CHAPTER 1

1. INTRODUCTION

1.1 *Staphylococcus aureus*

Staphylococcus aureus is one of the most prevalent and clinically significant pathogens worldwide, causing a variety of illnesses ranging from mild superficial skin eruptions to life-threatening infections with bacteremia, endocarditis, pneumonia and toxic shock syndrome (Lowy, 1998). *S. aureus* is notorious for its ability to become resistant to antibiotics. Infections caused by *S. aureus* used to respond to β-lactam and related group of antibiotics but the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has posed a serious therapeutic challenge. In the 1940s, medical treatment for *S. aureus* infections became routine and successful with the discovery and introduction of antibiotics, such as penicillin (Lowy, 2003).

Methicillin was introduced in 1959 to treat the infections caused by penicillin resistant *S. aureus*. Methicillin-resistant *S. aureus* (MRSA) was first identified in 1961 (Jevons et al., 1963) and has become the most common cause of nosocomial and community infections worldwide (Deresinski, 2005). In 1961, there were reports from the United Kingdom of *S. aureus* isolates that had acquired resistance to methicillin (methicillin-resistant *S. aureus*, MRSA). MRSA isolates were soon recovered from other European countries, and later from Japan, Australia, and the United States (Jevons, 1961 & Barrett et al., 1968). Infections that are caused by antibiotic-resistant strains often occur in epidemic waves initiated by one or a few successful clones.

S. aureus is a member of commensal resident or transient microflora and readily colonizes the anterior nares in humans. Healthcare workers act as important sources for transmission of many bacterial agents including MRSA. Many infections caused by *S. aureus* occur in persons with prior nasal carriage. This carriage is an important risk factor for nosocomial *S. aureus* infections among patients undergoing surgery, hemodialysis, implantation of intravascular devices, and among HIV-infected patients (Kluytmans et al., 1997).

Historically MRSA associated with hospitals and other healthcare settings, has now emerged as a widespread cause of community infections also. Community-associated MRSA (CA-MRSA) can spread rapidly among healthy individuals. Outbreaks of CA-MRSA infections have been reported worldwide, although the prevalence of CA-MRSA varies geographically (Boucher et al., 2008).

The prevalence of MRSA infections has been increasingly reported. The increase in the incidence of infections due to *S. aureus* is partially a consequence of advances in patient care and also due to pathogen's ability to adapt to a changing environment. Infection due to *S. aureus* imposes a high and increasing burden on healthcare resources. A growing concern is the emergence of MRSA infections in patients with no apparent risk factors. MRSA in tonsils may serve as a potential source for the spread of these organisms to other body sites as well as to other individuals. The growing problem in the Indian scenario is that MRSA prevalence has increased from 12% in 1992 to 80.83% in 1999 (Verma et al., 2000). Prevalence of MRSA in Nepal has increased significantly in last two decades. Studies from various hospitals reported the prevalence ranging from 15.4%-69% (Rai et al., 1990, Subedi et al., 2005, Tiwari et al., 2009). MRSA is prevalent in many hospitals and often reflects the difficulties in hospitals and the health service generally, in terms of the control and prevention of healthcare-associated infection (Humphreys, 2008). Multidrug resistant bacteria, such as MRSA, are endemic in healthcare settings in the United States and many other countries of the world. Nosocomial transmission of MRSA serves as a source of hospital outbreaks. Reports of vancomycin resistant *S. aureus* strains in the United States emphasize the need for better control of MRSA and other multidrug resistant bacteria within healthcare settings (Henderson, 2006).

Despite high prevalence of MRSA in developing countries, studies on clinical and economic impact are limited. Infections caused by MRSA increase cost of healthcare, duration of hospitalization and sometimes intensive care admission resulting into additional financial burden.

The methicillin resistance gene (*mecA*) encodes a methicillin-resistant penicillin-binding protein that is not present in susceptible strains and is believed to have been acquired from a distantly related species. *mecA* is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), which has four forms differing in size and genetic composition.

More recently, a divergent form of the *mecA* gene, known as *mecC* (previously mecALGA251), was identified in isolates from both animals and humans (Garcia et al., 2011). Many MRSA isolates are multidrug resistant and are susceptible only to glycopeptide antibiotics such as vancomycin and investigational drugs. Decreasing susceptibility to glycopeptides has been reported and is a matter of great concern for public health (Hiramatsu et al., 1997 & CDC, 1997). Treatment options for MRSA infections include doxycycline, minocycline, clindamycin, trimethoprim-sulfamethoxazole (TMP-SMX), chloramphenicol, rifampin and linezolid. Macrolides, lincosamides and streptogramin B (MLSB) antibiotics act by inhibiting bacterial protein synthesis.

Lincosamides like clindamycin were promising option for clinicians to treat skin and soft tissue infections caused by MRSA. Emergence of clindamycin resistance during therapy created doubt among the clinicians for continuation of the therapy. Resistance to macrolides among *S. aureus* is either by target site modification or by the action of an energy dependent pump that expels the drug from the cell of bacteria (Leclercq, 2002). The MLSB resistance results from reduction of affinity due to N-6 –dimethylation of an adenine residue in the 23S rRNA. Methylation of 23S rRNA is encoded by *ermC* and *ermA* genes (Roberts et al., 1999).

1.1.1 Panton Valentine leukocidin (*PVL*)

Pathogenicity of *S. aureus* is related to a number of virulence factors that allow organism to adhere, avoid the immune system and cause harmful effects to the host. One of the important cytotoxin produced by some strains of *S. aureus* is Panton Valentine leukocidin (*PVL*), encoded by two genes, *lukS-PV* and *lukF-PV* responsible for leukocyte destruction and tissue necrosis (Genestier et al., 2005). Panton Valentine leukocidin is a member of the synergohymenotropic toxin family that induces pores in the membranes of cells. *PVL* was named after Sir Philip Noel Panton and Francis Valentine who associated it with soft tissue infections in 1932 (Prevost et al., 1995 & Panton et al., 1932). *PVL* producing MRSA usually cause mild skin or soft tissue infections, however, severe cases of necrotizing pneumonia and other deep seated infections such as infective endocarditis, osteomyelitis, enterocolitis and sepsis have also been reported (Maltezou et al., 2006). It is present in majority of community associated MRSA isolates and rarely present in hospital isolates (Vandenesch et al., 2003). Epidemiological data suggest that high virulence of community acquired MRSA is associated with *PVL* genes but direct evidence of association of *PVL* to pathogenesis has been limited (Li et al., 2010). Some reports have suggested that, certain strains of CA-MRSA may be more virulent than HA-MRSA (Boyle et al., 2007 & Said-Salim et al., 2003). Experimental studies on animals have demonstrated that, *PVL* positive strains were more virulent as compared to *PVL* negative strains (Diep et al., 2010).

The strains *PVL* positive CA-MRSA have spread throughout the world. The prevalence of *PVL* among *S. aureus* and MRSA isolates from various clinical specimens has been found to vary significantly with geographical area and population. The high prevalence of *PVL* has been reported (57%) from the isolates of West African countries compared to 9.7% in England (Breurec et al., 2010 & Shallcross et al., 2010). Global increase in the prevalence of *PVL* among community MRSA isolates is a matter of great concern and challenge for the treating clinicians. The prevalence of *PVL* genes among MRSA isolates has not been adequately reported from Nepal.

1.1.2 Biofilm

Biofilm is a complex structure consisting of colonies of bacteria that secrete mucilaginous protective coating. It contains viable and nonviable microorganisms that adhere to the surface and are trapped within a matrix of extracellular polymeric substance (EPS). Biofilm formation is one of the most important defense mechanisms of microbes against host defenses and the effect of antimicrobial agents (Fischbach et al., 2009 & Rodvoldet al., 2014). Bacteria and fungi are known to produce biofilm and *S. aureus* is considered as one of the important biofilm producing human pathogens. Biofilm-associated microbial infections are usually chronic and frequently occur in hospitals. Staphylococci including *S. aureus* are known to form biofilm on various biomaterials. Staphylococci can persist in clinical setting and gain increased resistance to antimicrobials by formation of biofilm. Formation of biofilm is believed to be an important contributory factor in microbial survival *in vivo* and *in vitro*. The polysaccharide intracellular adhesion, encoded by *ica* genes has been shown to be associated with biofilm formation by Staphylococci (De Silva et al., 2002). Role of *α*-toxin in biofilm formation has also been demonstrated (Caiazza et al., 2003).

Biofilm formation is a multistep process starting with transient adherence to a surface. Subsequently, specific bacterial adhesins, referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) promote the actual attachment (Foster et al., 1998). During the accumulation phase, bacteria stick to each other and production of extracellular polymeric substances (EPS) and/or incorporation of host derived components, such as platelets, takes place, resulting in a mature biofilm. Development of biofilm in *S. aureus* is the controlled by genetic locus Staphylococcal accessory regulator (sarA), which controls the intracellular adhesin (ica) operon and agr regulated pathways (O'Gara, 2007). Biofilm formation in methicillin-resistant *S. aureus* (MRSA) is predominantly regulated by surface adhesins, which are repressed under agr expression. On the other hand, biofilm formation in methicillin-susceptible *S. aureus* (MSSA) is dependent more on cell to cell adhesion by the production of icaADBC-encoded polysaccharide intercellular adhesin (PIA), also referred as poly-N-acetyl

glucosamine (PNAG) or slime (O'Neill et al., 2007). However, the role of the ica locus of *S. aureus* is not as clear as that of *Staphylococcus epidermidis* (Izano et al., 2008). Bacteria embedded in biofilms are often difficult to eradicate with standard antibiotics and more resistant to host immune responses (Patel, 2005). Biofilm producing MRSA is known to be more difficult to control, providing greater resistance to antibacterial agents than MRSA not embedded in biofilm.

1.2 Rationale

S. aureus is notorious among bacterial pathogens for its ability to develop resistance to antibiotics. Infections caused by antibiotic-resistant strains are more severe and difficult to treat. Methicillin-resistant *S. aureus* (MRSA) is a major human pathogen worldwide and infections are associated with increased morbidity and mortality as compared to other bacterial pathogens.

It is important to understand the trends in the antibiotic resistance pattern of MRSA in a given hospital setting periodically to minimize the irrational use of antibiotics like vancomycin when other antibiotics could be prescribed. The findings of this study would be beneficial in formulating policies to combat the challenges of treatment and control of MRSA infections. Association of *PVL* among MRSA isolates may increase the virulence of the organism and difficult to treat. Detection of *PVL* genes among MRSA isolates would help to differentiate community acquired MRSA from hospital acquired MRSA.

Methicillin-resistant *S. aureus* (MRSA) is well established nosocomial pathogen and frequently colonizes healthcare workers. Screening of healthcare workers for MRSA may reduce the nosocomial transmission of MRSA among patients and hospital staff. Similarly, sampling from hospital environment sometimes may be useful to trace the source of infection and also in preventing spread of potential pathogen. Determination of antibiotic resistance profile of the pathogens isolated from various units guides clinician to start empirical therapy in suspected cases of hospital acquired infections.

Increasing number of prosthetic implants urges the need of strategies to combat the biofilm forming bacteria including *S. aureus*. Molecular characterization of MRSA would be helpful for epidemiological studies, understanding pathogenesis, virulence factors, classification of community and hospital acquired MRSA, treatment strategies and in the development of newer drugs.

1.3 Objectives

1.3.1 General objective

To study Phenotypic and molecular characterization of methicillin resistant *S. aureus* isolates from various clinical specimens and hospital environment.

1.3.2 Specific objectives

1) To isolate and identify *S. aureus strains* from various clinical specimens and hospital environment.

2) To perform antibiotic susceptibility testing of the isolates.

3) To determine Minimal Inhibitory Concentration (MIC) of cefoxitin.

4) To perform biofilm assay among *S. aureus*/MRSA isolates.

5) To perform genotypic characterization of drug resistant genes pertaining to antimicrobial resistance (*mecA* gene) and detection of Panton Valentine leukocidin (*PVL*) genes among MRSA isolates.

6) To explore correlation between phenotypic and genotypic characteristic features pertaining to antimicrobial resistance.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Staphylococci

Staphylococci, Micrococci and Stomatococci belong to family Micrococcaceae. Staphylococci are Gram positive, non-motile, non-sporing, occasionally capsulated, aerobic or facultative anaerobic with exception of one species *Staphylococcus saccharolyticus* which is a true anaerobe. Staphylococci divide incompletely in three perpendicular planes to form pairs, tetrads, short chains and clusters. The arrangement of the cocci helps to distinguish Micrococci and Staphylococci from Streptococci, which usually grow in chains.

Genus Staphylococcus consists of 36 species and 18 subspecies (Gotz, 2006). The majorities of species are harmless and inhabit normally on skin and mucus membranes of human and animals. Staphylococci are worldwide in distribution and form small component of soil microflora. Staphylococci cause wide variety of clinical illness among human population ranging from mild skin infections to severe life threatening illnesses depending upon virulence of infective strain, anatomical site of infection and immune status of the host. *S. aureus* is considered as the most common species associated with human infections while *S. epidermidis* and *S. saprophyticus* are opportunistic pathogens.

2.1.1 Taxonomy

DNA-ribosomal RNA (rRNA) hybridization and comparative oligonucleotide analysis of 16S rRNA has demonstrated that Staphylococci form a coherent group at the genus level. Taxonomy of Staphylococci is based on 16S rRNA sequences and most of the Staphylococcal species fall in 11 clusters. These include: *S. aureus* group, *S. auricularis* group, *S. carnosus* group, *S. epidermidis* group, *S. hemolyticus* group, *S. hycus* group, *S. lugdunensis* group, *S. saprophyticus* group, *S. sciuri* group, *S. simulans* group and *S. warneri* group.

2.1.2 Historical review of Staphylococci

Staphylococcus was first identified in 1880 in Aberdeen, Scotland, by Alexander Ogston in pus sample from surgical abscess of knee joint. He operated the abscess of one of his patients, collected pus, made a stained smear and examined under a microscope.

"My delight may be conceived when there were revealed to me beautiful tangles, tufts and chains of round organisms in great numbers, which stood out clear and distinct among the pus cells and debris" (Elek, 1959)

Based on microscopic observations, Ogston hypothesized that acute abscesses were caused by Micrococci. He injected pus from acute abscesses into guinea pigs and mice, demonstrated that new abscesses were formed, followed by signs of septicemia. Ogston reported his results during the Ninth Surgical Congress in Berlin (1880) and firmly established Staphylococci as the etiological agent of suppurative abscess (Ogston, 1881). In 1884, Anton J. Rosenbach, a German surgeon, isolated two strains of Staphylococci, which he named based on pigmented colonies on solid culture media: *S. aureus* (from the Latin aurum for gold) and *Staphylococcus albus*, now called *Staphylococcus epidermidis* (from the Latin albus for white) (Rosenbach, 1884).

2.2 *Staphylococcus aureus*

S. aureus are Gram positive cocci, one micrometer in diameter and form grape like clusters. On blood or nutrient agar, after overnight incubation at 37ºC, it forms colonies of size 1-3 mm in diameter. Colonies are smooth, low convex, glistening, densely surrounded by zone of hemolysis on blood agar. Many strains produce beta hemolysis while some of the strains are non-hemolytic also. Apart from hemolysis, pigmentation is another important characteristic of *S. aureus*, ranges from cream to golden yellow on nutrient agar. This property of pigment production helps in the differentiation of *S. aureus* from other Staphylococci. *S. aureus* can tolerate high concentration of sodium chloride (up to 10%) that is inhibitory to many of other bacteria (Becton & Dickinson,

2005). On mannitol salt agar (MSA) and MacConkey's agar, the colonies are yellow and pink due to acid production by fermentation mannitol and lactose.

2.3 Biochemical characteristics

S. aureus ferments a range of sugars including mannitol which has diagnostic importance. *S. aureus* produces number of enzymes such as catalase, coagulase, gelatinase, alkaline phosphatase, Esterase, staphylokinase, proteinase, hyaluronidase, deoxyribonucleases (DNase) and compounds like acetoin.

2.4 Resistance to physical and chemical agents

S. aureus possess high degree of resistance to physical and number of chemical agents among the non-spore forming bacteria. They survive well in the environment under both moist and dry conditions for variable duration. *S. aureus* can survive many months on nutrient agar slopes under refrigeration. It withstands moist heat at 60°C for 30 minutes. It is readily killed by phenolic and hypochlorite disinfectants. It is sensitive to antiseptic preparations such as hexachlorophene, chlorhexidine, and providone-iodine. Antibiotic resistant strains are believed to have less susceptibility to some of these agents than ordinary strains (McLure et al., 1992).

2.5 Extracelluar product

Virulence of *S. aureus* results from combined effect of many factors such as extracellular proteins and polysaccharides, expressed during infection. *S. aureus* express surface proteins that facilitate attachment of the organism to host proteins such as laminin and fibronectin to form extracellular matrix. Fibronectin is present on epithelial and endothelial surfaces as well as a component of blood clots. In addition, most strains express a fibrinogen/fibrin binding protein (the clumping factor) which promotes attachment to blood clots and traumatized tissue.

2.5.1 Capsular polysaccharide

The majority of clinical isolates of *S. aureus* express a surface polysaccharide of either serotype 5 or 8. This is called a microcapsule because it can be visualized only by electron microscopy after antibody labeling, unlike the copious capsules of other bacteria which are visualized by light microscopy. *S. aureus* isolated from infections expresses high levels of polysaccharide but rapidly loses it upon laboratory subculture. The function of the capsule is not clear but it probably prevents phagocytosis (Foster, 1996).

2.5.2 Protein A

Protein A is 42 KD surface protein originally found on the cell wall of *S. aureus* isolates. It is of importance in biochemical research because of its ability to bind immunoglobulins, (especially immunoglobulin G) at the Fc region. *S. aureus* utilizes Protein A, along with other proteins and help in survival of the organism against host defenses. In serum, Protein A acts as an immunological disguise, resulting into disruption of opsonization and phagocytosis. Apart from this, Protein A has number of biological properties including chemotactic and anti-complementary effect. It also induces platelet damage and hypersensitivity. Mutant strains of *S. aureus* without protein A are more efficiently phagocytozed *in vitro* (Goodyear et al., 2003).

2.5.3 Toxins and enzymes

S. aureus cause number of human infections through its ability to multiply and spread in tissues as well as by production of many extracellular substances. These extracellular substances include various enzymes and toxins. Production of some of the extracellular substances is controlled by plasmids while some are under the control of both chromosomal and extrachromosomal mechanisms. The important enzymes are: catalase, coagulase, hyaluronidase (spreading factor), staphylokinase, proteinases (lipase, B-lactamase), phospholipase and DNase.

2.5.4 Exotoxins

These are cytolytic toxins consisting of four hemolysins (alpha, beta, gamma and delta) and leucocidin.

2.5.4.1 Alpha hemolysin (alpha toxin)

It is the most important among hemolysins produced by *S. aureus*, causes lysis of rabbit erythrocytes but less active against sheep and human red cells. In humans, platelets and monocytes are particularly sensitive to α-toxin due to their high affinity sites which allow toxin to bind at physiologically relevant concentrations **(**Bantel et al., 2001). It also has leucocidal, cytotoxic, dermonecrotic and neurotoxic effect. A complex series of secondary reactions cause release of number of cytokines which trigger production of inflammatory mediators. These events cause the symptoms of septic shock during severe *S. aureus* infections. Alpha-toxin is also one of the key virulence factors in pneumonia caused by *S. aureus* (Bubeck et al., 2007).

2.5.4.2 Beta toxin

Beta toxin is a sphingomyelinase which damages lipid rich membranes of bacterial cells. The majority of human isolates of *S. aureus* do not express β-toxin. In contrast, the majority of bovine mastitis isolates express β-toxin, suggesting its importance in the pathogenesis of mastitis. A study reported association of beta toxin in 72% of bovine mastitis isolates, 11% of healthy human nasal isolates, and in 13% of human septicemia isolates (Aarestrup et al., 1999).

2.5.4.3 Delta toxin

The delta-toxin is a very small peptide toxin produced by most of the strains of *S. aureus*. It is also produced by *S. epidermidis* and *S. lugdunensis*. The role of delta-toxin in pathogenesis is unknown.

2.5.4.4 Gamma toxin

The gamma toxin and the leukocidins are two-component protein toxins that damage membranes of susceptible bacterial cells. The proteins are expressed separately but act together to damage bacterial cell membrane and contribute in the pathogenesis.

2.5.5 Leukocidin (Panton Valentine leukocidin)

The history of Panton Valentine leukocidin (*PVL*) began in 1894, approximately 24 years after Sir Alexander Ogston and Louis Pasteur had reproduced human pyogenic infections in mice and rabbits by inoculating pus-derived cultures (Pasteur, 1880). Leucocidin, one of the toxins produced by *S. aureus*, has ability to kill exposed WBCs but its role in pathogenesis remains uncertain. Philip Panton and Francis Valentine, working at the Hale clinical laboratory of London Hospital, selected 22 strains of *S. aureus* isolated from variety of severe human infections and compared their toxic properties (Panton et al., 1932). The authors observed no correlation between hemolytic and leucotoxic activity, indicating that leucocidin was distinct from hemolysins. Out of these 22 isolates, seven produced high leucocidin and low hemolysin activities, indicating that at least 30% of the selected isolates produced this true leucocidin. However, *S. aureus* with leucocidin are capable of very active intracellular multiplication whereas nonpathogenic organisms tend to die inside the cell. Antibodies to leucocidin may play a role in resistance to recurrent staphylococcal infections.

Apart from its role in pathogenesis, there is a strong epidemiological association between *PVL* and the emergence of CA-MRSA infections. *PVL* is rarely associated with MSSA and HA-MRSA isolates (Johnsson et al., 2004 & Chini et al., 2006). In a study of 593 *S. aureus* isolates from France, *PVL* was absent in HA-MRSA isolates but was associated with all CA-MRSA isolates (Dufour et al., 2002). Another study across the globe showed, *PVL* was ubiquitous in a large number of CA-MRSA isolates (Vandenesch et al., 2003).

The presence of *lukS-PV* and *lukF-PV* (the cotranscribed genes for *PVL*) was detected in 172 strains of *S. aureus* collected from patients with a variety of clinical syndromes (Lina et al., 1999). *PVL* was significantly associated with community-acquired pneumonia (85% of strains), compared with hospital-acquired pneumonia (0%). However, some of the reports have shown that association of *PVL* with hospital acquired MRSA isolates (Bhutia et al., 2012).

2.5.6 Exfoliative (Epidermolytic) toxins

This toxin is responsible for generalized desquamation in the Staphylococcal Scalded Skin Syndrome (SSSS). This condition was also known as Ritter's disease, dermatitis exfoliative neonatorum, or pemphigus neonatorum. Exfoliative toxin is responsible for scalded skin syndrome in neonates, with widespread blistering and loss of the epidermis (Ladhani et al., 1999). Two antigenically distinct forms of the toxin are exfoliative toxin A (ETA) and exfoliative toxin B (ETB). The exfoliative toxin associated with scalded skin syndrome, causes cleaving between the living layers and the superficial dead layers of epidermis. This separation is through the stratum granulosum of the epidermis. This is probably why healing occurs with little scarring although the risks of fluid loss and secondary infections are increased. Staphylococcal Scalded Skin Syndrome affects large parts of the body and the lesions are often sterile. A localized form of SSSS, restricted to the sites of infection, is recognized as “bullous impetigo”. Staphylococcal exfoliative toxin B has been shown to specifically cleave desmoglein 1, a cadherin found in desmosomes in the epidermis.

2.5.7 Superantigens

2.5.7.1 Enterotoxin

Enterotoxin produced by *S. aureus* acts as superantigen. Staphylococcal enterotoxin has six different serotypes (A, B, C, D, E and G) responsible for diarrhea and

vomiting. Enterotoxins are heat stable, remain stable during cooking and when ingested are responsible for staphylococcal food poisoning. Systemic manifestation of enterotoxins may result in toxic shock syndrome (TSS).

