalence of biofilm production of *Staphylococcus epidermidis* isolated from case and control groups.
Table no 10. Overall biofilm production of *Staphylococcus epidermidis* isolated from case and control groups. In case group, 13 (40.62%) *Staphylococcus epidermidis* produced biofilm. In healthy control group, 5 (16.66%) *Staphylococcus epidermidis* produced biofilm.

Figure no 10. Overall biofilm production of *Staphylococcus epidermidis* isolated from case and control groups.

Table no 11. Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by Modified microtiter plate test. In this test sensitivity, specificity, positive predictive value and negative predictive value were 40.62%, 83.33%, 72.22% and 56.81% respectively.

Figure no 11. Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by Modified microtiter plate test.

Table no 12. Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from case and control groups. Among 32 clinical isolates and 30 isolates from control group, no hemolysin production was detected.

Figure no 12. Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from case group.

Figure no 13. Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from control group.
Table 10. Overall biofilm production of *Staphylococcus epidermidis* isolated from case and control groups

<table>
<thead>
<tr>
<th>Test Results</th>
<th>Case</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive biofilm production</td>
<td>13 (40.62)</td>
<td>05 (16.66)</td>
<td>18 (29.03)</td>
</tr>
<tr>
<td>Negative biofilm production</td>
<td>19 (59.37)</td>
<td>25 (83.33)</td>
<td>44 (70.96)</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>30</td>
<td>62</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentages.

Detection of biofilm production of *S. epidermidis* isolated from case group is significantly different from that of control group.

Chi – squire value = 4.31, p value = <0.05, degree of freedom (df) = 1
Figure 10. Overall biofilm production of *Staphylococcus epidermidis* isolated from case and control groups
Table 11. Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by modified microtiter-plate test

<table>
<thead>
<tr>
<th>Test Results</th>
<th>Case</th>
<th>Control</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive biofilm production</td>
<td>13 (a)</td>
<td>05 (b)</td>
<td>40.62</td>
<td>83.33</td>
<td>72.22</td>
<td>56.81</td>
</tr>
<tr>
<td>Negative biofilm production</td>
<td>19 (c)</td>
<td>25 (d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>32 (a+c)</td>
<td>30 (b+d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.B.: Sensitivity, Specificity, Positive predictive value and negative predictive value were calculated by the following formula

\[ a = \text{True positive}, \ b = \text{False positive}, \ c = \text{False negative} \text{ and } d = \text{True negative}. \]

Sensitivity = \( \frac{a}{a+c} \times 100 = 40.62\% \)

Specificity = \( \frac{d}{b+d} \times 100 = 83.33\% \)

Positive predictive value = \( \frac{a}{a+b} \times 100 = 72.22\% \)

Negative predictive value = \( \frac{d}{c+d} \times 100 = 56.81\% \)
Figure 11. Sensitivity, specificity, positive predictive value and negative predictive value of biofilm production of *Staphylococcus epidermidis* by modified microtiter-plate test
Table 12. Prevalence of hemolysin production of *Staphylococcus epidermidis* isolated from case and control groups

<table>
<thead>
<tr>
<th>Source of specimens</th>
<th>No hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em> isolated from cases (n=32)</td>
<td>32 (100)</td>
</tr>
<tr>
<td><em>S. epidermidis</em> isolated from controls (n=30)</td>
<td>30 (100)</td>
</tr>
<tr>
<td>Total (n=62)</td>
<td>62 (100)</td>
</tr>
</tbody>
</table>

Figures in the parentheses indicate percentages.
Figure 12. Hemolytic property of *Staphylococcus epidermidis* isolated from case group
Figure 13. Hemolytic property of *Staphylococcus epidermidis* isolated from control group
List of references


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