2.5.7.2 Toxic Shock Syndrome Toxin (TSST)

Staphylococcal toxic shock syndrome is relatively rare condition (0.06 cases per 1, 00,000 cases) (CDC, 1996). This toxin is responsible for fever, shock, and multisystem involvement including hypotension, myalgia, vomiting, diarrhea, mucosal hyperemia and erythematous rash which desquamates subsequently (Chesney, 1989). Toxic Shock Syndrome Toxin-1 is expressed systemically and is weakly related to enterotoxins but it does not have emetic activity. TSST-1, is the most common toxin responsible for majority (95%) of cases associated with menstruation and 40-60% of the nonmenstrual cases (Descloux et al., 2008 & Garbe et al., 1985). Enterotoxins B and C are responsible for 50% of non-menstrual cases of TSS. Toxic Shock Syndrome can occur as a sequel to any staphylococcal infection if an enterotoxin or TSST-1 is released systemically, and the host lacks appropriate neutralizing antibodies. During usual antigen presentation, there is stimulation of only 1 in 10,000 T cell while superantigens stimulate T cells non-specifically without normal antigenic recognition resulting in stimulation of one in five T cells. Cytokines released in large amounts, cause the symptoms of TSS.

2.5.8 Other extracellular proteins

2.5.8.1 Coagulase

It is an extracellular protein which binds to prothrombin to form a complex called staphylothrombin. The protease activity which is characteristic of thrombin gets activated in the complex, resulting in the conversion of fibrinogen to fibrin clots. Coagulase is considered as a traditional marker for rapid identifying *S. aureus* in the routine clinical microbiology laboratory practice. There is no definite evidence of association of coagulase in the pathogenesis of staphylococcal diseases, however, experiments demonstrated that the bacteria could protect themselves from host defenses by causing

localized clotting (Tortora et al., 2013). It has recently been shown that the coagulase can bind fibrinogen as well as thrombin, at least when it is extracellular. Genetic studies have shown unequivocally that coagulase and clumping factor are distinct entities.

2.5.8.2 Staphylokinase

Many strains of *S. aureus* express a plasminogen activator known as staphylokinase. A complex formation between staphylokinase and plasminogen activates plasmin-like proteolytic activity which causes lysis of fibrin clots. The mechanism of action is similar to streptokinase, which has therapeutic application for patients with coronary thrombosis (Banerjee et al., 2004).

2.5.8.3 Other enzymes

S. aureus can express number of enzymes like proteases, lipase, deoxyribonuclease (DNase) and fatty acid modifying enzyme (FAME). The first three enzymes have minor role in pathogenesis while, FAME may be important in abscesses formation. FAME has been found to be associated with modification of anti-bacterial lipids and help in prolong survival of bacteria at the site of infection. The thermo stable DNase is an important diagnostic test for identification of *S. aureus* (Foster, 1996).

2.5.9 Biofilm

Biofilm is densely packed communities of microbial cells that develop on living or inert surfaces. A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of polysaccharide material.Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms. Many bacterial species including Staphylococci form biofilms. Biofilm unite the microbes and offer protection from the action of human immune system as well as from the effect of antimicrobial agents (Fischbach et al., 2009 & Rodvoldet al., 2014).

Thousands of years ago, humans realized that they could survive better in a dangerous world if they form communities. They realized that a community is far more likely to survive through division of labor: one person makes food, another collects resources, others protect the community against invaders. Working together in this manner requires communication and cooperation. Inhabitants of a community live in close proximity and create various forms of shelter in order to protect themselves from external threats. We build houses that protect our families and larger buildings that protect the entire community. Grouping together inside places of shelter is a logical way to enhance survival. Biofilm provides survival advantages to the bacteria including easy access to food, nutrients, protection from host enzymes, natural antimicrobials, toxic compounds and more importantly to number of antibiotics. Depending upon the organism and type of antimicrobial, biofilm forming bacteria can be thousand times more resistant than free swimming bacteria (Gilbert et al., 1997).

Biofilm formation takes place on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water plumbing as well as natural aquatic systems. Biofilms are composed primarily of microbial cells and extracellular polymeric substance (EPS) matrix. EPS accounts for 50% to 90% of the total organic carbon of biofilms (Flemming et al., 2000) and can be considered as the primary matrix material of the biofilm. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as in case of EPS of Gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pryruvates confer the anionic property (Sutherland, 2001). This property is important as it allows association of divalent cations (calcium and magnesium), which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Flemming et al., 2000). In the case of some Gram-positive bacteria, such as the staphylococci, the chemical composition of EPS may be quite different and may be primarily cationic. The slime of coagulase-negative bacteria consists of a teichoic acid mixed with small quantities of proteins (Hussain et al., 2001).

Biofilm formation is a slowly developing multistep phenomenon. Initial step in the formation of biofilm is attachment of bacteria to the surface called cell adhesion facilitated by cell adhesion molecules. After adhesion, bacteria begin to build up matrix that holds the biofilm together. Some external biofilms like chronic wound, dental plaques can be removed manually while internal biofilms are difficult to eradicate because of their inaccessibility and heightened drug resistance. Biofilms grow slowly, in diverse locations, and overt symptoms are often delayed. However, biofilm forming bacteria can spread in numerous ways that allow them to easily infect new tissues. Biofilms may move collectively, by rippling or rolling across the surface, or by detaching in clumps. Sometimes, in a dispersal strategy referred to as "swarming/seeding", a biofilm colony differentiates to form an outer "wall" of stationary bacteria, while the inner region of the biofilm "liquefies", allowing planktonic cells to "swim" out of the biofilm and leave behind a hollow mound. During unfavorable conditions like nutritional depletion, microbe in the biofilm can enter into viable but non-cultural state and wake up periodically from the state of dormancy. The expression of 800 genes has been shown to be altered when single bacterial species joins biofilm (Sauer et al., 2002).

2.6 Epidemiology

S. aureus is a major cause of infections in both community and healthcare facilities, and is increasingly showing resistance to multiple antimicrobial agents. Asia is among the regions with the highest incidence of methicillin-resistant *S. aureus* (MRSA) in the world (Chen et al., 2014). Along with MRSA, vancomycin-intermediate *S. aureus* (VISA) strains and vancomycin-resistant *S. aureus* (VRSA) strains are also being identified in certain countries of this region (Chen et al., 2014). Changing epidemiology and updated information on epidemic *S. aureus* strains in local and neighboring countries is essential for the prevention and control of this pathogen. These informations are equally important for clinicians dealing with treatment of staphylococcal diseases.

MRSA is one of the most prevalent pathogens in all healthcare facilities. The incidence of MRSA infections varies significantly between various countries, and has changed drastically over a period of time. Most of the reports on MRSA have been from certain relatively high-income countries, including Taiwan, Japan, Korea, Hong-Kong SAR, and Singapore. The complete information regarding exact prevalence of MRSA is poorly available from majority of the resource-limited countries in Southeast Asia and South Asia, which substantially has limited understanding of the epidemiology of staphylococcal diseases.

After the emergence of the first MRSA strain in the UK, increasing numbers of MRSA outbreaks were reported in Europe and the USA in the 1960s. MRSA was rarely documented before 1980 in East Asia (Chen et al., 2014). The occurrence of nosocomial MRSA infections remained at a low rate, and 0.2– 0.9 episodes were identified per 1000 discharges in a hospital in the early 1980s. Japan was an exception; *S. aureus* with low-level resistance to methicillin was first identified in the early 1960s, but with a very low incidence (<3%).

By the late 1970s, MRSA became a significant problem in the United States, with large number of outbreaks in several tertiary care hospitals. MRSA is a major pathogen worldwide and is endemic in many American and European hospitals with more frequent

colonization in intensive care units (Boyce, 1990 & Kerttula et al., 2004). National Nosocomial Infection Surveillance (NNIS) System data reported a steady rise in the prevalence of nosocomial methicillin-resistant *S. aureus* (MRSA) infections among ICU patients. *S. aureus* infections are increasingly reported around the world. CDC reported approximately 125,969 hospitalizations annually for *S. aureus* infections in the year 1999–2000, including bloodstream infections and pneumonia (Kuehnert et al., 2005) and 43.2% isolates were MRSA. A large surveillance program of nosocomial bloodstream infections in the United States showed that MRSA isolates increased from 22% in 1995 to 57% in 2001 (Wisplinghoff et al., 2004). In an analysis of US inpatients, nearly 400,000 admissions due to *S. aureus* infection per year were reported in 2003 (Noskin et al., 2007). The National Health Care Safety Network in the United States estimated that hospitalized patient acquire two million healthcare associated infections per year, of which MRSA contributes significantly (Edwards et al., 2007). Approximately 19,000 deaths/year reported in the United States due to MRSA, surpassing those due to HIV-AIDS. MRSA infections have high economic burden with estimated cost in billions of dollars (Edwards et al., 2007).

Community-associated MRSA infections were initially described among children with bloodstream infections without prior health care exposure (NNIS, 2004). Community acquired-MRSA has been increasingly reported as the cause of skin infections and abscesses among previously healthy adults and as a cause of bloodstream infections among patients in healthcare settings (Daum, 2007). Geographic variation in the United States has been observed, with more MRSA infections seen in the South (Kuehnert et al., 2005). Similarly, in Europe, considerable variation exists in the incidence of MRSA, with only 0.5% in Iceland but 44% in Greece from 1999 to 2002 (Tiemersma et al., 2004).

Between 1999 and 2005, the estimated number of hospitalizations associated with MRSA infections in the United States were more than double (Klein et al., 2007). According to the Centers for Disease Control and Prevention (CDC), there were estimated 94,360 invasive MRSA infections in the US with approximately 18,650 deaths (Klevens et al.,

2007). Other reports estimate over one million infected with MRSA in US with more than 10,000 deaths. In a study conducted in the year 2003-2004, approximately 29% of the US population was colonized with *S. aureus* with 1.5% MRSA. Another hospital based study with universal screening upon admission of all patients showed colonization of MRSA as high as 12% (Brendan, 2005).

MRSA infections and colonization have steadily increased world wide over the years among both hospital and community settings. In 1974, only 2% of all *S. aureus* infections were MRSA which increased upto 22% in 1995 and 64% in 2004 with an estimate of over 70% of all *S. aureus* are now MRSA (Brendan, 2005). In the community, MRSA contributes approximately 60% of all skin and soft tissue infections. MRSA pooses high economic impact on patient, community and healthcare system with estimated $9.7 billion, without considering indirect costs related to patient pain, illness, and time spent in the hospital (Klein et al., 2007).

In recent years, the epidemiology of MRSA has changed from infections acquired primarily in health care settings to infections acquired in the community (Chambers et al., 2009). CA-MRSA strains are genetically distinct from HA-MRSA strains and are thought to have evolved separately.

Annual death rates in the United States for selected infectious diseases (Boucher et al., 2008)

Infectious disease	No. of deaths (estimated)	Year
MRSA infection	19,000	2005
AIDS	15,798	2004
Tuberculosis	662	2004
Viral hepatitis	5793	2002

MRSA causes significant human infections with variable morbidity and mortality. Asian countries have been reported to show the highest rates of MRSA from hospitals. Prevalence rates of MRSA in hospitals of Korea, Japan, Taiwan and China were reported to be 70%–80% (Lee et al., 2000 & Voss et al., 1995). A high prevalence of MRSA in the Asian region might be partly due to the spread of a few epidemic clones. The rate of MRSA in East Asia increased remarkably from the year 1980 to 2000. In Taiwan, the proportion of MRSA among all nosocomial *S. aureus* isolates increased from 20.2% in 1981–1986 to 64.8% in 1993–1998, and 69.3% in 1999 (Hsueh et al., 2000).

In 1990, a nationwide study including 43 hospitals of Japan showed 58.6% of clinical *S. aureus* isolates were MRSA (Kimura et al., 1992). Nationwide surveillance in Korea in 1998 showed a mean MRSA rate of 72% for all clinical *S. aureus* isolates from 25 hospitals (Lee et al., 2000). A study of seven hospitals in Korea reported an average MRSA rate of 77.6% for nosocomial *S. aureus* isolates during 2004–2006. The most recent report of the Regional Resistance Surveillance (RRS) programme showed that 73% of the clinical *S. aureus* isolates from two hospitals in Korea were MRSA in 2011 (Mendes et al., 2013). Korea has the highest MRSA rate among the 12 surveillance countries in the RSS programme.

In Southeast Asia, epidemiological data on MRSA were collected from multinational surveillance programmes, with participation of limited numbers of hospitals from each participating country (Bell et al., 2002 & Christiansen et al., 2004). Surveillance report suggested that MRSA infections are common in this region, and accounted for a significant proportion of nosocomial infections. The Asian Network for Surveillance of Resistant Pathogens (ANSORP) study showed MRSA rates of 38.1% in Philippines, 57% in Thailand and 74.1% in Vietnam during 2004–2006 (Song et al., 2011). The most recent data from multinational study of the RSS programme revealed that the proportion of MRSA among clinical *S. aureus* isolates ranged from 28% in Indonesia to 59% in the Philippines (Mendes et al., 2013). A multicenter study sampling six hospitals in Singapore further showed average MRSA rates of 35.3% among all clinical *S. aureus* isolates and 46.7% among *S. aureus* isolates from patients in intensive-care units (Hsu et

al., 2007). The clinical significance and concern regarding emergence of MRSA was recognized between 1980s and in the 1990s. MRSA has become growing problem in the Indian scenario with its prevalence increased from 12% in 1992 to 80.83% in 1999 and is now endemic (Verma et al., 2000). The incidence of MRSA varies from 25 % in Western part of India (Patel et al., 2010) to 50 percent in South India (Gopalakrishnan et al., 2010). Prolonged hospital stay, indiscriminate use of antibiotics, lack of awareness, receipt of antibiotics before coming to the hospital are possible predisposing factors of emergence and spread of MRSA. Incidence of Community acquired MRSA (CA-MRSA) has also been increasingly reported from India.

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Indian Network for Surveillance of Antimicrobial Resistance (INSAR) was formed with support from the World Health Organization at various medical colleges and hospitals in India. This network was aimed to monitor antimicrobial resistance and review the magnitude of problem in India. Initially few organisms of public health importance were selected for monitoring prevalence and antimicrobial resistance patterns. All participating laboratories shared their antimicrobial susceptibility data and provided technical support to other members.

In a study from north India, the prevalence of MRSA was 46% and majority MRSA were found multidrug resistant (Arora et al., 2010). The prevalence of MRSA from Chennai, India, reported 40-50% (Gopalakrishnan et al., 2010). A high prevalence of MRSA (35% in ward and 43% in ICU) was observed from blood culture specimens in a study from Delhi (Wattal et al., 2010). The prevalence of MRSA is not uniform and varies with geographical location as well as hospitals of the same region. A study from Delhi reported wide variation of MRSA prevalence in nosocomial SSTI ranged from 7.5 to 41.3% among three tertiary care teaching hospitals (Gadepalli et al., 2009).

The proportion of MRSA among nosocomial *S. aureus* strains has been reported very high in many Asian countries (Molton et al., 2013). The prevalence of MRSA in China has reached 50 to 70% based on previous laboratory-based surveillance data (Xiao et al.,

2011). Another study found that the mean prevalence of MRSA across China was over 50% in 2005 and in Shanghai over 80% (Wang et al., 2008).

In 1991, 62.61% MRSA prevalence was reported from Bangladesh where methicillin was not yet introduced (Khan et al., 1991). However, 47.2% MRSA was reported in an investigation on clinical *S. aureus* isolates in 2002 (Rahman et al., 2002). These prevalence rates of MRSA were higher than some developed countries like Austria 21.6%, Belgium 25.1%, Spain 30.3%, and France 33.6% (Herwaldt et al., 1996).

Laboratory based antimicrobial resistance surveillance (AMR) was started in Nepal in 1999 with aim of monitoring the resistance trend of selected bacterial pathogens which include *Salmonella* species, *Shigella* species, *Vibrio cholerae*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria gonorrhoea*. National public health laboratory (NPHL) in association of World Health Organization (WHO) conducts various training programs and collects data of the participating laboratories from different parts of Nepal. MRSA has been recently included in the list of selected infectious pathogens in Nepal. Scenario of MRSA in various parts of Nepal has changed over a period of time. Due of lack of trained staff and unavailability of basic infrastructure, there are challenges in the healthcare facilities to perform proper antibiotic susceptibility testing. Therefore data regarding prevalence of MRSA from all parts of Nepal is not available. Earlier studies reported prevalence of 15.4%-26.0% (Subedi et al., 2005 & Kumari et al., 2008). Newer studies from various hospitals of Nepal reported higher prevalence of 26%-69% (Sanjana et al., 2010, Tiwari et al., 2009, Khanal et al., 2010 & Pandey et al., 2012).

Study from Eastern Nepal, reported that 26.14% of the isolates were MRSA (Kumari et al., 2008). Seventy percent isolates of MRSA were from inpatient departments and amongst them only 10% were from intensive care units (ICUs). More than 65% of MRSA were found to be resistant to penicillin, cephalosporins, ciprofloxacin, gentamicin

erythromycin and tetracycline, while 47.96% of them were resistant to amikacin. Study from Western Nepal, reported 69.1% of isolates as MRSA, of which 37 (33.1%) were community acquired and 75 (66.9%) were hospital acquired. All MRSA strains were resistant to penicillin, and 91.9%, 87.4%, 77%, and 55.5% were resistant to amoxicillin, ampicillin, trimethoprim/sulfamethoxazole, and cephalexin, respectively (Tiwari et al., 2009).

A hospital based study from Chitwan, Nepal, reported overall prevalence of MRSA 68% (408/600). MRSA infection was higher among patients above 30 years of age. Tibeto-Burman had high MRSA infection rate (72.5%) than Indo-Aryans (56.8%) and the rate was significantly higher among males (75.0%) than females (63.4%) ($p<0.05$). MRSA isolation rate was higher from wound (76.9%), followed by purulent exudates (67.7%) and abscesses (64.1%) (Khanal et al., 2010). Another study from the same region reported lower MRSA prevalence of 43.1% without any vancomycin resistant isolate. (Ansari et al., 2014). Another study from Kathmandu, Nepal, reported MRSA prevalence of 26.12% (Pandey et al., 2012). The rate of multidrug resistance was 75.86% for MRSA and 6.09% for MSSA. All the staphylococcal isolates were resistant to penicillin but none to vancomycin. In another hospital based study from Kathmandu, reported the prevalence of MRSA 52.9% (Shrestha, 2013).

2.7 Coagulase negative Staphylococci

Group of Staphylococci other than *S. aureus* are classified as coagulase negative Staphylococci and can cause variety of human infections. *Staphylococcus epidermidis* is the most important coagulase-negative staphylococcus (CNS) and major cause of infections associated with prosthetic devices and catheters (Foster, 1996). *Staphyloccus epidermidis* occasionally causes urinary tract infections among young ladies. These Staphylococci also cause peritonitis in patients receiving continuous ambulatory peritoneal dialysis and endocarditis in patients with prosthetic valves. Other species such as *S. haemolyticus, S. warneri, S. hominis, S. capitis, S. intermedius, S. schleiferi* and *S. simulans* are uncommon pathogens. *Staphylococcus lugdunesis* is a newly recognized species, probably more pathogenic than are other CNS, with cases of endocarditis and other infections being reported (Foster, 1996). Lower incidence of infections by these organisms is believed to be associated with difficulties in identification.

In contrast to *S. aureus*, little is known about of pathogenesis of *S. epidermidis* infections. A characteristic of clinical isolates of *S. epidermidis* is the production of "slime." Some researchers believe that slime is an *in vitro* manifestation of the ability to form a biofilm *in vivo* (for example on the surface of a prosthetic device), and thus is a virulence marker (Foster, 1996). *In vitro*, slime formation takes place in liquid medium as a biofilm on the surface of the growth vessel. The chemical composition of this slime is probably influenced by the type of growth medium used. One study with defined medium showed that the slime was predominantly secreted teichoic acid, a polymer normally found in the cell wall of staphylococci. Some polysaccharides in slime from bacteria grown on solid medium are derived from the agar.

2.8 MRSA colonization among healthcare workers

Methicillin-resistant *S. aureus* (MRSA) is the most commonly identified drug-resistant pathogen in hospitals around the world. Staphylococci form part of normal flora of various anatomical sites of human body. The anterior nares are the most common site of MRSA colonization, although other body sites, such as the hands, skin, axillae, and intestinal tract are frequently involved (Caiazza et al., 2003).

Healthcare workers (HCWs) play important role in the transmission of MRSA, but more frequently act as vector (Wang et al., 2011). The commonest mode of MRSA transmission is through contaminated hands especially among healthcare workers (Cimolai, 2008). Colonized individuals are generally asymptomatic and duration of colonization varies among the population. Three types of MRSA carrier have been observed: non-carriers, persistent carriers (who are chronically colonized with the same strain), and intermittent carriers (who are colonized with varying strains for short time periods) (Albrich et al., 2008). Another form of short-term carriage is transient carriage, which is identified during or after a work shift and in most cases lost before the next shift (Cookson et al., 1989). Persistent and intermittent carrier rates in adults vary from 20-50%. A large study found that 24% were persistent carriers and 57% were intermittent carriers of *S. aureus* whilst 20% were never colonized (Gould et al., 1954).

Nasal carriage of *S. aureus* has been associated with an increased risk of infection. (Safdar et al., 2008). However, it is unclear whether the risk of infection is higher for the colonized individual when carriage is persistent. Approximately 5% of colonized HCWs develop clinical infections (Albrich et al., 2008) and symptomatic MRSA infections among HCWs have been described in several case reports (Haamann et al., 2011).

Nasal carriage rates of MRSA have been reported to be 0.8 to 3.0% among adults in the community. Among healthcare workers in hospital setting, nasal carriage rate ranges from 6 to 17.8% (Eveillard et al., 2004 & Cesur et al., 2004). The MRSA colonization among the patients, staff and hospital environment of a Teaching Hospital in Kathmandu, Nepal has reported 29.1% (Rai et al., 1990). Another study from Kathmandu, Nepal, reported nasal carriage rate of 43.8% among healthcare personnel of a Medical College Teaching Hospital (Pant et al., 2007).

2.9 Staphylococcal diseases

Staphylococcal infections are one of the most common human infections. Almost all the systems of human body are affected by staphylococcal infections. *S. aureus* was the commonest bacterium isolated from inpatients of 300 clinical microbiology laboratories in the United Sattes from 1998 to 2005 (Styers et al., 2005). Strains of *S. aureus* capable of invading intact normal skin are rare and most are able to cause infection only if they enter through breaks in the skin. *S. aureus* causes localized as well as systemic infections in human. Strains causing localized infections have tendency to cause systemic infections and result into serious consequences. Spontaneous bacteremia may occur without an evident septic focus, particularly in patients debilitated by chronic hepatitis, diabetes mellitus, organ transplantation etc. Common staphylococcal infections are

2.9.1 Skin and soft tissue infections

Skin and soft tissue infections (SSTIs) are one of the most common clinical entities of variable presentation caused by wide range of microorganisms. SSTIs involve microbial invasion of layers of skin and underlying soft tissues. Severity of SSTIs ranges from mild infections such as pyoderma to severe life threatening conditions such as necrotizing fasciitis. The estimated incidence rate of SSTIs is 24.6 per thousand people per year (Ellis et al., 2006). As majority of cases of SSTIs resolve within 7 to 10 days, estimated prevalence is variable.

Skin acts as principal barrier which protects microbial invasion. It constantly interacts with the diverse population of microorganism in external environment, leading to colonization of skin. The colonizing flora consists of pathogenic, non pathogenic and opportunistic pathogens. The common skin colonizers are Gram-positive bacteria such as

S. epidermidis, *Corynebacterium* species, *S. aureus and Micrococcus* species (Grice et al., 2009). Among these, *S. aureus* and *Streptococcus pyogenes* significantly contribute to SSTIs. Recent epidemiological studies have shown an increase in the rate of skin and soft tissue infections caused both by healthcare-associated and CA-MRSA (Eady et al., 2003). These infections can be minor and self-limiting, such as furunculosis, moderately severe, such as abscesses to the life-threatening Staphylococcal scalded skin syndrome. Common staphylococcal skin and soft tissue infections include following conditions:

2.9.1.1 Cellulitis

Celluliis is an infection involving the skin and tissues below the skin surface. It begins as a small area of redness, pain, swelling, and warmth on the skin. Infection spreads locally and person may feel feverish and ill. Cellulitis can affect any area of the body, but skin on lower legs is most commonly affected. Cellulitis, if left untreated spread to other parts resulting into serious consequences. *S. aureus* and *S. pyogenes* are two most common bacteriological agents associated with cellulitis, with occasional involvement of other bacteria (Khan, 2016).

2.9.1.2 Impetigo

Impetigo is highly contagious superficial skin infection mostly occurs in young children, but can sometimes affect adults. Commonly affected anatomical sites include face, hands, or feet. An impetigo skin infection begins as a small blister or pimple, and then develops a honey-colored crust. Impetigo doesn't usually cause pain or fever, although the blisters may rupture and can spread to other parts.

2.9.1.3 Folliculitis

Folliculitis is an infection of the hair follicles, characterized by formation of tiny white-headed pimples at the base of hair shafts, sometimes with a small red area around each pimple. Commonly affected areas are axilla, beard, scalp, thigh and inguinal regions (Singh et al., 2010).

2.9.1.4 Furuncle

Furuncle is commonly known as a boil, is a swollen, red, painful lump on the skin, usually due to an infected hair follicle. The lump usually fills with pus, grow larger and more painful until it ruptures and drains. Folliculitis usually progresses to furuncles. Furuncles most often appear on the face, neck, buttocks, armpits, and inner thighs. A cluster of several furuncles is called carbuncle (Ibler et al., 2014).

2.9.1.5 Wound infections

Surgical wounds are a prime target for infection by MRSA, especially in hospitalized patients. In some units of the hospital like surgical wards, this problem is endemic and challenging for clinicians to eradicate the organism. Wound infections generally manifest two or more days after the injury or surgery. The signs of a wound infection include redness, pain, swelling, and warmth which are similar to cellulitis. Patients may develop fever and feel sick in general. Pus or a cloudy fluid can drain from the wound with yellow crust (Harbarth et al., 2008).

2.9.1.6 Decubitus ulcers

Decubitus ulcers also known as pressure ulcers often occur on the skin covering bony areas. Chronic cutaneous ulcers can be colonized and infected by MRSA. Patients in medical institutions and long-term care facilities are at higher risk of these infections.

2.9.2 Respiratory infections

S. aureus including MRSA are associated with number of respiratory infections which include: tonsillitis, pharyngitis, sinusitis, otitis media, bronchopneumonia, lung abscess

and pneumonia. Lower respiratory tract infections caused by MRSA are increasing in recent years. It can occur with healthy individuals, but more common in persons with chronic respiratory conditions such as bronchiectasis, cystic fibrosis and immune-compromised patients (Giron et al., 2009). Necrotizing pneumonia, a severe presentation can lead to respiratory failure and death (d'Azevedo et al., 2009). Patients with prolonged intubation or undergone tracheostomy are at increased risk of developing MRSA respiratory infections, especially in intensive care units.

Pneumonia caused by MRSA has higher mortality rate as compared to other respiratory infections. Staphylococcal pneumonia has been a changing clinical entity since initially reported in the late 19th and early 20th Centuries (Defres et al., 2009). It was recognized in young healthy military personnel during World War I as a post-influenza pneumonia with a rapid onset of symptoms. In the 1950s, cases of Staphylococcal pneumonia began to be reported without prior influenza infection. These patients usually had some predisposing risk factors, such as cardiopulmonary disease, alcoholism, diabetes mellitus, or had acquired an infection in hospital. These strains include both MSSA as well as MRSA. The mortality in the antibiotic era ranges from 20% in young adults to 30–50% in post-influenza cases and 83% in patients with bacteraemic primary pulmonary pneumonia (Hausmann et al., 1956 & Watanakunakorn, 1987). Clinical presentation of MRSA pneumonia is similar to those caused by Gram-negative organisms, and has associated mortality of 55.5% regardless of early appropriate therapy (Rubenstein et al., 2008).

Ventilator Associated Pneumonia (VAP) due to MRSA has significant excess morbidity and mortality regardless of appropriate antimicrobial treatment and patient characteristics. Ventilator Associated Pneumonia caused by MRSA also had an increased length of ICU stay compared with controls (Rello et al., 2005).

2.9.3 Urinary tract infections

S. aureus including MRSA has been reported less frequent cause urinary tract infection in general population (Demuth et al., 1979). Instrumentation of urinary tract such as indwelling catheter increases the risk of colonization by *S. aureus* (Coll et al., 1994). MRSA can ascend from urethral meatus or via blood stream to cause bacterriuria. The majority of cases of *S. aureus* bacteriuria are asymptomatic (Demuth et al., 1979). As bacteriuria commonly associated with long-term urinary catheterization, the clinical significance of isolation of *S. aureus* from such patients remains unclear.

In a multicenter, community-based study conducted in Great Britain, *S. aureus* accounted for only 0.5% of isolates (Barrett et al., 1994). A similar laboratory-based study conducted in France revealed that *S. aureus* accounted for only 1.3% of isolates from urine specimens collected from the community population (Goldstein, 2000). A study from Pokhara, Nepal, reported 5% of *S. aureus* isolates from the cases of urinary tract infections (Ghosh et al., 2012). However, multiplication of MRSA in urinary tract may lead to complications such as severe pyelonephritis and occasionally urosepsis.

2.9.4 Systemic infections

S. aureus is the most frequently occurring bacterial pathogen among clinical isolates from hospital inpatients in the United States and is the second most prevalent bacterial pathogen among clinical isolates from outpatients. *S. aureus* bloodstream infections are among the most prevalent and difficult to treat (Wisplinghoff et al., 2004). The incidence of *S. aureus* bacteremia, particularly caused by methicillin-resistant *S. aureus* (MRSA) strains, has increased in recent years in the United States and in some European countries (Shorr et al., 2006 & EARSS management team, 2007). Bacteremia associated with MRSA has high mortality rates and increases cost and resource burden on health care system (Steinberg et al., 1996). Furthermore, MRSA bacteremia may also lead to severe life threatening complications like infective endocarditis and metastatic infections (Troidle et al., 2007). Endocarditis caused by *S. aureus* has higher mortality rates as compared to endocarditis caused by other bacteria (Miro et al., 2005).

MRSA can potentially infect any organ or system of body but invasive staphylococcal infections are relatively less common. In some patients, staphylococcal skin infections can lead to more serious invasive infections with higher mortality rates as compared to superficial infections. In the hospital setting, intensive antibiotic use and iatrogenic instrumentations promote topical and hematogenous dissemination of MRSA. These could result in meningitis, epidural abscess, neonatal sepsis, mastitis and toxic shock syndrome.

2.9.5 Bone and joint infections

Haematogenous spread of MRSA can disseminate to bones causing acute osteomyelitis or joints and their surrounding structures, resulting in septic arthritis (Ahamed, 2009 & Yamagishi et al., 2009). Articular and periarticular injections can also induce iatrogenic MRSA septic arthritis (Rhee et al., 2008). Surgical intervention, such as joint replacement procedures, also increases the risk of serious MRSA infections.

2.10 Antibiotic resistance

Antibiotic resistance is the ability of bacteria to resist the effect of drugs. Antibiotic resistance is a global concern. Infections caused by drug resistant bacteria do not respond to the standard treatment, resulting in prolonged illness and may require toxic and costly alternatives. Antibiotic resistance among bacteria may occur naturally and due to human action. Different mechanisms of antibiotic resistance include: alteration of the target site with decreased affinity for the antibiotic, enzymatic inactivation of antibiotic, accelerated drug efflux and a by-pass mechanism in which an alternative drug-resistant version of the target is expressed (Foster, 1996). Antibiotic inactivation mechanisms are more commonly associated with bacterium that gains resistance overtime from human action. An example of this can be traced through *S. aureus*, which resists antibiotics by deactivating β-lactam binding proteins.

Since the beginning of the antibiotic era, *S. aureus* has responded to the introduction of new drugs by rapidly acquiring resistance. Antibiotic era started with the Alexander Fleming's discovery of penicillin in 1928 (American Chemical Society, 2015). In the 1940s, penicillin was introduced for the treatment of infection. Penicillin was found to be a miracle drug to treat uniformly fatal infections. Antibiotic resistance in *S. aureus* was uncommon when penicillin was first introduced. In 1942, strains of *S. aureus* resistant to penicillin had been detected in hospitals. Emergence of antibiotic resistance among *S. aureus* had undergone series of changes over a period of time. Approximately 80% of both hospital and community- acquired *S. aureus* isolates were found penicillin resistant within last two decades (Appelbaum, 2007). Resistance to penicillin was due to production of enzyme beta lactamases which cleaves the beta lactam ring of the drug and make it ineffective.

Most clinical isolates of *S. aureus* are resistant to benzylpenicillin, due to the production of enzyme beta-lactamase that binds to the antibiotic and destroys its activity by opening the beta-lactum ring. The alternative drug for penicillin resistant Staphylococci was introduced in the form of methicillin with penicillinase-stable penicillins in 1961 (Jevons, 1961). This victory over penicillin resistant Staphylococci was brief as emergence of methicillin-resistant *S. aureus* (MRSA) was recorded in the year of the drug's launch. Methicillin resistance is conferred by the *mecA* gene, which encodes a penicillin-binding protein (PBP2A) with decreased affinity for *β*-lactam antibiotics. Methicillin resistance is a complex property and multiple mechanisms are involved (Fung et al., 1991). Strains of MRSA that are beta-lactamase negative can appear penicillin sensitive, methicillin resistant on testing (Richardson et al., 1990). Therefore, isolates of *S. aureus* should be considered as either sensitive or resistant to methicillin for clinical purposes, regardless of underlying mechanism. It is important to appreciate that methicillin resistance implies resistance to all beta-lactum antibiotics, including the cephalosporins, even though disc sensitivity tests may show zones of inhibition. The term superbug has been coined for MRSA primarily based on its antimicrobial resistance.

In the year 1962, MRSA was detected in a British hospital. During the next 10 years, multidrug resistant *S. aureus* became widespread in Europe, Australia and the United States. In 1970s, Denmark, Netherlands, and other European countries established infection-control regulations; MRSA rates begin to drop there while holding steady or climbing in the United States. Today, MRSA strains are found worldwide, and most are multidrug resistant.

Study of earlier isolates of MRSA showed that a key genetic component responsible for resistance, *mecA,* is not native to the *S. aureus* genome. The staphylococcal chromosome cassette *mec* (SCC*mec*) has been characterized as a novel, mobile resistance element that differs from both transposons and bacteriophages (Ito et al., 2001). MRSA generally spreads through clones; however, it is known that the *mec* genes have been transmitted between *S. aureus* strains and, possibly, between other staphylococcal species (Berger et al., 2002).

2.11 MRSA testing methods

Laboratory screening of MRSA provides valuable information to clinicians to guide the therapy. Therefore, it is important to adopt correct method of MRSA testing in the clinical microbiology laboratory. Various methods have been recommended for methicillin susceptibility testing and often conflicting 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 and behave differently under different test conditions (Tomasz et al., 1991). Number of factors has been found to be associated with the expression of resistance. Three most common factors which may interfere with expression of methicillin resistance are sodium chloride concentration in the medium, the temperature and duration of incubation.

MRSA detection can be performed by disc diffusion method, MIC determination and molecular methods, which detects *mecA* gene. Disc diffusion methods remain the most widely used in routine clinical microbiology laboratories, although some commercial systems for detection of methicillin resistance are also available. Automated methods are increasingly used in developed countries.

2.11.1 Disc diffusion methods

Traditionally, the results of test against methicillin or oxacillin have been representative of all beta-lactam agents (Acar et al., 1970). However, methicillin is no longer being manufactured. Different combination discs have been recommended for screening of MRSA. Cephamycins were used extensively in Japan in the early 1980s, and as a result some MRSA and MSSA isolates became resistant to cefoxitin **(**Okonogi et al., 1989). Cefoxitin is a better inducer of *mecA* gene and tests using cefoxitin give more accurate

and reproducible results as compared to oxacillin. Therefore, cefoxitin has been proposed for detection of methicillin resistance by disc diffusion method.

2.11.2 Epsilometer (E) test

The E test method gives Minimal Inhibitory Concentration (MIC) values and test results are affected by similar conditions as described in diffusion methods. Test is performed by inoculating standard bacterial suspension (density equivalent to 0.5–1.0 McFarland standards) on Mueller Hinton agar with 2% NaCl, with a sterile swab and incubation at 35°C for 24 hours (Huang et al., 1993). The E test has an advantage over other MIC methods in that it is as easy to set up as the disc diffusion test.

2.11.3 Agar screening method

This method has been recommended for routine screening and confirmation of resistance suspected by disc diffusion methods. The test is performed by inoculating the test organism of density of a 0.5 McFarland standard on Mueller Hinton agar containing 4% NaCl and 6 mg/L oxacillin with a spot or a streak of the organism. Plates are incubated at 35°C for 24 hours and any growth other than a single colony is indicative of resistance (NCCLS, 2003).

2.11.4 Chromogenic agar

Chromogenic agar contains media substrate that changes color when *S. aureus* grows on medium. MRSA differentiation can be achieved by incorporation of antibiotics like cefoxitin into the medium. Such medium allows rapid identification of MRSA from primary isolation plates within 24-48 hours, obviating the need for subcultures and further identification (Malhotra et al., 2008).

2.11.5 Latex agglutination

This is rapid slide latex agglutination test which detects PBP2a and available commercially. At first, extraction of PBP2a from bacterial suspensions is carried out,

followed by agglutination with latex particles coated with monoclonal antibodies to PBP2a. The test is very sensitive and specific for *S. aureus*, grown on non-selective medium but may not be reliable for colonies grown on media containing high concentration of NaCl (Brown et al., 2001). The advantage of this method is no special equipments are required and is suitable for confirmation of resistance or equivocal tests in routine clinical laboratories. Isolates producing small amounts of PBP2a may give weak or negative agglutination reactions.

2.11.6 Automated methods

Laboratory testing of methicillin/oxacillin resistance can be performed by number of automated systems which include Vitek/Vitek2 (BioMerieux), Phoenix (Becton Dickinson) and Microscan (Dade Behring). Results of the test generally reliable although small number of incorrect results has been reported (Spanu et al., 2004).

2.11.7 Quenching fluorescence method

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 unutilized oxygen in the broth. This method is reliable but requires several hours of incubation during the test procedure (Louie et al., 2000).

2.11.8 Molecular methods

High-level resistance of *S. aureus* to penicillins is related to the presence of the *mecA* gene that provides genotypic method of detection of methicillin resistance. This method allows specific characterization of this resistance mechanism. The earliest molecular methods for the detection of *mecA* relied on either radiolabelled or digoxigenin (DIG)-labeled DNA probes (Archer et al., 1990). The non-radioactive DIG-labeled probe as well as the radioactive label, enabling the safer utilization of the test system in a diagnostic laboratory. Disadvantage of this method is delayed reporting due to number of time consuming manipulations.

More recently, PCR-based methods have been used routinely by reference laboratories as their standard method for detecting the *mecA* gene (Bignardi et al., 1996). This technique also detects susceptible strains carrying a nonfunctional or non-expressed *mecA*. However, this method fails to detect non *mecA* mediated borderline resistance, although these are uncommon (Murakami et al., 1991).

2.12 Classification of MRSA

MRSA can be classified into two major groups based on environment of acquisition: Hospital-acquired MRSA (HA-MRSA) and Community-acquired MRSA (CA-MRSA).

2.12.1 Hospital acquired MRSA

The Centers for Disease Control and Prevention (CDC) has defined healthcare-associated infections (HAIs) as infections acquired after admission of the patient, without any evidence of infection or incubation at the time of entry into the healthcare setting (Horan et al., 2008). These are group of MRSA which are acquired from the hospital setting during the period of patients hospital stay. Hospital acquired MRSA infections are contracted from the environment or staff of a healthcare facility. The increased risk of MRSA infection among hospitalized patients is due to inhibitory effect of antibiotics on normal flora, giving MRSA strain an advantage to multiply. Similarly, many patients in the hospitals have breaks in the skin (surgical wounds, intravenous lines, catheters) that can allow bacteria to enter into deeper tissues including blood stream. Therefore, HA-MRSA is commonly associated with post-operative wound infections, or infections resulting from implanted devices and catheters. Typically, patients infected with HA-MRSA are immune-compromised and the resulting infections are generally more invasive.

HA-MRSA has increased during the past decade due to a number of factors including an increased number of immunocompromised patients and increase in the number of invasive procedures. Failures in infection control measures such as hand washing prior to patient contact and removal of non-essential catheters have also been significantly associated with spread of HA-MRSA infections. Many hospital-acquired infections caused by MRSA exhibit increased antimicrobial resistance as compared to community

strains. HA-MRSA infections are associated with prolonged hospital stay, increased mortality, and increased costs. In 2003, 64.4% of *S. aureus* infections in ICUs were found to be associated with methicillin-resistant strains (Siegel et al., 2007).

The most common manifestations of HA-MRSA infections are:

- Surgical wound skin and skin structure infection (SSSI)
- Osteomyelitis and septic arthritis as complications of orthopedic surgery, including prosthetic device infections
- Bacteremia
- Healthcare and ventilator-associated pneumonia.

2.12.2 Community acquired MRSA

Community acquired MRSA are contracted outside any type of healthcare setting. As per the definition of CDC, any infection diagnosed in outpatient or within 48 hours of hospitalization without evidence of traditional risk factors of MRSA infections such as receipt of hemodialysis, surgery, hospitalization during previous year, presence of indwelling catheter or percutaneous device or previous record of isolation of MRSA (Morrison et al., 2006). Infections caused by CA-MRSA affect healthy individuals, usually manifests as skin infections, such as pimples or boils, cellulitis and are more serious than minor skin irritation. Traditionally, MRSA has been considered a major nosocomial pathogen, but in the past decade, it has emerged as significant pathogen in the community as well. The first case of CA-MRSA infection in the United States was reported in 1980 (Saravolatz et al., 1982**)**. Until the 1990s, infections of MRSA among community members without exposure to the healthcare setting were uncommon (one exception is injection drug users). An outbreak of CA-MRSA infections occurred between 1989 and 1991 among indigenous Australians in Western Australia without healthcare contact (Udo et al., 1993).

.CA-MRSA strains are genetically and phenotypically distinct from HA-MRSA. CA-MRSA strains often produce a cytotoxin called Panton-Valentine leukocidin (*PVL*). The *PVL*-producing CA-MRSA appears to be associated with increased risk of transmission, complications and hospitalization (Nathwani D et al., 2008). CA-MRSA infections are caused by newly emerging strains unlike those responsible for HA-MRSA and are capable of infecting healthy persons without having any links to healthcare systems. CA-MRSA infections typically manifest as skin or soft tissue infections, but can develop into more invasive, life-threatening conditions. CA-MRSA generally spread from person to person by direct contact with the skin, respiratory droplets or via inanimate objects. Staphylococci that are shed into the environment may survive for long period in dust and may transmit the infection. Skin scales may contaminate if they become airborne during activities such as bed-making if the affected person is heavily colonized or has a conditions which causes shedding of high numbers of organisms.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site

This study was conducted at Manipal Teaching Hospital (MTH), Phulbari, Pokhara, Nepal. Manipal Teaching Hospital is 825 bedded multispeciality teaching hospital of Manipal College of Medical Sciences (MCOMS), situated in the Western Region of Nepal. The hospital is a major healthcare provider for the region. The institution caters to the population of about ten of the fifteen districts of Western Development Region of Nepal. The population of these ten districts as per 2001 census was approximately 2 million (Easow et al., 2010). The hospital has an average daily patient load of 700 outpatients and 350 in-patients with seasonal variations.

3.2 Duration of study

This study was conducted for a period of three years from 2012-2015.

3.3 Sample size

A total of 400 non repeated isolates of *S. aureus* were collected from 3051clinical samples and 311 environmental samples.

3.4 Inclusion Criteria

All isolates of *S. aureus* from various clinical specimens and environmental samples from various units of the hospital (Operation Theater and Intensive Care Units).

3.5 Exclusion criteria

Staphylococci other than *S. aureus* (Coagulase negative Staphylococci).

3.6 Sample collection, processing and identification of *S. aureus*

3.6.1 Specimen collection

Various clinical samples like pus (from abscess, drainage, ear discharge, wound swab etc.), sputum, blood, body fluids and urine were collected in sterile containers from patients who visited Manipal Teaching Hospital (both inpatients and out patients). Collected samples were immediately transported to the laboratory for further processing.

3.6.1.1 Collection of nasal swab from hospital staff

Nasal swabs were collected by inserting sterile swab sticks (moistened with 0.85% sterile normal saline) about 2 cm inside into the anterior nostril. It was rotated against the anterior nasal mucosa. Using same swab, procedure was repeated in other nostril. After collection, swabs were placed back into the tube and immediately processed in the laboratory.

3.6.1.2 Sampling from hospital environment

Additional samples from hospital environment including Operation Theater and Intensive Care Units were also collected. Sites of Operation Theater included: bed outer, bed inner, light outer, light inner, wall and floor. Environmental samples from seven different Operation Theaters were collected. Air settle plates from different rooms of Operation Theater were also examined. Sample collection sites of intensive care unit included: phototherapy bed, ventilator, radiant warmer, incubators, laryngoscope, floor, head box, bed side locker, suction tip, and nebulizer. Sampling was done by rubbing the sterile swab moistened with sterile normal saline over the sampling site and then swabs were inoculating test tubes containing peptone water. Air settle plates were exposed for 30 minutes in respective sites and then incubated at 37ºC.

3.6.2 Processing of specimen

Specimens were immediately inoculated on fresh 5% sheep Blood agar, Chocolate agar, MacConkey agar, Nutrient agar, Brain heart infusion broth, Peptone water (Hi Media, Mumbai, India) depending on type of specimen and incubated at 37ºC for 24-48 hours.

Clinical specimen and culture media used

Specimen	Culture media
Pus & wound swab	Blood agar, Chocolate agar, MacConkey agar
Sputum, Throat/Nasal swab,	Blood agar, Chocolate agar, MacConkey agar
Body fluids	Blood agar, Chocolate agar, MacConkey agar
Urine	Blood agar, MacConkey agar
Blood	Biphasic media (Brain heart infusion broth and Brain heart infusion agar)
Environmental samples	Peptone water, Blood agar, Nutrient agar

Smears from the specimen were made and subjected to Gram's stain to demonstrate pus cells and bacterial morphology.

3.6.3 Identification of *S. aureus* isolates

Identification of *S. aureus* was done based on following observations (Mackie & McCartney, 1996).

3.6.3.1 Colony morphology

S. aureus colonies are round, 1-2 mm in diameter, lactose fermenting (pink) on MacConkey agar. On Blood agar, many of the strains show beta hemolysis with clear zone of hemolysis around the colonies while few were non hemolytic.

3.6.3.2 Gram's stain

Gram's stain was performed from clinical specimens as well as colonies for demonstration of Gram positive cocci arranged in clusters.

Following biochemical tests were performed for identification of *S. aureus*:

3.6.3.3 Catalase test

It is based on the principle that enzyme catalase causes liberation of water and oxygen from hydrogen peroxide. All members of the staphylococci are catalase positive, whereas members of the genus streptococcus are catalase negative. Catalase test was performed by transferring bacterial colony using sterile glass rod onto slide containing 3% hydrogen peroxide. Presence of bubbles of gas indicates positive reaction. Colonies of *S. aureus* ATCC 25923 and *Enterococcus* species were used as positive and negative quality control strains respectively.

3.6.3.4 Coagulase test

Gram positive cocci that are catalase positive belong to the family Micrococcaceae, which include the staphylococci. Coagulase test is used as a screening test for *S. aureus* which are almost always coagulase positive. Although some other species of staphylococci (*S. intermedius, S. hycus*) may also give positive coagulase reaction, these species are not commonly associated with human infections. Strains of *S. aureus* produce two different types of coagulase, which include free and bound coagulase. Coagulase test was performed by slide and tube coagulase method.

3.6.3.4.1 Slide coagulase test

This method detects bound coagulase which is also known as clumping factor. Clumping factor cross links α and β chain of fibrinogen in plasma to form fibrin clots resulting into clump formation visible by naked eyes. The slide agglutination test for clumping factor is very rapid but up to 15% of *S. aureus* strains are negative. Therefore, isolates negative in slide tests were subjected to tube coagulase test.

Procedure

Slide coagulase test was performed by emulsifying a staphylococcal colony in a drop of sterile normal saline on a clean and grease free glass slide with a minimum of spreading. Those isolates did not form a smooth, milky suspension, were not processed further and considered as coagulase negative staphylococci. Similarly, suspensions of positive and negative control strains were also made to check reactivity of the plasma. Transfer of plasma over the suspension of test organism as well as positive and negative controls was performed by dipping straight inoculating wire into plasma. A coarse clumping visible to the naked eye within 10 seconds was recorded as positive and absence of clumping within 10 seconds was considered as negative. All slide coagulase test negative strains were subjected to tube coagulase test as definitive test for *S. aureus*.

3.6.3.4.2 Tube coagulase test

This method detects free coagulase which reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. Thrombin converts fibrinogen to fibrin leading to clotting of plasma.

Procedure

Three sterile test tubes were taken and labeled "test", "positive control" and "negative control". Dilution of human plasma (1 in 6 dilutions) in saline (0.85% NaCl) was prepared and 1 ml volume of the diluted plasma was transferred into three labeled tubes (test, positive control and negative control). 0.2 ml of overnight broth culture of test organism and control organisms was added to 1 ml of diluted plasma in respective tubes. Mixture of diluted plasma and bacterial suspension was incubated at 37^{o}C in water bath. The tubes were examined at 1, 2 and 4 hour for clot formation by inclining the tube. Positive results were indicated by gelling of the plasma, which remains in place even after inverting the tube. Test showing no clots after 4 hours were further incubated

overnight at room temperature and examined for clot formation. No clot inside the tube was considered as negative result.

3.6.3.5 DNase test

Deoxyribonuclease (DNase) or DNA hydrolysis test is used to determine the ability of an organism to hydrolyze DNA and utilize it as a source of carbon for growth. DNase agar (Hi Media, Mumbai, India) was used to test the ability of an organism to produce deoxyribonuclease or DNase.

S. aureus grows in the medium produces Deoxyribonuclease and breaks down DNA into smaller fragments. When the DNA is broken down, it no longer binds to the methyl green, and green color fades and the colony is surrounded by a colorless zone. DNase test was performed by heavy spot inoculation of staphylococcal colonies on DNase agar along with positive (*S. aureus*) and negative control (*S. epidermidis*). Plates were incubated at 37ºC for 18-24 hours. 1N HCl was added over the plates and observed for clearing around the spot of inoculation. A clear zone around the spot was considered as positive test.

3.6.3.6 Mannitol fermentation test

Mannitol fermentation property was tested by using mannitol salt agar (Hi Media, Mumbai, India). It contains a high concentration (8%) of salt (NaCl), making medium selective for Staphylococci. High concentration of NaCl is inhibitory to most of other bacteria (CLSI, 2012). It is also a differential medium for mannitol-fermenting staphylococci, containing carbohydrate mannitol and the indicator phenol red for detection of acid production during mannitol-fermentation by staphylococci. (CLSI, 2011) *S. aureus* produce yellow colonies with yellow zones, whereas other staphylococci produce small pink or red colonies with no color change in the medium.

Hospital and community associated *S. aureus* isolates were categorized based on the following criteria: Isolates cultured from clinical specimens that were obtained after 72 hours of admission of the patients or from patients with a history of hospitalization within six months were considered as hospital-acquired *S. aureus* strains; Isolates which were cultured within 72 hours of hospitalization, from outpatient department (OPD) or patients with no history of hospitalization within six months were categorized as community- acquired strains. The clinical information on the patients' clinical background which was used to set the criteria for classification of community and hospital acquired MRSA was obtained from the medical records.

Hospital and community acquired MRSA: HA-MRSA and CA-MRSA were classified as per the standarad guidelines. Any infection diagnosed in outpatient or within 48 hours of hospitalization without evidence of traditional risk factors of MRSA infections such as receipt of hemodialysis, surgery, hospitalization during previous year, presence of indwelling catheter or percutaneous device or previous record of isolation of MRSA were considered as CA-MRSA (Morrison et al., 2006). Infections acquired after admission of the patient, without any evidence of infection or incubation at the time of entry into the healthcare setting were considered as HA-MRSA (Horan et al., 2008).

3.7 Phenotypic characterization of *S. aureus*

This includes Antibiotic Susceptibility Testing (AST), determination of MIC and detection of beta lactamases.

3.7.1 Antibiotic Susceptibility testing

Antibiotic Susceptibility testing of the isolates was performed by Kirby-Bauer disc diffusion method on Muller-Hinton agar plates ((Hi Media, Mumbai, India). Few isolated colonies of *S. aureus* were suspended in sterile peptone water and incubated at 37ºC. Turbidity of the suspension was compared and adjusted to match with the 0.5 McFarland standard. The bacterial suspension was spread over MHA plates by lawn culture.

Following antibiotic discs (Hi Media, Mumbai, India) were tested, with six discs on a 90mm diameter Petridis: penicillin (10 U), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), clindamycin (2 µg), cefazolin (30 µg), amoxicillin-clavulanic acid (20/10 µg), tetracycline (15 µg), trimethoprim/sulfamethoxazole(1.25/23.75 µg) and vancomycin (30 µg).

Antibiotic susceptibility of quality control strain was inoculated simultaneously along with the test organisms. Reading of the plates was carried out after overnight incubation at 35°C, using the CLSI guidelines (CLSI, 2011).

Multidrug resistance (MDR): *S. aureus* showing resistance to at least one agent from three or more antimicrobial categories were labeled as multidrug resistant (Magiorakos et al., 2012).

3.7.2 Screening tests for MRSA

Primary screening of MRSA was carried out by testing the organism against oxacillin (1 µg) and cefoxitin (30 µg).

One microgram oxacillin disc and 30 microgram cefoxitin disc were tested on Mueller Hinton agar containing 4% NaCl. Incubation temperature was 35°C for 24 hours. Zone diameter of the test strain was measured in millimeter with a scale. The isolates were considered methicillin resistant as per the following interpretive criteria (CLSI, 2011).

Interpretive criteria for oxacillin MIC tests

	Susceptible	Intermediate	Resistant
MIC	≤ 2 µg/ml	N/A	≥ 4 µg/ml

Interpretive criteria for oxacillin disc diffusion tests

	Susceptible	Intermediate	Resistant
Diameter of zone of inhibition	≥ 13 mm	11-12 mm	≤ 10 mm

Interpretive criteria for cefoxitin disc diffusion test

	Susceptible*	†	Resistant**
Diameter of zone of inhibition	≥ 22 mm		≤ 21 mm

Interpretive criteria (in µg/ml) for cefoxitin MIC tests

	Susceptible	Intermediate	Resistant
MIC	≤ 4 µg/ml	N/A	≥ 8 µg/ml

* Report as oxacillin susceptible

** Report as oxacillin resistant

†There is no intermediate category with the cefoxitin disk diffusion test.

3.7.3 Detection of MIC (Minimal inhibitory concentration)

Minimal inhibitory concentration was carried out by cefoxitin E test strip as per CLSI guidelines (CLSI, 2012). Standard inoculum was prepared by few colonies of *S. aureus* into sterile normal saline. Turbidity of the suspension was compared with 0.5 McFarland standard and adjusted accordingly. Sterile cotton swabs were dipped into the prepared suspension and swabs were rotated several times against the inside of the test tube to remove excess liquid. Dried Mueller Hinton plates were inoculated with the swabs by repeated streaking motion. After 5 minutes of inoculation E test strip was applied over the medium and plates were incubated at 35°C. After overnight incubation, results were recorded and interpreted was done as per the above mentioned criteria.

3.7.4 Detection of inducible clindamycin resistance

Detection of inducible clindamycin resistance was performed by D-test. All the isolates resistant to erythromycin were subjected to D-test as per CLSI guidelines (CLSI, 2012). The erythromycin disc was placed at distance of 15mm (edge to edge) from clindamycin disc on Mueller Hinton agar plate as for standard disc diffusion test. A flattening of the zone of inhibition in the area between the discs with D shaped appearance after 18–24 hours of incubation was considered to give an indication of inducible clindamycin resistance.

3.8 Genotypic characterization of MRSA isolates

3.8.1 DNA extraction

DNA extraction was performed by Phenol-Chloform method as described by Sambrook et al., 1989, with minor modifications.

All the preserved isolates of Methicillin Resistant *S. aureus* (MRSA) were first sub cultured on nutrient agar. Approximately 570µl Tris buffer with Tritonex was taken in Eppendorf tube and isolated colonies of MRSA were mixed. Mixed/Vortexed the mixture and 30µl of 10% Sodium Dodecyl Sulphate (SDS) was added to the above mixture. 3µl of Proteinase K (20mg/ml) was added, mixed and kept at 37ºC for overnight incubation. Then 100µl of 5M NaCl was added, mixed/Vortexed and 80µl of Cetyl Trimethyl Ammonium Bromide (CTAB) was added. Mixture was incubated at 60ºC in water bath for 10 minutes.

Equal volume of Phenol Chloform Isoamyl alcohol (25:24:1) was added to above mixture, vortexed and centrifuged at 10,000 rpm for 10 minutes. After centrifugation, supernatant solution (aqueous solution) was separated in another Eppendorf tube and equal volume of Chloroform and Isoamyl alcohol (24:1) mixture was added to the supernatant solution. Mixed/Vortexed and centrifuged at 10,000 rpm for 10 minutes. Supernatant solution (aqueous solution) was separated in another Eppendorf tube. Equal volume of Isopropanol was added to above solution and kept at room temperature for 5 minutes. Mixed/Vortexed and centrifuged at 10,000 rpm for 10 minutes. Supernatant solution was discarded and 200 µl of 70% Ethanol was added into the deposit. Mixture was centrifuged at 10,000 rpm for 10 minutes. Supernatant was discarded and tubes were

kept open inside incubator for 30 minutes to allow the evaporation of ethanol. Finally, 50 µl of TE buffer (PH 8) was added and tubes were stored inside deep freeze at -20ºC. Molecular characterization of MRSA was performed at Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University (BHU), India.

3.8.2 Detection of *mecA* gene

The *mecA* gene is located on mobile gene element, known as the Staphylococcal cassette chromosome mec. Primers used for detection of *mecA* gene: mecA1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and mecA2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A) primers, as described earlier (Geha et al., 1994).

Master Mix preparation

10X Buffer	2.5 µl
DNTP mix	2µl
Primer 1	1.5 µl
Primer 2	1.5 µl
Taq polymerase	0.33 µl
Distilled water	

DNA thermocycler (Biometra, Germany) was programmed for initial denaturation at 94ºC for 4 min; 30 cycles of amplification (denaturation at 94ºC for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 30 seconds); and a final extension at 72°C for 2 minutes. To visualize, 10 µl of the PCR amplicon was loaded in 1.2% agarose gel in TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) containing 0.5 µl/ml of ethidium bromide and visualized by Gel documentation system (Alfa Imager 2200, Alfa Innotech Corporation, USA).

3.8.3 Detection of *mecA* and *PVL* genes by multiplex Polymerase Chain Reaction (PCR)

DNA extraction of the MRSA isolates was performed by using chloroform: phenol extraction method (Sambrook et al., 1989). Primers used for *mecA* gene were MecA1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and MecA2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A) (Geha et al., 1994). Primers used for detection of *PVL* genes were Luk-PV-1 (ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A) and Luk-PV-2 (GCA TCA AGT GTA TTG GAT AGC AAA AGC) (McClure et al., 2006). DNA thermocycler (Biometra, Germany) was programmed for initial denaturation at 94 °C for 4 minutes; 30 cycles of amplification (denaturation at 94 °C for 45 seconds, annealing at 56 °C for 45 seconds, and extension at 72 °C for 30 seconds); and a final extension at 72 °C for 2 minutes. To visualize, 10 µl of the PCR amplicon was loaded with dye in 1.2% agarose gel containing ethidium bromide followed by electrophoresis at 100 volts for one hour and visualized by using UV transillumination at 310 nm. Images of the test were obtained by gel documentation system (Alfa Imager 2200, Alfa Innotech Corporation, USA). Fragments of DNA 310 bp corresponded with *mecA* gene and 433 bp corresponded amplification of a fragment to the *PVL* genes.

3.9 Detection of biofilm production

Biofilm formation among staphylococci was detected by microtitre plate assay, with minor modifications (Christensen et al., 1985). Bacterial suspension of each strain was prepared in trypticase soya broth (Hi media) and incubated overnight at 37°C. Further, 1:100 dilution of overnight broth was prepared in trypticase soya broth and 200ul of diluted broth was added to microtitre plate wells along with 10 controls containing equal volume of trypticase soya broth. Microtitre plate was then covered with aluminum foil and incubated at 37°C in the incubator. After incubation, bacterial suspension was taken out from the wells using micropipette and the wells were washed 3 times with phosphate buffer saline. 200 µl of picric acid solution (Boine's fixative) was added to each well and plate was incubated for 5 minutes at room temperature. All the wells were washed with

sterile distilled water and 200 µl of Hucker's crystal violet was added to all the wells. Plate was again incubated for 5 minutes at room temperature. The dye was taken out and wells were washed 5 times by adding sterile distilled water drop by drop.

The optical density of empty wells was taken at 470 nm using ELISA reader. The optical densities of test organisms and blank controls (trypticase soya broth with bacteria) were obtained. Cut off value was calculated as three standard deviations above the arithmetic mean of ten blanks (3xSD + mean optical density of ten blanks).

3.10 Data analysis

Percentage resistance against various antibiotics amongst MSSA and MRSA groups was compared by using Pearson's Chi-square test. A p-value of <0.05 was considered as statistically significant. Similarly, pattern of resistance amongst OPD Vs ward isolates were compared using above statistical methods.

3.11 Ethical clearance

All the samples included in this study were from routine clinical specimens received at the Microbiology laboratory for investigations. None of the sample included in this study was collected separately for the study purpose from the patients. Permission to conduct the study was obtained from the Institutional Ethical Committee.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Clinical and microbiological profile of *S. aureus* (MRSA & MSSA)

S. No.	Specimen	Number of samples (%) (n=3362)	Number of *S.aureus* isolates (%) (n=400)	Number of MRSA isolates (%) (n=139)

A total of 400 *S. aureus* strains were isolated from various clinical specimens (n=3051) and hospital environmental samples (n=311). Details of clinical specimens and relative distribution of MRSA are shown in Table 1. Majority of *S. aureus* isolates were cultured from pus and wound swabs followed by blood, urine, sputum, hospital environmental samples, throat swabs and body fluids.

Table 1: Frequency of *S. aureus* and MRSA in various specimens

1	Pus & wound swab	410 (12.2)	288 (70.2)	98 (23.9)
2	Blood	1135 (33.7)	46 (4)	14 (1.5)
3	Urine	984 (29.2)	28 (2.8)	12 (1.3)
4	Sputum	346 (10.3)	16 (4.6)	6 (1.7)
5	Hospital environment (OT/ICU)	311 (9.2)	15 (4.3)	7 (2.2)
6	Throat swab	94 (2.8)	5 (5.3)	0 (0.0)
7	Body fluids	82 (2.4)	2 (2.4)	2 (2.4)

Primary screening of MRSA performed by oxacillin and cefoxitin disc diffusion method detected 128 and 141 MRSA respectively. Out of 400 isolates 139 (34.7%) were *mecA* positive and confirmed as MRSA. Photograph 1 shows *mecA* gene (310bp) detection by PCR.

Out of total 3051patients, 1654 were male and remaining 1397 were female. MRSA was isolated in 4.2% (71/1654) male patients, 4.3% (61/1397) among female patients and 2.2% (7/311) were from environmental samples. Out of 139 MRSA, 71 were isolated from male, 61 from female patients and the remaining 7 were isolated from environmental samples.

Detailed clinical information related to the patients was obtained from medical record. Table 2 shows various clinical conditions associated with MRSA infections.

Table 2: Clinical conditions associated with MRSA infections

S. No.	Clinical diagnosis	Number of samples	Number of MRSA	Percentage (%)
1	Cellulitis	180	57	31.6
2	Breast abscess	107	14	13

3	Burn wound	36	13	36.1
4	Post operative wound	34	5	14.7
5	Diabetic foot	32	5	15.6
6	Gluteal abscess	21	4	19
7	Bacteraemia/Septicemia	935	12	1.3
8	Urinary tract infection	881	12	1.3
9	Pneumonia/LRTI	346	06	1.7
10	Osteomyelitis	10	02	20
11	Peritonitis	13	01	7.7
12	Pleural effusion	12	01	8.3
13	Pharyngitis/Tonsilitis	94	0	0

S. No.	Department	Total samples	Number of MRSA isolates (n=139)	Percentage (%)
1	Surgery	476	80	16.8
2	Medicine	1482	14	0.9
3	Pediatrics	526	11	2.1
4	ICU	125	09	7.2
5	ENT	94	07	7.4
6	OBG	303	06	1.9
7	Post-operative	45	05	11.1

The majority of the *S. aureus* isolates were cultured from the clinical cases of cellulitis followed by breast abscess, burn wound etc as shown in Table 2.

The majority of MRSA and MSSA were from pus samples received from the surgery department. Department/Unit wise distribution of MRSA isolates is shown in Table 3.

8	Hospital environment	311	07	2.2

Table 3: Department/Unit wise distribution of MRSA isolates.

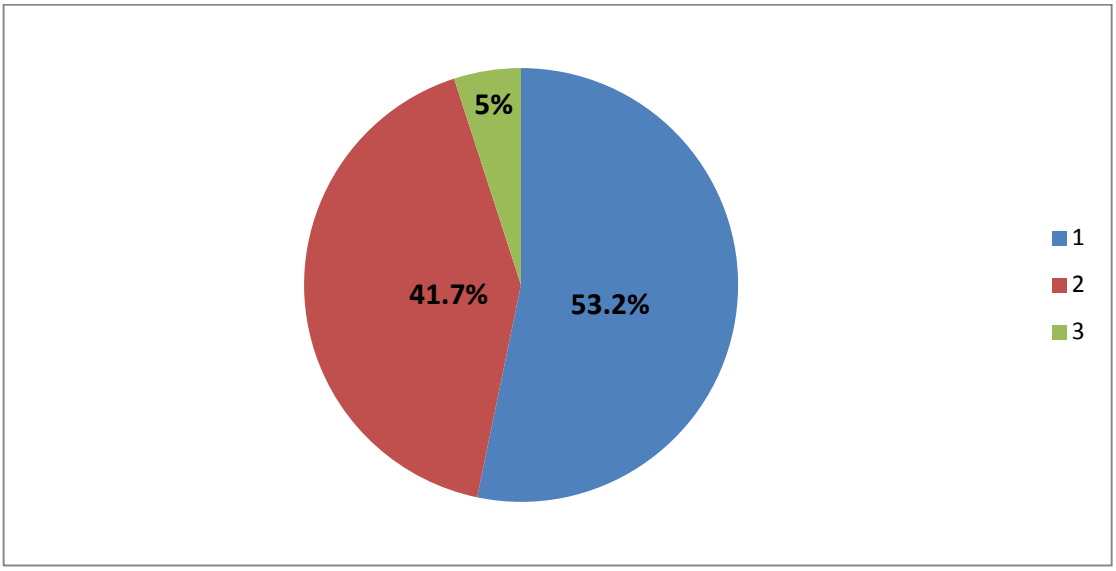

Figure 1: Distribution of sources of MRSA isolates (1: MRSA isolated from Inpatients, 2: MRSA isolated from out patients, 3: MRSA isolated from hospital environment)

A total 74 (53.2%) of the MRSA isolated from ward patients 58 (41.7%) from OPD patients (Figure 1) and the remaining 7 (5%) isolates were from hospital units (OT and ICUs).

Out of 139 MRSA isolates, 56 (40.3%) were found to be hospital associated MRSA and remaining 83 (59.7%) were community associated MRSA according to the criteria mentioned above.

Out of 112 hospital staff tested, only 8 (7.1%) were found to be MRSA nasal carriers. Details of distribution of *S. aureus*/MRSA isolates among hospital staff is shown in Table 4.

Table 4: Distribution of *S. aureus*/MRSA isolates among hospital staff

Antibiotic resistance pattern of the isolates showed that, majority of *S. aureus* were

S. No.	Antibiotic	Resistant isolates (%) (n=400)	Resistant MSSA isolates (%) (n=261)	Resistant MRSA isolates (%) (n=139)	*Chi square (p value)

S. No.	Health care workers	Total number	Number of *S. aureus* isolates	Number of MRSA
1	Staff nurse	40	06	4
2	Doctor	25	04	2
3	Students	22	03	1
4	Attender/Cleaning staff	25	01	1

resistant to penicillin. Among MRSA isolates, the majority were resistant to erythromycin, ciprofloxacin and cotrimoxazole.

Table 5 shows overall resistance pattern of *S. aureus* with comparison between resistance pattern of MRSA and MSSA isolates. Our study revealed that, the majority of MRSA (73.3%) isolates were multidrug resistant as per the standard criteria mentioned above. Resistance to penicillin was highest (93.7%) followed by erythromycin (53%), ciprofloxacin (48%), cotrimoxazole (44%) amoxycillin-clavulanic acid (35.4%) and gentamicin. Antibiotics like cefazolin, amikacin tetracycline and clindamycin were found effective. No vancomycin resistant isolate was found (Figure 2).

Table 5: Antibiotic resistance pattern of MSSA and MRSA isolates

S. No.	Antibiotic	MRSA isolates (%)	MRSA isolates (%) OPD	MRSA isolates (%) Ward	*Chi square (p value)
1	Penicillin	375 (93.7)	236 (90.4)	139 (100)	14.02 (<0.001)
2	Erythromycin	212 (53)	110 (42.1)	102 (73.4)	35.524 (<0.001)
3	Ciprofloxacin	192 (48)	80 (30.6)	112 (80.5)	90.568 (<0.001)
4	Cotrimoxazole	176 (44)	83 (31.8)	93 (66.9)	45.364 (<0.001)
5	Cefoxitin	141 (35.2)	0	141 (35.2)	--
6	Amoxycillin-clavulanic acid	142 (35.4)	40 (15.3)	102 (73.4)	133.504 (<0.001)
7	Cefazolin	52 (13)	11 (4.2)	41 (29.5)	51.257(<0.001)
8	Gentamicin	86 (21.5)	20 (7.6)	66 (47.5)	85.206 (<0.001)
9	Clindamycin	17 (4.2)	02 (0.8)	15 (10.8)	22.400 (<0.001)
10	Amikacin	15 (3.7)	01 (0.4)	14 (10)	23.589 (<0.001)
11	Tetracycline	15 (3.7)	5 (1.9)	10 (7.2)	7.001 (0.008)
12	Vancomycin	0	0	0	--

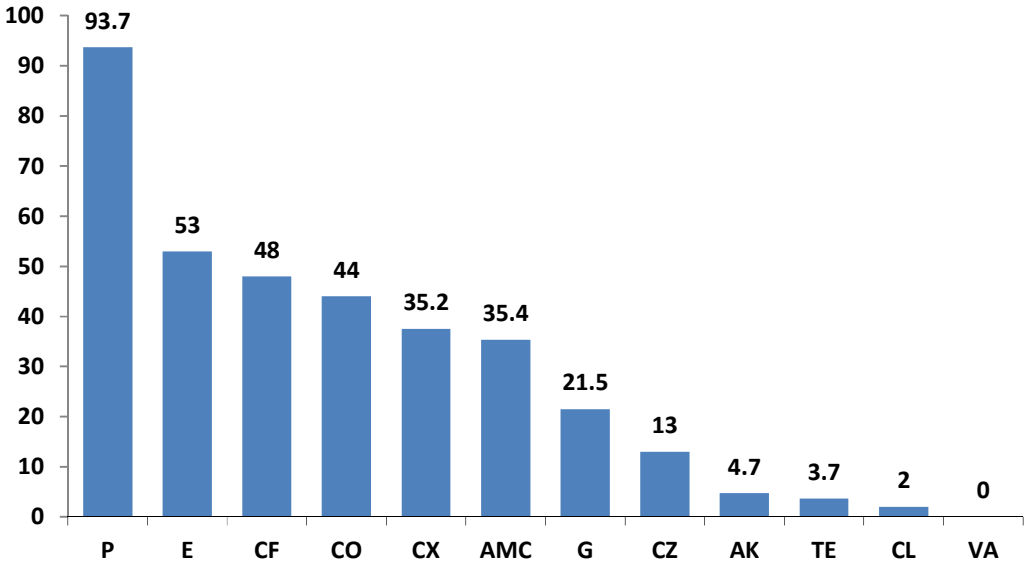

Figure 2: Antibiotic resistance pattern of *S. aureus* (n=400) isolates.

Table 6: Antibiotic resistance pattern of MRSA and its distribution in OPDs and wards

P: Penicillin, E: Erythromycin, CF: Ciprofloxacin, CO: Cotrimoxazole, CX: Cefoxitin, AMC: Amoxycillinclavulanic acid, G: Gentamicin, CZ: Cefazolin, AK: Amikacin, TE: Tetracycline, CL: Clindamycin, VA: Vancomycin.

		(n=139)	(n=58)	(n=81)	
1	Penicillin	139 (100)	58 (100)	81 (100)	--
2	Erythromycin	98 (70.5)	31 (53.4)	67 (82.7)	13.922 (<0.001)
3	Ciprofloxacin	112 (80.5)	38 (65.5)	74 (91.3)	14.420 (<0.001)
4	Cotrimoxazole	93 (66.9)	35 (60.3)	58 (71.6)	1.935 (0.164)
5	Amoxycillin-Clavulanic acid	102 (73.3)	37 (68.9)	65 (80.2)	4.684 (0.0230)
6	Cefazolin	41 (29.5)	11 (18.9)	30 (37)	5.308 (0.021)
7	Gentamicin	66 (47.5)	22 (37.9)	44 (54.3)	3.641 (0.041)
8	Clindamycin	15 (10.8)	6 (10.3)	9 (11.1)	0.021 (0.886)
9	Amikacin	14 (10)	4 (6.9)	10 (12.3)	1.108 (0.293)
10	Tetracycline	10 (7.2)	3 (5.1)	7 (8.6)	0.609 (0.435)
11	Vancomycin	0	0	0	--

Resistance pattern of MRSA and its relative distribution in ward and OPD patient revealed that MRSA isolated from ward patients have significantly higher antibiotic resistance as compared to OPD isolates (Table 6)

Table 7: Cefoxitin MIC values and *mecA* positivity among MRSA isolates

S No	Cefoxitin MIC (μg/ml)	Number of isolates	Number of *mecA* positive
1	8	14	14
2	12	65	65
3	16	52	52
4	24	06	06
5	32	02	02

Cefoxitin MIC results were correlated with results *mecA* gene by PCR. A detail of cefoxitin MIC values and correlation with *mecA* gene is depicted in Table 7.

The majority (73.4%) of MRSA isolates were found multidrug resistant while only 26.6% isolates of MRSA were non MDR (Figure 3).

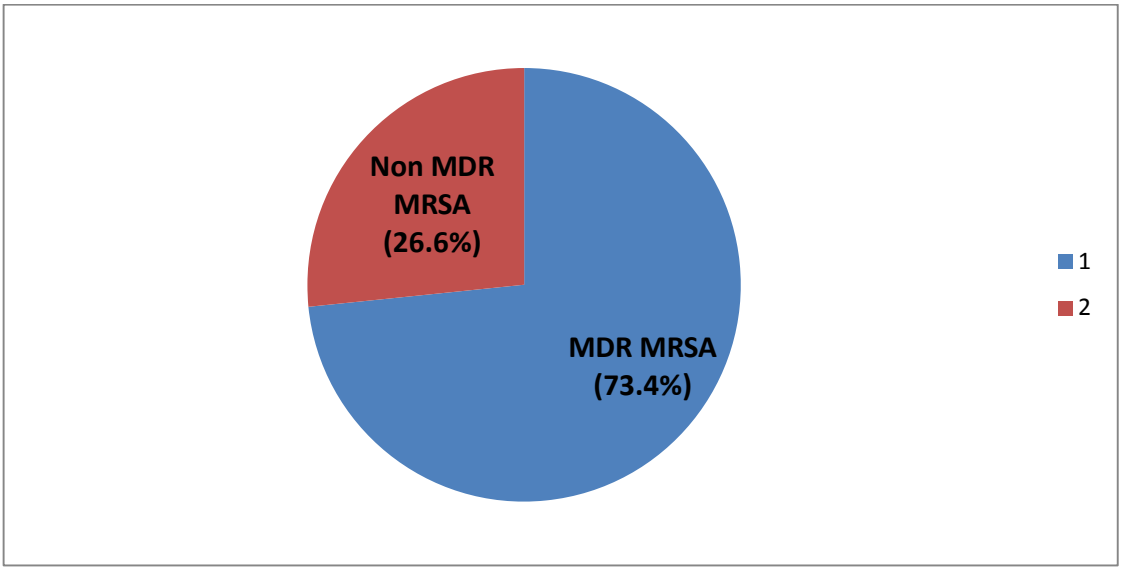

Figure 3: Distribution of MDR MRSA (1) and Non MDR MRSA (2)

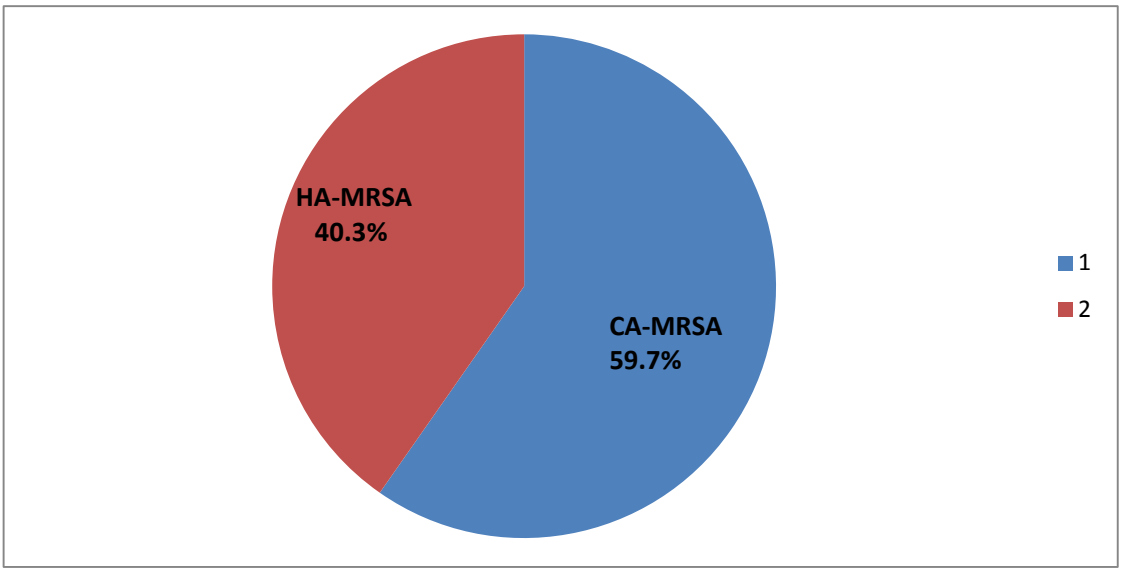

Figure 4: Distribution of community acquired MRSA (1) and hospital acquired MRSA (2)

Out of 139 MRSA isolates, 83(59.7%) were classified as CA-MRSA and 56 (40.3%) were HA-MRSA (Figure 4) as per above mentioned criteria.

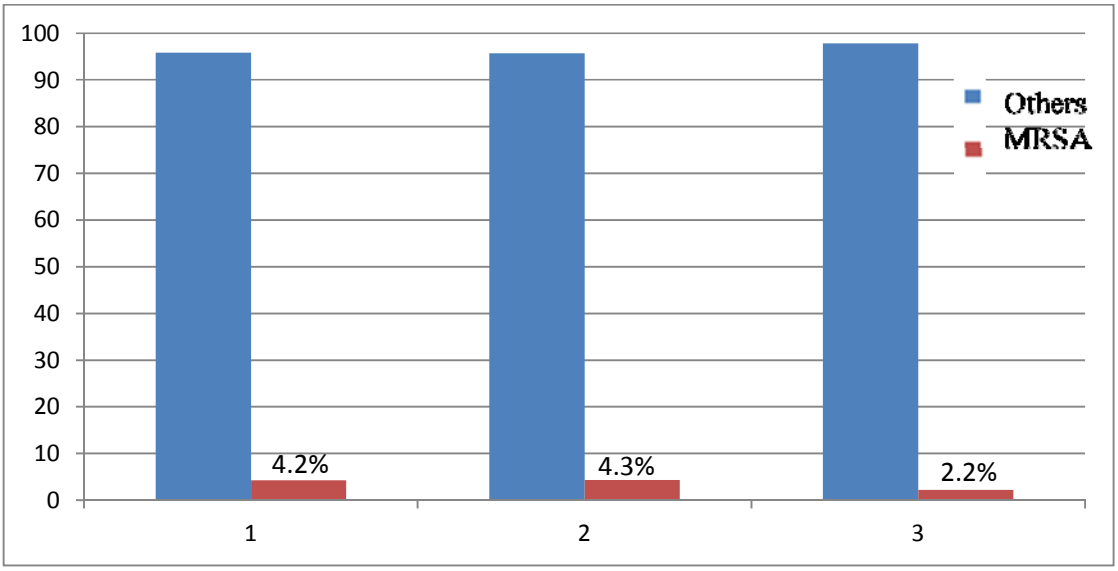

Figure 5: MRSA distribution among male (1), female (2) and hospital environmental isolates (3)

Out of total 139 MRSA isolates, 4.2% (71/1654) were isolated from male patients, 4.3% (61/1397) were from female patients and 2.2% (7/311) were from hospital environmental samples (Figure 5).

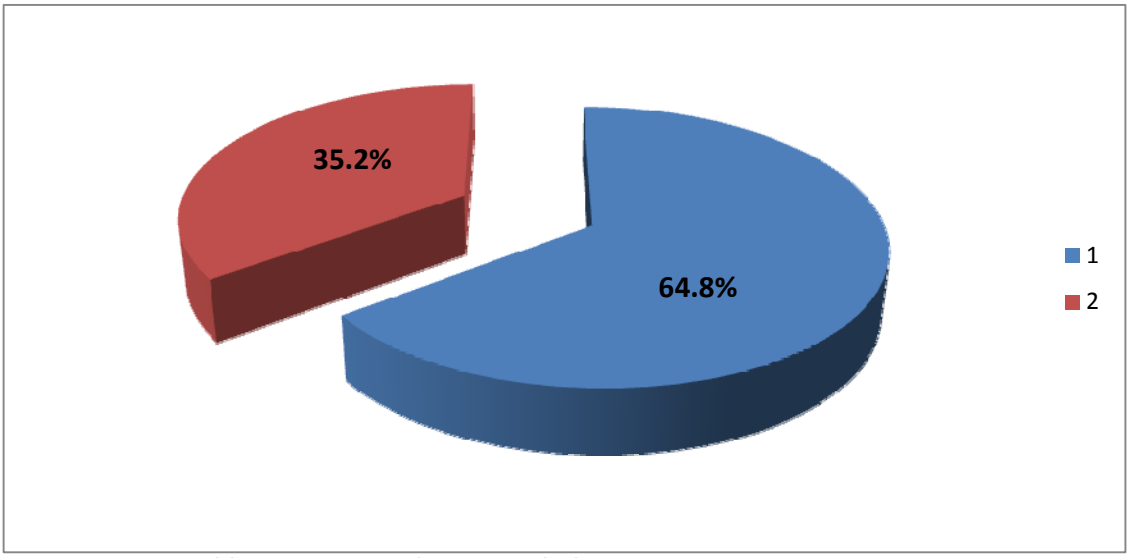

Figure 6: D-test positive MRSA (1) and MSSA (2) isolates.

Out of 212 erythromycin resistant *S. aureus* isolates, inducible clindamycin resistance was detected in 54 (25.4%) isolates by D-test. Out of 54 D-test positive isolates, 35 (64.8%) isolates were MRSA while remaining 19 (35.2%) were MSSA (Figure 6).

4.2 Distribution of Panton Valentine leukocidin (*PVL*) genes among MRSA

Panton Valentine leukocidin (*PVL*) genes were detected in 79 (56.8 %) of the 139 MRSA. Photograph 2 shows *mecA* (310bp) and *PVL* genes (433bp) detection by multiplex PCR. Distribution of *PVL* genes among MRSA isolates in different specimen is shown in Table 8. The majority of the *PVL* positive strains were isolated from pus samples from surgery department. Out of 79 *PVL* positive MRSA, 48(60.8 %) were from male and 31(39.2 %) were from female patients. All seven MRSA obtained from environmental samples did not have *PVL* genes. Out of the 139 MRSA, 56 (40.3%) were found to be hospital associated and the remaining 83 (59.7%) isolates were classified community associated MRSA. Among these 83 CA-MRSA, 75 (90.4%) were *PVL* positive while *PVL* was detected only in 4 out of 56 (7.1%) hospital associated MRSA strains.

Table 8: Distribution of *PVL* genes among MRSA isolates in different specimens

S. No.	Specimen type	Total number of MRSA	*PVL* positive (%)
1	Pus	98	74 (75.5)
2	Blood	14	02 (14.3)
3	Urine	12	02 (16.6)
4	Sputum	06	01 (16.6)
5	Hospital environment	07	0 (0.0)
6	Body fluids	02	0 (0.0)

Table 9 shows antibiotic resistance pattern of *PVL* positive and *PVL* negative MRSA isolates. Antibiotic resistance among *PVL* negative MRSA isolates was higher than the *PVL* positive MRSA, however, the difference was not statistically significant except for erythromycin. Out of 139 MRSA isolates, 102 were multidrug resistant of which, 50 were *PVL* positive and remaining 52 were *PVL* negative. Overall percentage of *PVL* positive MDR MRSA was 63.3% (50/79) while *PVL* negative MDR MRSA was 86.6% (52/60).

Table 9: Antibiotic resistance pattern of *PVL* positive and *PVL* negative MRSA isolates.

S. No.	Antibiotic	*PVL* positive MRSA (n=79) Frequency (%)	*PVL* negative MRSA (n=60) Frequency (%)	P value
1	Penicillin	79 (100)	60 (100)	--
2	Erythromycin	52 (65.8)	50 (83.3)	0.021
3	Ciprofloxacin	61 (77.2)	51 (85)	0.251
4	Cotrimoxazole	48 (60.7)	45 (75)	0.077
5	Amoxycillin Clavulanic acid	55 (69.6)	47 (78.3)	0.250
6	Gentamicin	34 (43)	32 (53.3)	0.229
7	Cefazolin	20 (25.3)	21 (35)	0.215
8	Clindamycin	08 (10.1)	07 (11.6)	0.772
9	Amikacin	06 (7.6)	08 (13.3)	0.266
10	Tetracycline	4 (5)	06 (10)	0.265
11	Vancomycin	0	0	--

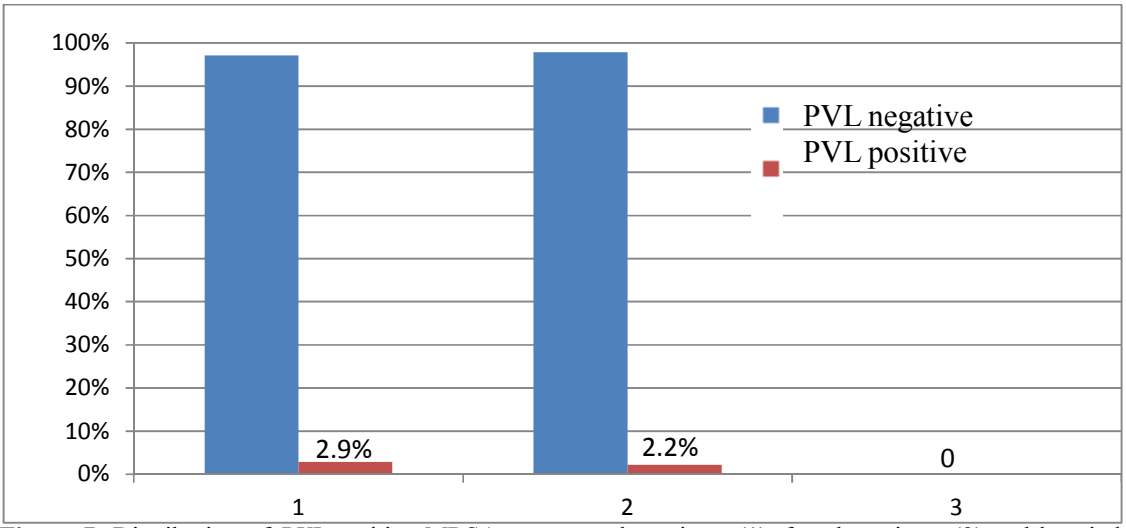

Figure 7: Distribution of *PVL* positive MRSA among male patients (1), female patients (2) and hospital environmental isolates (3).

Distribution of *PVL* among male patients was 2.9% (48/1654) and female 2.2% (31/1397). No *PVL* was detected among hospital environmental isolates (Figure 7).

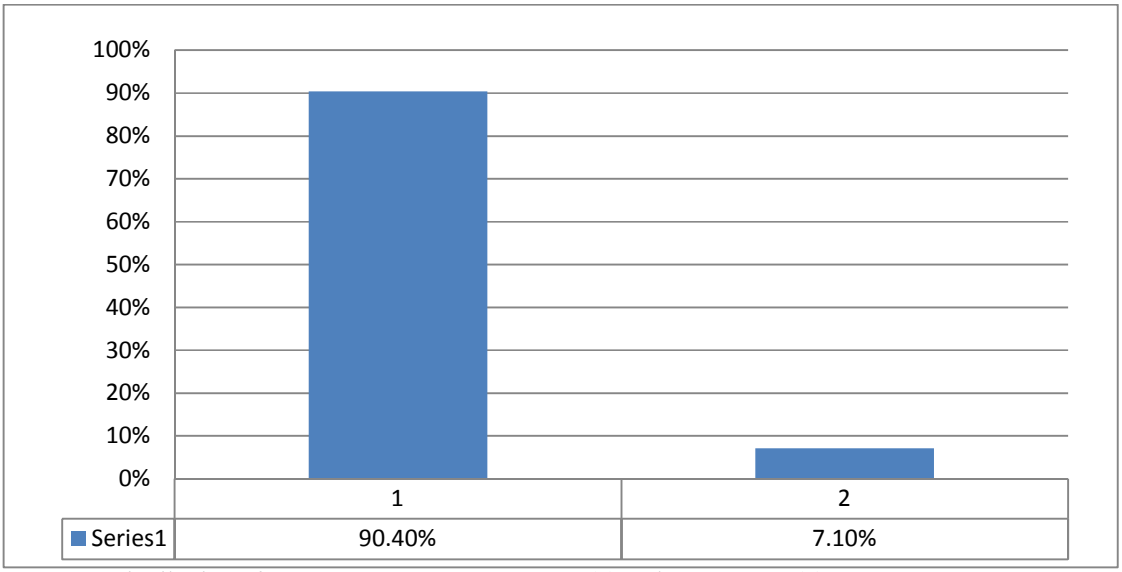

Figure 8: Distribution of *PVL* genes among CA-MRSA (1) and HA-MRSA (2)

The majority of CA-MRSA strains were found positive for *PVL* genes while only 7.1% HA-MRSA strains were associated with *PVL* genes (Figure 8).

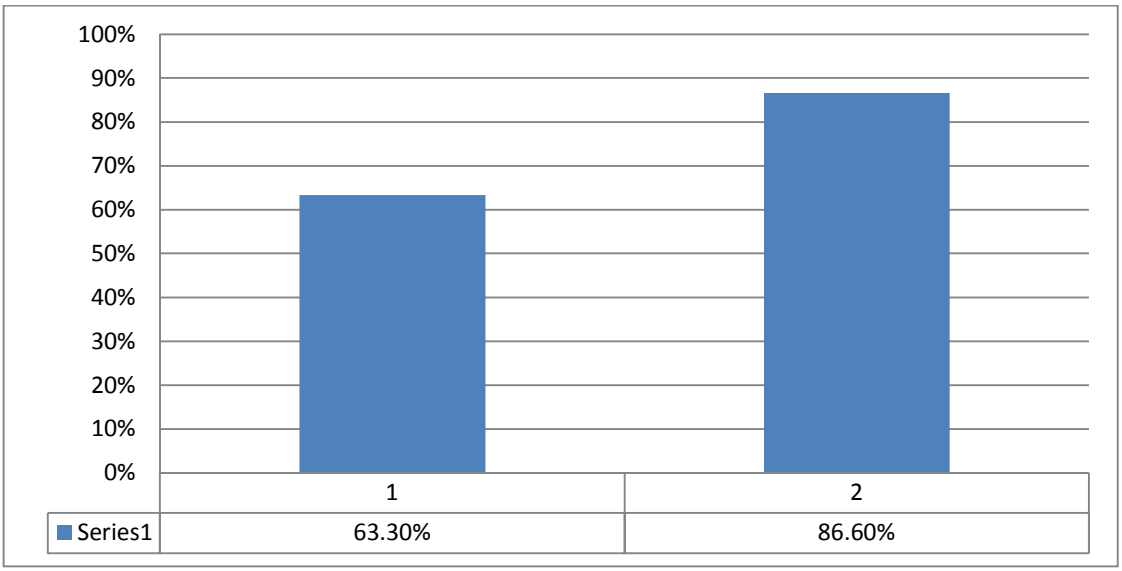

Figure 9: Comparison of *PVL* positive MDR MRSA (1) and *PVL* negative MDR MRSA (2)

The relative percentage of MDR MRSA was found high among *PVL* negative isolates as compared to *PVL* positive isolates (Figure 9)

4.3 Biofilm assay among MRSA and MSSA

A total of 148 isolates of *S. aureus* were tested for biofilm assay. Of these, 94 (63.5%) were MRSA and 54 (36.5%) were MSSA. Out of the total of 94 MRSA isolates, 39 (41.5%) were biofilm producers. On the contrary, only 9 (16.7%) of the 54 MSSA were found to be biofilm producers and the difference was statistically significant (Table 10; χ^2= 10.44; p<0.05).

Table 11 depicts the distribution of multidrug resistance amongst the biofilm producers and non-biofilm producers. Out of the 48 biofilm producing organisms a vast majority 40 (83.3%) were MDR as compared to only 46 (46%) amongst the 100 biofilm non-producers. This difference was found to be statistically significant (p<0.001).

Significant difference was not observed in the biofilm producers and non-producers when we analyzed the sources of these isolates. Altogether 40 (83.3%) out of 48 biofilm positive bacteria were the ones isolated from pus samples alone (Table 12). Of the 105 *S. aureus* isolates from pus, 40 (38.1%) were biofilm producers and 65 (61.9%) were non producers. A large proportion 32 (80%) of these 40 biofilm producers were the isolates from cases of deep seated infections suggesting thereby that biofilm producing *S. aureus* had the potential to produce serious deep infection probably being protected from the adverse effects of the antibiotics and defense mechanism of the body.

Table 10: Biofilm production among MRSA and MSSA isolates

Methicillin sensitivity	Biofilm production		Total
	Positive (%)	Negative (%)	
MRSA	39*(41.5)	55 (58.5)	94
MSSA	09*(16.7)	45 (83.3)	54
Total	48(32.4)	100(67.6)	148

*χ^2= 10.44; p<0.05

Table 11: Multidrug resistance shown by biofilm producing and non-biofilm producing *S. aureus*

S No	Biofilm production	Drug resistance		Total
		MDR (%)	Non-MDR (%)	
1	Biofilm producers	40* (83.3)	8 (16.7)	48
2	Non-biofilm producers	46* (46)	54 (54)	100
3	Total	86 (58.1)	62 (41.9)	148

*χ^2= 18.52; p<0.001

Table 12: Biofilm producers and non-producers *S. aureus* isolated from various clinical specimens

S No	Specimen (Number)	Biofilm producer (%)	Non biofilm producer (%)
1	Pus (105)	40 (38.1)	65 (61.9)
2	Blood (21)	3 (14.3)	18 (85.7)
3	Sputum (6)	1 (16.7)	5 (83.3)
4	Urine (4)	1 (25.0)	3 (75.0)
5	Environmental (12)	3 (25)	9 (75)
6	Total (148)	48 (32.4)	100 (67.6)

4.4 DISCUSSION

4.4.1 Drug resistance among *S. aureus* and MRSA

S. aureus is one of the most common human pathogen associated with pyogenic infections. In this study of 400 *S. aureus* isolates, 288 (72%) were isolated from pus samples indicating their key role in pyogenic soft tissue and wound infections. Increasing drug resistance among bacterial pathogens and dwindling availability of newer antimicrobial is worrisome. Among Gram positive bacteria, *S. aureus* is notorious for developing resistance against various antimicrobial agents.

Antimicrobial resistance is a global threat and MRSA has emerged as an important human pathogen with wide range of antibiotic resistance. Global scenario of MRSA is not uniform and great variation in its prevalence has been observed. Earlier reports of MRSA from Nepal reported prevalence of 15.4%-29% (Subedi et al., 2005, Rai et al., 1990 & Kumari et al., 2008). Recent studies from various hospitals of Nepal show higher prevalence 39.6%-69% (Sanjana et al., 2010 & Tiwari et al., 2009) as compared to our study (Bhatta et al., 2015).

Most of the MRSA related studies conducted in Nepal used only cefoxitin and/or oxacillin for screening (Subedi et al., 2005, Rai et al., 1990 & Kumari et al., 2008). In our study, methicillin resistance status was confirmed by detecting *mecA* gene. As per our results, cefoxitin disc diffusion method gives satisfactory results when zone diameter is <19mm. If zone diameter is between 19-22mm, then results are difficult to interpret and need confirmation by more sensitive and specific methods like MIC and *mecA* gene detection. This may be one of the reasons of under reporting of MRSA in our study as compared to other studies from Nepal (Ansari et al., 2014 & Tiwari et al., 2008). Prevalence of MRSA at Manipal teaching hospital was 34.7% which is comparable with study from Chitwan, Nepal (MRSA prevalence 39.6%). MRSA prevalence in our study is comparable (38.4%) with the study from northern India (Tiwari et al., 2008).

A small percentage of the MSSA isolates (9.6%) were found susceptible to penicillin. Significant difference in antibiotic resistance pattern was found among MRSA and MSSA isolates (P=<0.05) reflecting increased ability of MRSA to develop resistance against various antimicrobials as shown in Table 5. The majority of MSSA strains were sensitive to antibiotics like ciprofloxacin, erythromycin, cotrimoxazole, and amikacin except penicillin. Comparative study of resistance pattern of MRSA in ward and OPD patients showed higher resistance in ward isolates. Manipal Teaching Hospital, being referral hospital, majority of the ward patients are referred from other hospitals after admission of variable duration and primary treatment. Prolonged hospital stay and prior exposure to antibiotics could be one of the possible reasons associated with higher resistance among ward isolates as compared to OPD. Resistance to ciprofloxacin, amoxicillin- clavulanic acid and gentamicin was significantly higher among MRSA isolates from wards as compared to OPD (P=<0.05).

Vancomycin was the only drug to which 100% isolates were susceptible. However, the possibility of emergence of vancomycin resistance should always be kept in mind (Tiwari et al., 2008). Although, no vancomycin resistant *S. aureus* was found in our study, yet vancomycin should never be considered as first line drug, in view of the possibility of emergence of resistance. Thus, other drugs like clindamycin and amikacin which were found quite effective against MRSA in the present study would be better options for the management of such infections.

Overall resistance of *S. aureus* to antibiotics likes ciprofloxacin, erythromycin, co-trimoxazole and amoxicillin-clavulanic acid was found high. These antibiotics being cheaper and easy to administer were extensively used in past few years which have now been slowly replaced by newer antibiotics like cefixime and cefpodoxime. Use of expensive and injectable antibiotics like gentamicin and amikacin was less in small clinics and reflects on higher percentage of sensitive isolates. Clindamycin being more expensive and was not much in use in past years also showed relatively high sensitivity

against MRSA isolates. Higher rates of resistance to various antimicrobials in Western region of Nepal may be attributed to the low socio-economic status of the patients, lack of appropriate medical facilities, partial treatment and prescription of antibiotics without susceptibility testing, besides easy access to antibiotics across the counter. Indiscriminate use of antibiotics and delay in seeking medical treatment could be other reason for high rate of resistance. Manipal teaching hospital is a tertiary care center of the Western Nepal, many patients take the initial treatment in primary healthcare centers or in small pharmacy clinics before reporting to us. Patient usually come to this hospital when the disease becomes chronic, this could be another possible reason for higher percentage of drug resistance in our study. Due to lack of trained staff and unavailability of basic infrastructure, there are challenges in the healthcare facilities to perform AST in adequate manner. This could be one of the important factors responsible for indiscriminate use of antibiotics ultimately contributing to drug resistance.

In vitro susceptibility testing results provides valuable information to clinician for the treatment of microbial infections but sometimes *in vitro* tests may not reflect *in vivo* effectivity. Clindamycin, a lincosamide has been better treatment option for skin and soft tissue infections caused by MSSA and MRSA. One of the major problems with therapeutic use of clindamycin in staphylococcal infection is the possibility of presence of inducible resistance to clindamycin and possibility of clinical failure despite *in vitro* susceptible report. Resistance to clindamycin is highly variable and prevalence varies by geographical regions and even different hospitals of the same region (Fiebelkorn et al., 2003). Due to high rate of spontaneous mutation during therapy, organisms are likely to develop resistance to clindamycin (Prabhu et al., 2011). Knowledge regarding inducible clindamycin among the clinical microbiologists and clinicians is important to avoid false susceptibility against clindamycin during routine antibiotic susceptibility testing. Prevalence of inducible clindamycin resistance in our study was 25.4% which is higher than other studies conducted in Nepal (Ujwol et al., 2015., Ansari et al., 2014 & Mohapatra et al., 2009). Recent reports from India showed less percentage of inducible clindamycin resistance as compared to our study (Majhi et al., 2016 & Govindan et al.,

2014). The D- test is simple laboratory test for detection of inducible clindamycin resistance, therefore all erythromycin resistant isolates of *S. aureus* should be subjected to the D- test to rule out inducible clindamycin resistance and to prevent the clinical failures.

Isolation of MRSA from various units of the hospital is worrisome. There is the possibility of transmission of MRSA from hospital units (OT, ICUs) to patients, patients to healthcare professionals and vice versa during patient care, various diagnostic and therapeutic procedures. Therefore, regular surveillance, disinfection and/or fumigation with suitable agent at regular interval would minimize the colonization and transmission of MRSA. Performing routine bacteriological examination of hospital equipment and environment before and after disinfection on regular basis also provides valuable information about prevalence of bacterial pathogens in a particular unit as well as effectiveness of fumigation and disinfection. Determination of antibiotic resistance profile of the pathogens isolated from various units guides clinician to start empirical therapy in suspected cases of hospital acquired infections.

Similarly, isolation of MRSA from healthcare workers also increases the possibility of transmission of MRSA among the patients and other HCWs. Therefore, screening and treatment of carriers would minimize the risk of transmission of nosomial MRSA infections. Reports on nasal carriage of *S. aureus* among HCWs from different hospitals of Nepal showed higher carrier rates as compared to our study ranging from 20.3%-40.8% (Pant et al., 2007, Shakya et al., 2010, Sah et al., 2013 & Shrestha et al., 2009). However, nasal carriage of MRSA in our study was found higher than other studies conducted in Nepal (Khanal et al., 2015).

Cefoxitin disc diffusion method was found reliable marker for primary screening of MRSA isolates with 100% sensitivity and 98.6% specificity when considering *mecA* gene

results while oxacillin disc diffusion was found less sensitive (92%). Therefore, oxacillin disc diffusion method is no more recommended for screening of MRSA. Cefoxitin MIC results were found as specific as *mecA* gene results. Cefoxitin disc diffusion method is highly sensitive and specific when the zone diameter is ≤19mm. Isolates with zone diameter between 19-22 mm sometimes create confusion whether to report it as MRSA or MSSA. In our study, out of 15 doubtful cases of MRSA by cefoxitin disc diffusion method (zone diameter between 19-22mm), only eleven were found *mecA* positive. Out of four *mecA* negative isolates, two were found cefoxitin sensitive on subsequent retesting while two were found resistant. These isolates may be associated with issues with disc diffusion method and need for standardization or the resistance mechanism other than *mecA* and may indicate the possibility of *mecC* mediated resistance. Therefore, doubtful cases of MRSA need re-testing further confirmation by cefoxitin MIC and/or PCR for both mec genes in order get accurate results. In this way, cefoxitin disc diffusion method, being cheaper, easily available everywhere and easy to perform, should be the method of choice for screening of MRSA especially in smaller laboratories.

4.4.2 MRSA and *PVL* genes

Global emergence of MRSA is significant public health problem and challenge to clinicians and microbiologists. A number of factors contribute to the pathogenicity and drug resistance of *S. aureus*. The first *PVL* positive MRSA was observed in the late 1990 and these strains have become globally distributed in the recent years (Gravet et al., 1999). The role of *PVL* in enhancing virulence of *S. aureus* and their pathogenicity is controversial. Association of *PVL* increases the pathogenicity of *S. aureus* by necrosis, accelerating apoptosis and destruction of polymorphonuclear and mononuclear cells thereby contributing to morbidity and mortality (Lina et al., 1999). Some studies have shown no association of *PVL* with the virulence of the organism by demonstrating better clinical outcome of skin and soft tissue infections (Bae et al., 2009 & Campbell et al., 2008).

Community acquired MRSA strains are commonly associated with *PVL* genes, responsible for soft-tissue and deep dermal infections (Havaei et al., 2010 & Miller et al., 2005). Reports from various countries showed the increasing prevalence of *PVL* among MRSA isolates (Eckhardt et al., 2003 & Linde et al., 2005). Prevalence of *PVL* among MRSA isolates in this study was 56.8% (Bhatta et al., 2016) which is higher (39.7%) than recent study from Kenya (Omuse et al*.,* 2013). Study by G. Omuse et al., reported prevalence of *PVL* among both MRSA and MSSA isolates together while our study was restricted to *PVL* prevalence among MRSA isolates only, reflecting high prevalence of *PVL* among MRSA as compared to MSSA isolates. A study from India, reported overall 62.8% of *PVL* prevalence among MRSA and MSSA (MRSA: 85.1% and MSSA: 48.8%) which showed higher prevalence among MRSA than our findings (Kaur et al., 2012). Similar study reported prevalence of 64% *PVL* positive isolates among MRSA from Mumbai, India (D'Souza et al., 2010). Low prevalence of *PVL* was reported from other parts of world: 5% in France (Lina et al., 1999), 4.9% in UK (Holmes et al., 2005), 8.1% in Saudi Arabia (Moussa et al., 2008) and 14.3% in Bangladesh (Afroz et al., 2008), reflecting that the prevalence of *PVL* varies greatly between geographical locations and populations.

The majority (75.5%) of the *PVL* positive strains was found among isolates obtained from pus samples reflecting their key role in skin and soft tissue infections while association of *PVL* with isolates from other specimens was less. It may be hypothesized that high prevalence of *PVL* among MRSA isolated from pus may be associated with deposition of leucocytes locally at the site of infection as in most of pyogenic infections. As *PVL* can destroy leucocytes, its presence at the site of leucocyte deposition may play important role in the survival of bacteria and persistence of infection. Association of *PVL* in severe life threatening infections like blood stream infections was found less common in our study indicating poor association of *PVL* in invasive infections.

Isolates of MRSA have higher drug resistance and presence of *PVL* genes could increase their virulence. Community acquired MRSA are considered to be less dangerous than hospital strains especially in terms of drug resistance. However, presence of *PVL* genes among the majority of community acquired MRSA might indicate their potential pathogenicity. Results of antimicrobial susceptibility testing revealed higher resistance among *PVL* negative MRSA isolates as compared to *PVL* positive MRSA isolates, however the differences were not statistically significant except in case of erythromycin. Similarly, relative percentage of MDR MRSA among *PVL* negative (86.6%) isolates was found significantly higher than in *PVL* positive (63.3%) isolates (p value 0.002). These findings suggest that there is no association of *PVL* with MDR phenotypes. In other way, *PVL* positive isolates being community strains show lesser drug resistance.

Presence of *PVL* was considered as important marker for differentiation of HA-MRSA and CA-MRSA. Some studies have shown association of *PVL* genes among HA-MRSA isolates also (Shrestha et al., 2014 & Bhutia et al., 2012). Most of the studies including our study categorized HA-MRSA and CA-MRSA based upon the history of the patient or by getting information from medical record. However, information obtained from patient or from medical record may not be reliable all the time. To overcome this, we tested seven isolates of MRSA obtained from the environment of various units of the hospital

including wards and intensive care units. As these isolates are not known to be related to the specimen from patients and isolated from hospital environment, we might consider them as presumptive hospital strains. Therefore, as the seven isolates were found negative for *PVL* genes, this could indicate that *PVL* is not normally found in the hospital environment. However, these are not necessarily representative of hospital environment in general. Absence of *PVL* in inanimate objects of hospital environment may indicate limited role of antileucocytic activity outside the host.

In our study, we found association of *PVL* genes in four MRSA isolates which are hospital acquired as per the clinical criteria described above. As the majority of *PVL* positive strains represent community isolates, there is a possibility of transmission of these isolates from Out Patients to healthcare workers and patients. It is likely that these four *PVL* positive isolates might have been recently transmitted from a community source to hospital setting. Therefore, it may be hypothesized that, community strains when get entry into the hospital setting may slowly lose the *PVL* and may acquire drug resistant genes to counteract the antibiotic pressure for their survival. In this way, organism may lose its pathogenicity associated with *PVL* and may survive longer in patients by developing newer drug resistant mechanism.

4.4.3 Biofilm and MRSA

Biofilms are highly organized structured communities of bacterial cells enclosed in a self produced polymeric matrix adherent to an inert living surface. The ability to form biofilm was observed in a large number of bacteria, including *S. aureus*, *Pseudomonas aeruginosa* and coagulase negative Staphylococci (Klingenberg et al., 2005). Biofilm producing coagulase negative staphylococci (CNS) were shown to be adherent to artificial surfaces and were MDR (Klingenberg et al., 2005 & Gurung et al., 2013). The pathogenicity of CNS, particularly of *S. epidermidis,* in device related infections was documented to be due to slime production leading to attachment onto indwelling medical devices, eventually giving rise to biofilm formation (Costerton et al., 1999 & Xu et al., 2000).

Bacteria embedded inside biofilm frequently give rise to persistent and chronic infections often recalcitrant to treatment (Costerton et al., 1995). The results of the present study showed an overall biofilm positivity of 32.4% (48/148) amongst the *S. aureus* isolates and 41.5% amongst the MRSA isolates. Another study reported biofilm production in 34.6% of the MRSA isolates from nose, one of the common sites for *S. aureus* colonization (Rezaei et al., 2013). Similar study reported that 20.5% and 28.0% of the MRSA and MSSA isolates respectively from hospitalized patients were capable of forming biofilm on *in vitro* model (Smith et al., 2008).

Biofilm production by MRSA clinical isolates was documented in the past (Rezaei et al., 2013 & Ando et al., 2004), however the quantum of expression of such phenotypic character could be variable (O'Neill et al., 2007). A study from India, documented 26% of the MRSA isolates to be biofilm producer which is lower than our findings (Telang et al., 2010). Majority of the biofilm positive isolates in our study were from patients with deep seated infections such as gluteal abscesses, breast abscesses, cellulitis, osteomyelitis and 3rd degree burn wounds. Our findings could be a true reflection of the potential of

this organism to give rise to such serious, sometimes life threatening clinical conditions because of its virulence properties like biofilm production and methicillin resistance.

Nevertheless, the high rate of detection of MRSA from these deep body sites could suggest that invasive infections might be caused by a distinct population of *S. aureus* circulating in the hospital environment. These strains, probably armored with certain virulence factors as evidenced by our data that significantly higher number of MRSA as well as biofilm producers were MDR. Over and above, 44 (91.7%) of the 48 biofilm producers were from the samples like pus, blood and sputum. Pus samples which yielded the maximum number of biofilm producing organisms, had been collected from patients with deep seated abscesses, osteomyelitic lesions and rapidly spreading forms of cellulitis. This would imply that *S. aureus* capable of forming biofilms and producing infection at deeper parts of the body could have better survival advantage against antibiotic pressure and host defenses (Costeron et al., 1999). This has been amply evidenced by earlier workers while studying the isolates from cases of sepsis and bacteremia (Klingenberg et al., 2005, De silva et al., 2002 & Gurung et al., 2013). Unfortunately, we had limited number of blood isolates to draw any conclusion exclusively on the bacteremic patients. However it is quite evident from our results that a major proportion of non-biofilm producers (65 out of 105 from the pus samples) were obtained from samples collected from superficial wound infections rather than from deeper body parts.

It was demonstrated that crystal violet microtiter plate assay was more sensitive technique for detecting biofilm than demonstrating slime production by congo red agar test (Atshan et al., 2012 & Rezaei et al., 2013). In the pretext of the above, we adopted the crystal violet microtiter assay and found that 32.4% (48/148) of the *S. aureus* isolates were biofilm positive and on subsequent analysis it was found that 83.3% of our biofilm positive bacteria were MDR. In a recent investigation on resistance to multiple antibiotics exhibited by biofilm producing *S. epidermidis,* it was shown that 86%, 80%

and 86% of the organisms were resistant to oxacillin, gentamicin and fusidic acid respectively (Klingenberg et al., 2005). Somewhat similar picture was revealed amongst biofilm producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii;* 63-81% and 77-93% respectively among clinical isolates from the ICU patients showing resistance to multiple antibiotic classes (Gurung et al., 2013).

Irrespective of the clinical condition, such high degree of multidrug resistance prevailing among the biofilm producing organisms is alarming (De silva et al., 2002). This not only warrants urgent attention of strict control and surveillance measures to prevent spread of these pathogens among the hospitalized patients, but also pronounces an urgent need for rapid demonstration of other pathogenic markers in order to detect their possible virulence determinants within the least possible time period.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The prevalence of MRSA in the studied isolates was high. Monitoring antibiotic sensitivity pattern of MRSA isolates on regular basis, optimization of MRSA detection methods, implementation of preventive measures for MRSA spread, dissemination of data to clinicians and formulation of definite antibiotic policy may be helpful in reducing the incidence of MRSA infection and emergence of vancomycin resistant *S. aureus* as well. Vancomycin was the only drug to which 100% isolates were susceptible. However, the possibility of emergence of vancomycin resistance should always be kept in mind. Screening of erythromycin resistant isolates by D-test would detect inducible clindamycin resistance and minimize clinical failures associated with clindamycin therapy.

The prevalence of the *PVL* among the MRSA isolates was relatively high in this study, especially among isolates from pus samples. This suggests a possible role of *PVL* in pathogenesis of pyogenic infections, especially skin and soft tissue infections in community setting. The *PVL* positive MRSA isolates showed higher sensitivity against antibiotics as compared to *PVL* negative isolates indicating that *PVL* is not associated with drug resistance mechanisms. The presence of *PVL* among multidrug resistant bacteria like MRSA may be involved in virulence and scalates the challenges for clinicians. As expected, the majority of *PVL* positive MRSA were community-associated isolates, whereas only four MRSA from hospital related cases were positive for *PVL*. No *PVL* was detected in MRSA isolated from the hospital environment. In our view, the presence of *PVL* can be used as a reliable marker for CA-MRSA in resource limited settings like Nepal.

The prevalence of biofilm among MRSA isolates was high and majority of the biofilm producers were MDR as well. These findings suggest that the organisms having the potential to adhere could trigger the intriguing pathway of intercellular adhesion leading eventually to biofilm formation. As a consequence, biofilm, thus formed, would provide a niche for persistence of the bacteria resulting in chronic and recalcitrant infections. Early therapeutic intervention by employing molecular tools for detection of biofilm forming ability and multidrug resistance could be the probable strategy to combat such infections.

5.2 Recommendations

- Cefoxitin MIC test can be used for confirmation of doubtful MRSA isolates in resource limited laboratories.
- Erythromycin resistant isolates of *S. aureus* should be subjected to the D-test to rule out inducible clindamycin resistance and to prevent the clinical failures.
- Vancomycin should never be considered as first line drug, in view of the possibility of emergence of resistance. Thus other drugs like clindamycin and amikacin would be possible options for the management of such infections.
- Screening of HCWs and regular surveillance of sensitive units of the hospital like OT, ICU is necessary in order to prevent/reduce nosocomial transmission of MRSA infections.
- *PVL* can be used as a reliable marker for identification of CA-MRSA.
- Early therapeutic intervention for detection of biofilm forming ability and multidrug resistance is imperative to combat serious deep seated MRSA infections.

CHAPTER 6

6. SUMMARY

S. aureus is one of the most prevalent human pathogens worldwide, associated with variety of illnesses ranging from mild skin eruptions to life-threatening infections. *S. aureus* is notorious for its ability to become resistant to antibiotics. Methicillin was introduced in 1959 to treat the infections caused by penicillin resistant *S. aureus*. Methicillin-resistant *S. aureus* (MRSA) was first identified in 1961. The methicillin resistance gene (*mecA*) encodes a methicillin-resistant penicillin-binding protein that is not present in susceptible strains.

Pathogenicity of *S. aureus* is related to a number of virulence factors that allow organism to adhere, avoid the immune system and cause harmful effects to the host. One of the important cytotoxin produced by some strains of *S. aureus* is Panton Valentine leukocidin (*PVL*), encoded by two genes, *lukS-PV* and *lukF-PV* responsible for leukocyte destruction and tissue necrosis. Biofilm formation is one of the most important defense mechanisms of microbes against host defenses and the effect of antimicrobial agents. Bacteria and fungi are known to produce biofilm and *S. aureus* is considered as one of the important biofilm producing human pathogens. Historically MRSA was associated with hospitals and other healthcare settings, has now emerged as a widespread cause of community infections.

A total of 400 *S. aureus* isolates were cultured from various clinical specimens and from hospital environment. Primary screening of MRSA was performed by disc diffusion method (oxacillin and cefoxitin) and further confirmed by cefoxitin MIC and *mecA*gene detection by PCR. Antibiotic susceptibility testing was performed by Kirby Bauer disc diffusion method. Inducible clindamycin resistance was detected by D-test. *PVL* genes were detected by PCR using specific primers. Biofilm formation among MRSA and MSSA isolates was detected by microtitre plate method.

Out of 400 isolates 139 (34.75%) were *mecA* positive and confirmed as MRSA. Out of 139 MRSA isolates, 56 (40.3%) were found to be hospital associated MRSA and remaining 83 (59.7%) were community associated MRSA. Out of 112 hospital staff tested for MRSA nasal carrier, only 8 (7.1%) were found positive. Our study revealed that, the majority of MRSA (73.38%) isolates were multidrug resistant. Resistance to penicillin was highest (93.7%) followed by erythromycin (53%), ciprofloxacin (48%), cotrimoxazole (44%) and amoxicillin-clavulanic acid (35.4%). The MRSA isolates from ward patients showed significantly higher resistance as compared to OPD patients. No vancomycin resistant *S. aureus* was detected in our study. Out of 212 erythromycin resistant *S. aureus* isolates, inducible clindamycin resistance was detected in 54 (25.47%) isolates by D-test.

Panton Valentine leukocidin (*PVL*) genes were detected in 79 (56.8 %) of the 139 MRSA isolates. The majority of the *PVL* positive strains were isolated from pus samples from surgery department. Out of the 139 MRSA isolates, 56 (40.3%) were found to be hospital associated MRSA and the remaining 83 (59.7%) isolates were classified community associated MRSA. Among these 83 CA-MRSA, 75 (90.4%) were *PVL* positive while *PVL* was detected only in 4 out of 56 (7.1%) hospital associated MRSA strains. All seven MRSA isolates obtained from environmental samples were found negative for *PVL*. Antibiotic resistance among *PVL* negative MRSA isolates was higher than the *PVL* positive MRSA. Out of 139 MRSA isolates, 102 were multidrug resistant of which, 50 were *PVL* positive and remaining 52 were *PVL* negative. Overall percentage of *PVL* positive MDR MRSA was 63.3% (50/79) while *PVL* negative MDR MRSA was 86.6% (52/60).

A total of 148 isolates of *S. aureus* were tested for biofilm assay. Of these, 94 (63.5%) were MRSA and 54 (36.5%) were MSSA. Out of 148 isolates, 48 were found positive for biofilm. Out of total of 94 MRSA isolates, 39 (41.5%) were biofilm producers. On the contrary, only 9 (16.7%) of the 54 MSSA were found to be biofilm producers. Out of the

48 biofilm producing organisms a vast majority 40 (83.3%) were MDR as compared to only 46 (46%) amongst the 100 biofilm non-producers.

REFERENCES

Aarestrup, F.M., Larsen, H.D., Eriksen, N.H., Elsberg, C.S., and Jensen, N.E. (1999). Frequency of α- and β-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno and genotype and variation in phenotypic expression. *APMIS,* **107**(4)**,** 425-430.

Acar, J.F., Courvalin, P., and Chabbert, Y.A. (1970). Methicillin-resistant staphylococcemia: bacteriological failure of treatment with cephalosporins. *Antimicrob Agents Chemother,* **10**, 280–285.

Afroz, S., Kobayashi, N., Nagashima, S., Alam, M.M., Hossain, A.B.M.B., Rahman, M.A., Islam, M.R., Lutfor, A.B., Muazzam, N., Khan, M.A., Paul, S.K., Shamsuzzaman, A.K., Mahmud, M.C., Musa, A.K., and Hossain, M.A. (2008). Genetic Characterization of *Staphylococcus aureus* Isolates Carrying Panton Valentine Leukocidin Genes in Bangladesh. *Jpn J Infect Dis*, **61**(5), 393-396.

Ahamed, P.S. (2009). Osteomyelitis: A case report. *Aust Fam Physician,* **38**(7), 521–523.

Albrich, W.C., and Harbarth, S. (2008). Health-care workers: source, vector, or victim of MRSA? *Lancet Infect Dis*, **8**(5), 289–301.

Ansari, S., Nepal, H.P., Gautam, R., Rayamajhi, R., Shrestha, S., Upadhyay, G., Acharya, A., and Chapagain, M.L. (2014). Threat of drug resistant *Staphylococcus aureus* to health in Nepal. *BMC Infect Dis*, **14**, 157-162.

Appelbaum, P.C. (2007). Microbiology of Antibiotic Resistance in *Staphylococcus aureus*. *Clin Infect Dis*, **45**(3), 165-170.

Archer, G.L., and Pennell, E. (1990). Detection of methicillin resistance in staphylococci by using a DNA probe. *Antimicrob Agents Chemother*, **34**(9), 1720–1724.

Arora, S., Devi, P., Arora, U., and Devi, B. (2010). Prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in a Tertiary Care Hospital in Northern India. *J Lab Physicians*, **2**(2), 78–81.

Bae, I.G., Tonthat, G.T., Stryjewski, M.E., Rude, T.H., Reilly, L.F., Barriere, S.L., Genter, F.C., Corey, G.R., and Fowler, V.G. (2009). "Presence of Genes Encoding the Panton-Valentine leukocidin Exotoxin Is Not the Primary Determinant of Outcome in Patients with Complicated Skin and Skin Structure Infections Due to methicillin resistant *Staphylococcus aureus*: Results of a Multinational Trial," *J Clin Microbiol*, **47**(12), 3952-3957.

Banerjee, A., Chisti, Y., and Banerjee, U.C. (2004). Streptokinase-a clinically useful thrombolytic agent. *Biotechnol Adv,* **22**, 287 – 307.

Bantel, H., Sinha, B., Domschke, W., Peters, G., Schulze-Osthoff, K., and Janicke, R.U. **(**2001). α-Toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *J Cell Biol,* **155**(4)**,** 637-647.

Barrett, S.P., Savage, M.A., Rebec, M.P., Guyot, A., Andrew, N., and Shrimpton, S.B. (1994). Antibiotic sensitivity of bacteria associated with community-acquired urinary tract infection in Britain. *J Antimicrob Chemother*, **44**(3), 359-365.

Barrett, F.F., McGehee, R.F., and Finland, M. (1968). Methicillin- resistant *Staphylococcus aureus* at Boston City hospital. Bacteriologic and epidemiologic observations. *N Engl J Med*, **279**(9), 441–448.

Becton, Dickinson and Company (2005). (www.bacteriainphotos.com).

Bell, J.M., and Turnidge, J.D. (2002). High prevalence of oxacillin-resistant *Staphylococcus aureus* isolates from hospitalized patients in Asia-Pacific and South Africa: results from SENTRY Antimicrobial Surveillance Program, 1998–1999. *Antimicrob Agents Chemother*, **46**(3), 879–881.

Berger, B., and Rohrer, S. (2002). Factors influencing methicillin resistance in staphylococci. *Arch Microbiol*, **178**(3), 165-171.

Bhatta, D.R., Cavaco, L.M., Nath, G., Gaur, G., Gokhale, S., and Bhatta, D.R. (2015). Threat of multidrug resistant *Staphylococcus aureus* in Western Nepal. *Asian Pac J Trop Dis*, **5**(8), 617-621.

Bhatta, D.R., Cavaco, L.M., Nath, G., Kumar, K., Gaur, G., Gokhale, S., and Bhatta, D.R. (2016). Association of Panton Valentine Leukocidin (PVL) genes with methicillin resistant *Staphylococcus aureus* (MRSA) in Western Nepal: a matter of concern for community infections (a hospital based prospective study). *BMC Infect Dis*, **16**, 199-204.

Bhutia, K.O., and Singh, T.S.K. (2012). The prevalence and risk factors which are associated with *Staphylococcus aureus* and methicillin resistant *S. aureus* which harboured the Panton Valenti ne Leukocidin gene in Sikkim. *J Clin Diagn Res,* **6**(3), 393-399.

Bignardi, G.E., Woodford, N., Chapman, A., Johnson, P., and Speller, D.C.E. (1996). Detection of the mec-A gene and phenotypic detection of resistance in *Staphylococcus aureus* isolates with borderline or low-level methicillin resistance. *J Antimicrob Chemother*, **37**(1), 53–63.

Boucher, H.W., and Corey, G.R. (2008). Epidemiology of Methicillin Resistant *Staphylococcus aureus. Clin Infect Dis*, **46**(5), 344-349.

Boyce, J.M. (1990). Increasing prevalence of methicillin-resistant *Staphylococcus aureus* in the United States. *Infect Control Hosp Epidemiol*, **11**(12), 639-642.

Boyle, V.S., and Daum, R.S. (2007). Community-acquired methicillin resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Lab. Investig*, **87**(1), 3–9.

Brendan, H. (2005). MRSA Survivor Network. Statistics United States.

Breurec, S., Zriouil, S.B., Fall, C., Boisier, P., Brisse, S., Dijibo, S., Etienne, J., Fonkoua, M.C., Perrier, G.C.J.D., Pouillot, R., Ramarokoto, C.E., Randrianirina, F., Tall, A., Thiberge, J.M., Laurent, F., and Garin, B. (2010), "Epidemiology of Methicillin-Susceptible *Staphylococcus aureus* Lineages in Five Major African Towns: High Prevalence of Panton-Valentine Leukocidin Genes," *Clin Microbiol Infect*, **17**(2), 633-639.

Brown, D.F., and Walpole, E. (2001). Evaluation of the Mastalex latex agglutination test for methicillin resistance in *Staphylococcus aureus* grown on different screening media. *J Antimicrob Chemother*, **47**, 187–189.

Bubeck, W.J., Bae, T., Otto, M., Deleo, F.R., and Schneewind, O. (2007). "Poring over pores: α-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia". *Nat Med,* **13**(12), 1405–1406.

Caiazza, N.C., and O'Toole, G.A. (2003). Alpha-toxin is required for biofilm formation by *Staphylococcus aureus*. *J Bacteriol*, **185**(10), 3214-3217.

Campbell, S.J., Deshmukh, H.S., Nelson, C.L., Bae, I.G., Stryjewski, M.E., Federspiel, J.J., Tonthat, G.T., Rude, T.H., Barriere, S.L., Corey, R., and Fowler, V.G. (2008). "Genotypic Characteristics of *Staphylococcus aureus* Isolates from a Multinational Trial of Complicated Skin and Skin Structure Infections," *J Clin Microbiol*, **46**(2), 678-684.

Centers for Disease Control and Prevention (1996). Summary of notifiable diseases, United States. *Morb Mortal Wkly Rep,* **45**(53), 1–87.

Centers for Disease Control and Prevention (1997). *Staphylococcus aureus* with reduced susceptibility to vancomycin-United States. *Morbid Mortal Weekly Rep*, **46**, 765–766.

Centers for Disease Control and Prevention (2007). Information about MRSA for healthcare personnel. www.cdc.gov/ncidod/dhqp/ar_mrsa_healthcareFS.html.

Cesur, S., and Cokca, F.(2004). Nasal carriage of methicillin-resistant *Staphylococcus aureus* among hospital staff and outpatients. *Infect Control Hosp Epidemiol*, **25**, 169–171.

Chambers, H.F., and DeLeo, F.R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol*, **7**(9), 629-641.

Chen, C.J., and Huang, Y.C. (2014). New Epidemiology of *Staphylococcus aureus* infection in Asia. *Clin Microbiol Infect,* **20**(7), 605-623.

Chesney, P.J. (1989). Clinical aspects and spectrum of illness of toxic shock syndrome: overview. *Rev Infect Dis,* **11** (1), 1-7.

Chini, V., Petinaki, E., Foka, A., Paratiras, S., Dimitracopoulos, G., and Spiliopoulou, I. (2006). Spread of *Staphylococcus aureus* clinical isolates carrying Panton-Valentine leukocidin genes during a 3-year period in Greece. *Clin Microbiol Infect,* **12**(1), 29–34.

Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Malton, D.M., and Beachey, E.H. (1985). Adherence of coagulase negative Staphylococci to plastic tissue culture plates: a quantitative model to the adherence of Staphylococci to medical devices. *J Clin Microbiol*, **22**(6), 996-1006.

Christiansen, K.J., Bell, J.M., Turnidge, J.D., and Jones, R.N. (2004). Antimicrobial activities of garenoxacin (BMS 284756) against Asia-Pacific region clinical isolates from the SENTRY program, 1999 to 2001. *Antimicrob Agents Chemother*, **48**(6), 2049–2055.

Cimolai, N. (2008). The role of healthcare personnel in the maintenance and spread of methicillin-resistant *Staphylococcus aureus*. *J Infect Public Health*, **1** (2):78–100.

Clinical and Laboratory Standards Institute (2011). M100‑S21: Performance standards for antimicrobial susceptibility testing, 21st international supplements. Pennsylvania: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (2012). M100-S22: Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement. Pennsylvania: Clinical and Laboratory Standards Institute.

Coll, P.P., Crabtree, B.F., O'Connor, P.J., and Klenzak, S. (1994). Clinical risk factors for methicillin-resistant *Staphylococcus aureus* bacteriuria in a skilled-care nursing home. *Arch Fam Med,* **3**(4), 357-360.

Collee, J.G., Fraser, A.G., Marmion, B.P., and Simmons, A. (1996). Mackie and McCartney, Practical medical microbiology. 14th ed. New York: Churchill Livingstone.

Cookson, B., Peters, B., Webster, M., Phillips, I., Rahman, M., and Noble, W. (1989). Staff carriage of epidemic methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol*, **27**(7), 1471–1476.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999). Bacterial biofilm: a common cause of persistent infections. *Science*, **284**, 1388-1322.

D'Souza, N., Rodrigues, C., and Mehta, A. (2010). Molecular Characterization of Methicillin Resistant *Staphylococcus aureus* with Emergence of Epidemic Clones of Sequence type (ST) 22 and ST 772 in Mumbai, India. *J Clin microbial,* **48**(5), 1806-1811.

Daum, R.S. (2007). Clinical practice: skin and soft-tissue infections caused by methicillin-resistant *Staphylococcus aureus*. *N Engl J Med*, **357**, 380-390.

d'Azevedo, P.A., Inoue, F.M., Andrade, S.S., Tranchesi, R., and Pignatari, A.C. (2009). Necrotizing pneumonia due to methicillin-resistant *Staphylococcus aureus*. *Rev Soc Bras Med Trop*, **42**, 461–462.

De Silva, G.D.I., Kantzanau, M., Justice, A., Massey, R.C., Wilkinson, A.R., Day, N.P.J., and Peacock, S.J. (2002). The *ica* operon and biofilm production in coagulase negative Staphylococci associated with carriage and disease in a neonatal intensive care unit. *J Clin Microbiol*, **40**, 382-388.

Defres, S., Marwick, C., and Nathwani, D. (2009). MRSA as a cause of lung infection including airway infection, community-acquired pneumonia and hospital-acquired pneumonia. *Eur Resp J*, **34**(6), 1470-1476.

Demuth, P.J., Gerding, G.N., and Crossley, K. (1979). *S. aureus* bacteriuria. *Arch Intern Med,* **139**(1), 78-80.

Deresinski, S. (2005). Methicillin-resistant *Staphylococcus aureus*: an evolutionary, epidemiologic and therapeutic odyssey. *Clin Infect Dis*, **40**(4), 562-573.

Descloux, E., Perpoint, T., Ferry, T., Lina, G., Bes, M., Vandenesch, F., Mohammedi, I., and Etienne, J. (2008): One in five mortality in non-menstrual toxic shock syndrome versus no mortality in menstrual cases in a balanced French series of 55 cases. *Eur J Clin Microbiol Infect Dis,* **27** (1), 37-43.

Diep, B.A., Chan, L., Tattevin, P., Kajikawa, O., Martin, T.R., Basuino, L., Mai, T.T., Marbach, H., Braughton, K.R., Whitney, A.R., Gardner, D.J., Fan, X., Tseng, C.W., Liu, G.Y., Badiou, C., Etienne, J., Lina, G., Matthay, M.A., DeLeo, F.R., and Chambers, H.F. (2010), "Polymorphonuclear Leukocytes Mediate *Staphylococcus aureus* Panton-Valentine Leukocidin-Induced Lung Inflammation and Injury," *Proc Natl Acad Sci USA*, **107**(12), 5587-5592.

"Discovery and Development of Penicillin". *American Chemical Society. Retrieved 30 August 2015.*

Distribution of Population and Area by Development Regions and Districts (1981, 1991 and 2001). Available from http://www.cbs.gov.np/statistical_year_book_content.php. 2010.

Dufour, P., Gillet, Y., Bes, M., Lina, G., Vendenesch, F., Floret, D., Etienne, J., and Richet, H. (2002). Community-acquired methicillin-resistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. *Clin Infect Dis*, **35**(7), 819–824.

Eady, E.A., and Cove, J.H. (2003). Staphylococcal resistance revisited: community-acquired methicillin resistant *Staphylococcus aureus*—an emerging problem for the management of skin and soft tissue infections. *Curr Opin Infect Dis*, **16**, 103–124.

EARSS management team. European Antimicrobial Resistance Surveillance System annual report (2006). Bilthoven, The Netherlands: National Institute for Public Health and the Environment; 2007.

Eckhardt, C., Halvosa, J.S., Ray, S.M., and Blumberg, H.M. (2003). Transmission of methicillin-resistant *Staphylococcus aureus* in the neonatal intensive care unit from a patient with community-acquired disease. *Infect Control Hosp Epidemiol*, **24**(6), 460-461.

Edwards, J. R., Peterson, K.D., Andrus, M.L., Tolson, J.S., Goulding, J.S., Dudeck, M.A., Mincey, R.B., Pollock, D.A., and Horan, T.C. (2007). National Healthcare Safety Network (NHSN) report, data summary for 2006. *Am J Infect Control,* **35**, 290–301.

Elek, S.D. (1959). *Staphylococcus pyogenes* and its relation to disease. Edinburgh: E. & S. Livingstone, 3.

Ellis Simonsen, S.M., Van Orman, E.R, Hatch, B.E., Jones, S.S., Gren, L.H, Hegmann, K.T., and Lyon J.L. (2006). Cellulitis incidence in a defined population. *Epidemiol Infect*, **134** (2), 293–299.

Eveillard, M., Martin, Y., Hidri, N., Boussougant, Y., and Joly-Guillou, M.L. (2004). Carriage of methicillin-resistant *Staphylococcus aureus* among hospital employees: prevalence, duration, and transmission to households. *Infect Control Hosp Epidemiol*, **25**(2), 114–120.

Experimental vaccine protects mice against MRSA infection. Available from:http://www.medscape.com/viewarticle/586366?src=rss.

Fiebelkorn, K.R., Crawford, S.A., McElmeel, M.L., and Jorgensen, J.H. (2003). Practical disk diffusion method for detection of inducible clindamycin resistance in *Staphylococcus aureus* and coagulase‐negative staphylococci. *J Clin Microbiol*, **41**, 4740‐4744.

Fischbach, M.A., and Walsh, C.T. (2009). Antibiotics for emerging pathogens. *Science*, **325**(5944), 1089-1093.

Flemming, H.C., Wingender, J.G., and Mayer, C. (2000). Physico-chemical properties of biofilms. In: Evans LV, editor. Biofilms: recent advances in their study and control. Amsterdam: Harwood Academic Publishers; p. 19–34.

Foster, T. (1996). Staphylococcus. In: Baron S, editor. Medical Microbiology, 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; Chapter 12.

Foster, T.J., and Hook, M. (1998). Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol,* **6**(12), 484-488.

Fung, J., Huczko, E., Gradelski, E., Denbleyker, K., Bonner, D.P., and Kessler, R.E. (1991). Emergence of homogeneously methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol,* **29**, 2880-2883.

Gadepalli, R., Dhawan, B., Kapil, A., Sreenivas, V., Jais, M., Gaind, R., Chaudhry, R., Samantaray, J.C., and Udo, E.E. (2009). Clinical and molecular characteristics of nosocomial methicillin-resistant *Staphylococcus aureus* skin and soft tissue isolates from three Indian hospitals. *J Hosp Infect*, **73**(3), 253–263.

Garbe, P.L., Arko, R.L., Reingold, A.L., Graves, L.M., Hayes, P.S., Hightower, A.W., Chandler, F.W., and Broome, C.V. (1985). *Staphylococcus aureus* isolates form patients with non-menstrual toxic shock syndrome. Evidence for additional toxins. *JAMA*, **253**(17), 2538-2542.

Garcia, A.L., Holden, T.G., Lindsay, H., Webb, C.R., Brown, D.F.J., Curran, M.D., Walpole, E., Brooks, K., Pickard, D.J., Teale, C., Parkhill, J., Bentley, S.D., Edwards, G.F., Girvan, E.K., Kearns, A.M., Pichon, B., Hill, R.L., Larsen, A.R., Skov, R.L., Peacock, S.J., Maskell, D.J., and Homes, M.A. (2011). Methicillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect Dis*, **11**(8), 595-603.

Geha, D.J., Uhl, J.R., Gustaferro, C.A., and Persing, D.H. (1994). Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. *J Clin Microbiol,* **32**(7), 1768-1772.

Genestier, A.L., Michalete, M.C., Prévoset, G., Bellot, G., Chalabreysse, L., Peyrol, S., Thivolet, F., Etienne, J., Lina, G., Vallette, F.M., Vandenesch, F., and Genestier, L. (2005). *Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. *J Clin Invest,* **115**(11), 3117–3127.

Ghosh, A.N., Bhatta, D.R., Ansari, M.T., Tiwari, H.K., Mathuria, J.P., Supram, H.S., and Gokhale, S. (2013). Application of WHONET in the antimicrobial resistance surveillance of uropathogens : A first user experience from Nepal. *J Clin Diagn Res*, **7**(5), 845-848.

Gilbert, P., Das, J., and Foley, I. (1997). Biofilm susceptibility to antimicrobials. *Adv Dent Res,* **11**(1), 160–167.

Giron, R.M., Buendía, B., Pinedo, C., Casanova, A., Hoyos, N., and Ancochea, J. (2009). Methicillin-resistant *Staphylococcus aureus* in patients with cystic fibrosis. *Enferm Infect Microbiol Clin*, **27**(2), 85–88.

Goldstein, F.W. (2000). Antibiotic susceptibility of bacterial strains isolated from patients with community-acquired urinary tract infections in France. *Eur J Clin Microbiol Infect Dis*, **19**(2), 112-117.

Goodyear, C.S., and Silverman, G.J. (2003). Death by a B cell superantigen: *In vivo* VH-targeted apoptotic supra clonal B cell deletion by a Staphylococcal Toxin. *J Exp Med,* **197** (9), 1125–1139.

Gopalakrishnan, R., and Sureshkumar, D. (2010). Changing trends in antimicrobial susceptibility and hospital acquired infections over an 8 year period in a tertiary care hospital in relation to introduction of an infection control programme. *J Assoc Physicians India*, **58**(Suppl), 25–31.

Gotz, F. (2006). The genera Staphylococcus and Macrococcus. The prokaryotes. Springer: New York. pp. 5-75

Gould, J.C., and McKillop, E. (1954).The carriage of *Staphylococcus pyogenes var aureus* in the human nose. *J Hygiene*, **52**, 304–310.

Grice, E.A., Kong, H.H., Conlan, S., Deming, C.B., Davis, J., Young, A.C., Bouffard, G.G., Blakesley, R.W., Murray, P.R., Green, E.D., Turner, M.L., and Segre, J.A. (2009).Topographical and temporal diversity of the human skin microbiome. *Science*, **324**(5931), 1190–1192.

Haamann, F., Dulon, M., and Nienhaus, A. (2011). MRSA as an occupational disease: a case series. *Int Arch Occup Environ Health*, **84**(3), 259–266.

Harbarth, S., Fankhauser, C., Schrenzel, J., Christenson, J., Gervaz, P., Bandiera, C.C., Renzi, G., Vernaz, N., Sax, H., and Pittet, D. (2008). Universal screening for methicillin-resistant *Staphylococcus aureus* at hospital admission and nosocomial infection in surgical patients. *JAMA*, **299**(10), 1149–1157.

Hausmann, W., and Karlish, A.J. (1956). Staphylococcal pneumonia in adults. *BMJ*, **2**, 845–847.

Havaei, S.A., Moghadam, S.O., Pourmand, M.R., and Faghri, J. (2010). Prevalence of genes encoding bi-component leukocidins among clinical isolates of methicillin-resistant *Staphylococcus aureus*. *Iranian J Publ Health*, **39**(1), 8-14.

Henderson, D.K. (2006). Managing methicillin-resistant staphylococci: A paradigm for preventing nosocomial transmission of resistant organisms. *Am J Med*, **119**(6), 545-552.

Herwaldt, L.A., and Wenzel, R.P. (1996). Dynamics of hospital acquired infections. In: manual of clinical microbiology. 6th ed. Washington DC. *Am S Microbiol*., 169-181.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F.C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother,* **40**(1)**,** 135–136.

Holmes, A., Ganner, M., McGuane, S., Pitt, T.L., Cookson, B.D., and Kearns, A.M. (2005). *Staphylococcus aureus* isolates carrying Panton-Valentine leukocidin genes in England and Wales: frequency, characterization, and association with clinical disease. *J Clin Microbiol*, **43**(5), 2384-2390.

Horan, T.C., Andrus, M., and Dudeck, M.A. (2008). CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. *Am J Infect Control*, **36**(5), 309-332.

Hsu, L.Y., Tan, T.Y., Jureen, R., Koh, T.H., Krishnan, P., Lin, R.T.P., Tee, N.W.S., and Tambyah, P.A. (2007). Antimicrobial drug resistance in Singapore hospitals. *Emerg Infect Dis*, **13**(12), 1944–1947.

Hsueh, P.R., Chen, M.L., Sun, C.C., Chen, W.H., Pan, H.J., Yang, L.S., Chang, S.C., Ho, S.W., Lee, C.Y., Hsieh, W.C., and Luh, K.T. (2002). Antimicrobial drug resistance in pathogens causing nosocomial infections at a university hospital in Taiwan, 1981–1999. *Emerg Infect Dis*, **8**(1), 63–68.

Huang, M.B., Gay, T.E., Baker, C.N., Banerjee, S.N., and Tenover, F.C. (1993). Two percent sodium chloride is required for susceptibility testing of staphylococci with oxacillin when using agar-based dilution methods. *J Clin Microbiol*, **31**(10), 2683–2688.

Humphreys, H. (2008). Can we do better in controlling and preventing methicillin-resistant *Staphylococcus aureus* (MRSA)in intensive care unit (ICU)? *Eur J Clin Microbiol Infect Dis,* **27**(6), 409-413.

Hussain, M., Heilmann, C., Peters, G., and Herrmann, M. (2001). Teichoic acid enhances adhesion of *Staphylococcus epidermidis* to immobilized fibronectin. *Microb Pathog,* **31**(6)**,** 261-270.

Ibler K.S., and Kromann C.B. (2014). Recurrent furunculosis- challenges and managements: a review. *Clin Cosmet Investig Dermatol*, **7**, 59-64.

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, k., Tiensasitorn, C., and Hiramatsu, K. (2001). Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*, **45**(5), 1323–1336.

Izano, E.A., Amarante, M.A., Kher, W.B., and Kaplan, J.B. (2008). Differential roles of poly-N-acetyl glucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl Environ Microbiol*, **74**(2), 470-476.

Jevons, M. **(**1961). "Celbenin"-resistant Staphylococci. *Br Med J*, **1**(5219), 124–125.

Jevons, M.P., Coe, A.W., and Parker, M.T. (1963). Methicillin resistance in Staphylococci. *Lancet*, **281**(7287), 904-907.

Johnsson, D., Molling, P., Stralin, K., and Soderquist, B. (2004). Detection of Panton-Valentine leukocidin gene in *Staphylococcus aureus* by Light Cycler PCR: clinical and epidemiological aspects. *Clin Microbiol Infect*, **10** (10), 884–889.

Kaur, H., Purwar, S., Saini, A., Kaur, H., Karadesai, S.G., Kholkute, S.D., and Roy, S. (2012). Status of Methicillin Resistant *Staphylococcus aureus* Infections and Evaluation of *PVL* Producing Strains in Belgaum, South India. *JKIMSU*, **1**(2), 43-51.

Kerttula, A.M., Lyytikainen, O., Salmenlinna, S., and Vuopio-Varkila, J. (2004). Changing epidemiology of methicillin-resistant *Staphylococcus aureus* in Finland. *J Hosp Infect*, **58**(2), 109-114.

Khan, M.A., Mourshed, M.G., Khan, W.A., and Aziz, K.M.S. (1991). The emergence of methicillin resistant *Staphylococcus aureus* isolated from skin lesion. *Bangladesh J Microbiol*, **8**(1), 21-25.

Khan, Z. Z. (2016). Group A Streptococcal Infections. *Medscape, Drug and diasese.* Available at http://emedicine.medscape.com/article/228936-overview.

Khanal, L.K., and Jha, B.K. (2010). Prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) among skin infection cases at a hospital in Chitwan, Nepal. *Nepal Med Coll J,* **12**, 224-228.

Khanal, R., Sah, P., Lamichhane, P., Lamsal, A., Upadhaya, S., and Pahwa, V.K. (2015). Nasal carriage of methicillin resistant *Staphylococcus aureus* among health care workers at tertiary care hospital in Western Nepal. *Antimicob Resist Infect Control*, **4**, 39-43.

Kimura, A., Igarashi, H., Ushioda, H., Okuzumi, K., Kobayashi, H., and Otsuka, T. (1992). Epidemiological study of *Staphylococcus aureus* isolated from the Japanese National University and Medical College Hospitals with coagulase typing, and production of enterotoxins and toxic shock syndrome toxin-1. *Kansenshogaku Zasshi*, **66**(11): 1543–1549.

Klein, E., Smith, D.L., and Laxminarayan, R. (2007). Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005. *Emerg Infect Dis*, **13**(12), 1840–1846.

Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Craig, A.S., Zell, E.R., Fosheim, G.E., McDougal, L.K., Carey, R.B., and Fridkin, S.K. **(** 2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA,* **298**(15)**,** 1763-1771.

Klingenberg, C., Arag, E., Ronnestad, A., Sollid, J.E., Abrahamsen, T.G., Kjelden, G., and Flaegstad, T. (2005). Coagulase negative Staphylococcal sepsis in neonates: Association between antibiotic resistance, biofilm formation and the host inflammatory response. *Pediatr Infect Dis J*, **24**, 817-822.

Kluytmans, J., Van Belkum, A., and Verbrugh, H. (1997). Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev*, **10**(3), 505-520.

Kuehnert, M.J., Hill, H.A., Kupronis, B.A., Tokars, J.I., Solomon, S.L., and Jernigan, D.B. (2005). Methicillin-resistant–*Staphylococcus aureus* hospitalizations, United States. *Emerg Infect Dis*, **11**(6), 868–872.

Kumari, N., Mohapatra, T.M., and Singh, Y.I. (2008). Prevalence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in a Tertiary-Care Hospital in Eastern Nepal. *J Nepal Med Assoc*, **47**(170), 53-56.

Ladhani, S., Joannou, C.L., Lochrie, D.P., Evans, R.W., and Poston, S.M. (1999). Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome. *Clin Microbiol. Rev*, **12**(2), 224–242.

Lagace, W.P.R.S., Alfa M.J., Manickam K., and Karlowsky J. A. (2007). Thermostable DNase Is Superior to Tube Coagulase for Direct Detection of *Staphylococcus aureus* in Positive Blood Cultures. *J Clin Microbiol*, **45**(10), 3478–3479.

Leclercq, R. (2002). Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin Infect Dis,* **34**(4), 482–492.

Lee, K., Chang, C.L., Lee, N.Y., Kim, H.S., Hong, K.S., and Cho, H.C. (2000). Korean nationwide surveillance of antimicrobial resistance of bacteria in 1998. *Yonsei Med J*, **41** (4), 497–506.

Li, M., Cheung, G.Y.C., Hu, J., Wang, D., Joo, H.S., DeLeo, F.R., and Otto, M. (2010). Comparative analysis of virulence and toxin expression of global community associated methicillin resistant *Staphylococcus aureus* strains. *J Infect Dis*, **202**(12), 1866-1876.

Lina, G., Piemont, Y., Godail-Gamot, F., Bes, M., Peter, M.O., Gauduchon, V., Vendenesch, F., and Etienne, J. (1999). Involvement of Panton-Valentine leukocidin–producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis*, **29**(5), 1128–1132.

Linde, H., Wagenlehner, F., Strommenger, B., Drubel, I., Tanzer, J., Reischl, U., Raab, U., Holler, C., Naber, K.G.,Witte, W., Hanses, F., Salzberger, B., and Lehn, N. (2005). Healthcare-associated outbreaks and community-acquired infections due to MRSA carrying the Panton-Valentine leukocidin gene in southeastern Germany. *Eur J Clin Microbiol Infect Dis*, **24**(6), 419-422.

Louie, L., Matsumura, S.O., Choi, E. Louie, M., and Simor, A.E. (2000). Evaluation of three rapid methods for detection of methicillin resistance in *Staphylococcus aureus*. *J Clin Microbiol*, **38**, 2170–2173.

Lowy, F.D. (1998). *Staphylococcus aureus* infections. *N Engl J Med*, **339**(8), 520-532.

Lowy, F.D. (2003). Antimicrobial resistance: the example of *Staphylococcus aureus. J Clin Invest*, **111**(9), 1265–1273.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson, L.B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T. and Monnet, D.L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect,* **18** (3), 268-281.

Malhotra, K.S., Haccuria, K., Michiels, M., Leven, M., Poyart, C., Hryniewicz, W., Goossens, H., and MOSAR WP2 Study Team (2008). Current trends in rapid diagnostics for methicillin-resistant *Staphylococcus aureus* and glycopeptides-resistant *enterococcus* species. *J Clin Microbiol*, **46**(5), 1577-1587.

Maltezou, H.C., and Giamarellou, H. (2006). Community acquired methicillin resistant *Staphylococcus aureus* infections. *Int J Antimicrob agents*, **27** (2), 87-96.

McClure, J.A., Conly, J.M., Lau, V., Elsayed, S., Louie, T., Hutchins, W., and Zhang, K. (2006). A novel multiplex PCR assay for the detection of the Staphylococcal virulence marker, the Panton-Valentine Leukocidin genes and the simultaneous discrimination of the methicillin-susceptible from the resistant Staphylococci. *J Clin Microbiol*, **44**(3), 1141–1144.

McLure, A.R., and Gordon, J. (1992). *In-vitro* evaluation of povidone-iodine and chlorhexidine against methicillin-resistant *Staphylococcus aureus*. *J Hosp Infect*, **21**(4), 291-299.

Mendes, R.E., Mendoza, M., Singh, K.K.B., Castanheira, M., Bell, J.M., Turnidge, J.D., Lin, S.S.F., and Jones, R.N. (2013). Regional resistance surveillance program results for 12 Asia-Pacific nations (2011). *Antimicrob Agents Chemother,* **57**(11), 5721–5726.

Miller, L.G., Perdreau, R.F, Rieg, G., Mehdi, H., Perlroth, J., Bayer, A.S., Tang, A.W., Phung, T.O. and Spellberg, B. (2005). Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. *N Engl J Med*, **352**(14), 1445-1453.

Miro, J.M., Anguera, I., Cabell, C.H., Chen, A.Y., Stafford, J.A., Corey, G.R., Olaison, L., Eykyn, S., Hoen, B., Abtutyn, E., Raoult, D., Bayer, A., and Fowler, V.G. (2005). *Staphylococcus aureus* native valve infective endocarditis: report of 566 episodes from the International Collaboration on Endocarditis Merged Database. *Clin Infect Dis*, **41**(4), 507-514.

Molton, J.S., Tambyah, P.A., Ang, B.S., Ling, M.L., and Fisher, D.A. (2013). The global spread of healthcare-associated multidrug-resistant bacteria: a perspective from Asia. *Clin. Infect. Dis,* **56**(9), 1310–1318.

Morrison, M.A., Hageman, J.C., and Klevens, R.M. (2006). Case definition for community-associated methicillin-resistant *Staphylococcus aureus*. *J Hosp Infect*, **62**(2), 241.

Moussa, I.M., and Shibl, A.M. (2008). Molecular characterization of methicillin–resistant *Staphylococcus aureus* recovered from outpatient clinics in Riyadh, Saudhi Arabia. *Saudi Med J,* **30**(5), 611-617.

Mukhiya, R.K., Shrestha, A., Rai, S.K., Panta, K., Singh, R.N., Rai, G., and Prajapati, A. (2012). Prevalence of Methicillin-Resistant *Staphylococcus aureus* in hospitals of Kathmandu valley. *NJST,* **13**(2), 185-190.

Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., and Watanabe S. (1991). Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. *J Clin Microbiol*, **29**(10), 2240-2244.

Nathwani, D., Morgan, M., Masterton, R.G., Dryden, M., Cookson, B.D., French, G., and Lewis, D. (2008). Guidelines for UK practice for the diagnosis and management of methicillin-resistant *Staphylococcus aureus* MRSA infections presenting in the community. *J Antimicrob Chemother*, **61**(5), 976-994.

National Committee for Clinical Laboratory Standards (2003). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard M7-A6. NCCLS, Wayne, PA, USA.

National Nosocomial Infections Surveillance System (2004). National Nosocomial Infections Surveillance (NNIS) System report, data summary from January 1992 through June 2004. *Am J Infect Control,* **32**, 470-485.

Noskin, G.A., Rubin, R.J., Schentag, J.J., Kluytmans, J., Hedblom, E.C., Jacobson, C., Smulders, M., Gemmen, E., and Bharmal, M. (2007). National trends in *Staphylococcus aureus* infection rates: impact on economic burden and mortality over a 6-year period (1998–2003). *Clin Infect Dis,* **45** (9), 1132-1140.

O'Gara, J.P. (2007). ica and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS Microbiol Lett*, **270**(2), 179-188.

Ogston, A. (1881). Report upon micro-organisms in surgical diseases. *Brit Med J,* **1**, 369-375.

Okonogi, K., Nogi, Y., Kondo, M., Imada, A., and Yokota, T. **(**1989). Emergence of methicillin-resistant clones from cephamycin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother*, **24**(5)**,** 637-645.

Omuse, G., Shivachi, P., Kariuki, S., and Revathi, G. (2013). Prevalence of Panton Valentine leukocidin in carriage and infective strains of *Staphylococcus aureus* at referral hospital in Kenya. *Open J Med Microbiol*, **3**(1), 5-11.

O'Neill, E., Pozzi, C., Houston, P., Smyth, D., Humphreys, H., Robinson, D.A. and O'Gara, J.P. (2007). Association between methicillin susceptibility and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. *J Clin Microbiol*, **45**(5), 1379-1388.

Pandey, S., Raza, M.S., and Bhatta, C.P. (2012). Prevalence and antibiotic sensitivity pattern of methicillin resistant *Staphylococcus aureus* in Kathmandu Medical College-Teaching Hospital. *J Inst Med,* **34**, 13-17.

Pant, J., and Rai, S.K. (2007). Occurrence of *Staphylococcus aureus* in hospital environment and staffs in teaching hospital in Kathmandu, Nepal. *J Nepal Assoc Med Lab Sci*, **8**, 72-73.

Panton, N.P., and Valentine, F. (1932). Staphylococcal toxin. *Lancet*, **222**, 506-508.

Pasteur, L. (1880). De l'extension de la theorie des germes à l'étiologie de queques maladies communes. *C.R. Seances Soc Biol Paris,* **90**, 1035-1044.

Patel, A.K., Patel, K.K., Patel, K.R., Shah, S., and Dileep, P. (2010). Time trends in the epidemiology of microbial infections at a tertiary care centre in west India over last 5 years. *J Assoc Physicians India*, **8**(Suppl), 37–40.

Patel, R. (2005). Biofilms and antimicrobial resistance. *Clin Orthop Relat Res,* **437**, 41-47.

Prabhu, K., Rao, S., and Rao, V. (2011). Inducible clindamycin resistance in *Staphylococcus aureus* isolated from clinical samples. *J Lab Physicians*, **3** (1), 25‑27.

Prevost, G., Cribier, B., Couppie, P., Petiau, P., Supersac, G., Finck, B.V., Monteil, H., and Piemont, Y. (1995). Panton-Valentine leukocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by district genetic loci and have different biological activities. *Infect Immun*, **63**(10), 4121-4129.

Rahman, M., Hossain, M., Samad, T.M.A., Shahriar, M., and Zakaria, M.M. (2002). Prevalence of β-lactamase producing methicillin-resistant *Staphylococcus aureus* and antimicrobial sensitivity pattern. *Bangladesh Pharm J*, **12**(2), 1-4.

Rai, S.K., Talukdar, N.R., and Shrestha, H.G. (1990). Methicillin resistant *Staphylococcus aureus* in a tertiary medical centre, Nepal. *Ind J Med Microbiol*, **8**(3), 108–110.

Rello, J., Sole-Violan, J., Sa-Borges, M., Munoz, E., Sirgo, G., Olana, M., and Daiz E. (2005). Pneumonia caused by oxacillin-resistant *Staphylococcus aureus* treated with glycopeptides. *Crit Care Med*, **33**(9), 1983–1987.

Rhee, Y.G., Cho, N.S., Kim, B.H., and Ha, J.H. (2008). Injection-induced pyogenic arthritis of the shoulder joint. *J Shoulder Elbow Surg*, **17**(1), 63–67.

Richardson, J.F., Quoraishi, A.H., Francis, B.J., and Marples, R.R. (1990). Beta-lactamase-negative, methicillin-resistant *Staphylococcus aureus* in a newborn nursery: report of an outbreak and laboratory investigations. *J Hosp Infec*t, **16** (2), 109–121.

Roberts, M.C., Sutcliffe, J., Courvalin, P., Jensen, L.B., Rood, J., and Seppala, H. (1999). Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother*, **43**(12), 2823-2830.

Rodvold, K.A., and McConeghy, K.W. (2014). Methicillin resistant *Staphylococcus aureus* therapy: past, present and future. *Clin Infect Dis,* **58**(1), 520-527.

Rosenbach, A.J. (1884). Mikro-Qrganismenbei den Wund-Infections-Krankheiten des Menschen. Wiesbaden, J.F. Bergmann, 18.

Rubenstein, E., Kollef, M.H., and Nathwani D. (2008). Pneumonia caused by methicillin-resistant *Staphylococcus aureus*. *Clin Infect Dis,* **46**(5), 378–385.

Safdar, N., and Bradley, E.A. (2008). The risk of infection after nasal colonization with *Staphylococcus aureus*. *Am J Med*, **121**(4), 310–315.

Sah, P., Rijal, K.R., Shakya, B., Tiwari, B.R., and Ghimire, P. (2013). Nasal Carriage Rate of *Staphylococcus aureus* in hospital personnel of National Medical College and Teaching Hospital and their susceptibility pattern. *J Health Appl Sci*, **3**, 21–23.

Said-Salim, B., Mathema, B., and Kreiswirth, B.N. (2003). Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging pathogen. *Infect. Control Hosp Epidemiol,* **24**(6), 451–455.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* 2nd ed. New York: Cold Spring Harbor Laboratory Press.

Sanjana, R.K., Shah, R., Chaudhary, N., and Singh, Y.I. (2010). Prevalence and

antimicrobial susceptibility pattern of methicillin-resistant *Staphylococcus aureus* (MRSA) in CMS-teaching hospital: a preliminary report. *JCMS*, **6**(1), 1-6.

Saravolatz, L.D., Markowitz, N., Arking, L., Pohlod, D., and Fisher, E. (1982). Methicillin-resistant *Staphylococcus aureus*. Epidemiologic observations during a community-acquired outbreak. *Ann Intern Med,* **96**(1)**,** 11-16.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., and Davies, D.G. (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol*, **184**(4), 1140-1154.

Shakya, B., Shrestha, S., and Mitra, T. (2010). Nasal carriage rate of methicillin resistant *Staphylococcus aureus* among at National Medical College Teaching Hospital, Birgunj, Nepal. *Nepal Med Coll J*, **12**(1), 26–29.

Shallcross, L.J., Williams, K., Hopkins, S., Aldridge, R.W., Johnson, A.M., and Hayward, A. C. (2010). "Panton Valentine Leukocidin Associated Staphylococcal Disease: A Cross Sectional Study at a London Hospital, England," *Clin Microbiol and Infect*, **16**(11), 1644-1648.

Shorr, A.F., and Lodise, T. (2006). Burden of methicillin-resistant *Staphylococcus aureus* on healthcare cost and resource utilization. *ISMR Update*, **1**(2), 4-11.

Shrestha, B. (2013). Comparative prevalence of MRSA in two Nepalese tertiary care hospitals. *Open J Clin Diagn*, **3**, 67-73.

Shrestha, B., Pokhrel, B.M., and Mohapatra, T.M. (2009). *Staphylococcus aureus* nasal carriage among health care workers in a Nepal hospital. *Brazilian J Infect Dis*, **13**(5), 322.

Shrestha, B., Singh, W., Raj, V.S., Pokhrel, B.M., and Mahapatra, T.M. (2014). High Prevalence of Panton-Valentine Leukocidin (PVL) Genes in Nosocomial-Acquired *Staphylococcus aureus* Isolated from Tertiary Care Hospitals in Nepal. *BioMed Res Int,* **10**, 1155-1161.

Siegel, J.D., Rhinehart, E., Jackson, M., Chiarello, L., and Healthcare Infection Control Practices Advisory Committee (2007). Management of multidrug-resistant organisms in healthcare settings. *.Am J Infect Control*, **35**(10), 165-193.

Singh, B. D., and Tomecki K.J. (2010). Common skin infections. ClevelandClinicWebsite.www.clevelandclinicmeded.com/medicalpubs/diseasemanagemet/dermatology/common-skin-infections.

Song, J.H., Hsueh, P.R., Chung, D.R., Ko, K.S., Kang, C.I., Peck, K.R., Yeom, J.S., Kim, S.W., Chang, H.H., Kim, Y.S., Jung, S.I., Son, J.S., So, T.M., Lalitha, M.K., Yang, Y., Huang, S.G., Wang, H., Lu, Q., Carlos, C.C., Perera, J.A., Chiu, C.H., Liu, J.W., Chongthaleong, A., Thamlikitkul, V., Van, P.H., and ANSORP Study Group (2011). Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. *J Antimicrob Chemother*, **66**(5), 1061–1069.

Spanu, T., Sanguinetti, M., D'Inzeo, A., Ciccaglione, D., Romano, L., Leone, F., Mazzella, P., and Fadda, G. (2004). Identification of methicillin-resistant isolates of *Staphylococcus aureus* and coagulase negative staphylococci responsible for bloodstream infections with the Phoenix system. *Diagn Microbiol Infect Dis*, **48**(4), 221–227.

Steinberg, J.P., Clark, C.C., and Hackman, B.O. (1996). Nosocomial and community-

acquired *Staphylococcus aureus* bacteremias from 1980 to 1993: impact of intravascular devices and methicillin resistance. *Clin Infect Dis*, **23**(2), 255-259.

Styers, D., Sheehan, D.J., Hogan, P., and Sahm, D.F. (2006). Laboratory–based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. *Ann Clin Microbiol Antimicrob*, **9**(5), 2.

Subedi, S., and Brahmadathan, K.N. (2005). Antimicrobial susceptibility patterns of clinical isolates of *Staphylococcus aureus* in Nepal. *Clin Microbiol Infect,* **11**(3), 235-237.

Sutherland, I.W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology*, **147**(1), 3–9.

Tiemersma, E.W., Bronzwaer, S.L., Lyytikainen, O., Degener, J.E., Schrijnemakers, P., Bruinsma, N., Monen, J., Wittes, W., and Grundmann, H. (2004). Methicillin-resistant *Staphylococcus aureus* in Europe, 1999–2002. *Emerg Infect Dis*, **10**(9), 1627-1634.

Tiwari, H.K., Das, A.K., Sapkota, D., Sivarajan, K., and Pahwa, V.K. (2009). Methicillin resistant *Staphylococcus aureus*: prevalence and antibiogram in a tertiary care hospital in western Nepal. *J Infect Dev Ctries*, **3**(9), 681-684.

Tiwari, H.K., Sapkota, D., and Sen, M.R. (2008). High prevalence of multidrug-resistant MRSA in a tertiary care hospital on f northern India. *Infect Drug Resist*, **1**, 57-61.

Tomasz, A., Nachman, S., and Leaf, H. (1991). Stable classes of phenotypic expression in methicillin-resistant clinical isolates of Staphylococci. *Antimicrob Agents Chemother*, **35**, 124–129.

Tortora, G. J., Funke, B. R., and Case, C.L. (2013). *Microbiology: An Introduction* (11th ed.)

Troidle, L., Eisen, T., Pacelli, L., and Finkelstein, F. (2007). Complications associated with the development of bacteremia with *Staphylococcus aureus*. *Hemodial Int*, **11**(1), 72-75.

Udo, E.E., Pearman, J.W., and Grubb, W.B. (1993). Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. *J Hosp Infect,* **25**, 97–108.

Ujwol, B., Rijal, K.R., Neupane, B., Santu, S., Chaudhary, M., Acharya, D., Thapa, U.S., Adhikari, N., and Ghimire, P. (2016). Status of inducible clindamycin resistance among macrolide resistant *Staphylococcus aureus*. *Afr J Microbiol Res*, **10**(9), 280-284.

Vandenesch, F., Naimi, T., Enright, M.C., Lina, G., Nimmo, G.R., Heffernan, H., Liassine, N., Bes, M., Greenland, T., Reverdy, M., E., and Etienne, J. (2003). Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis*, **9**(8), 978–984.

Verma, S., Joshi, S., Chitnis, V., Hemwani, N., and Chitnis, D. (2000). Growing problem of methicillin resistant staphylococci - Indian scenario. *Indian J Med Sci*, **54**(12), 535-540.

Voss, A., and Doebbeling, B.N. (1995). The worldwide prevalence of methicillin-resistant *Staphylococcus aureus*. *Int J Antimicrob Agents*, **5**(2), 101–106.

Wang, H., Liu, Y., Sun, H., Xu, Y., Xie, X., and Chen, M. **(**2008). *In vitro* activity of ceftobiprole, linezolid, tigecycline, and 23 other antimicrobial agents against *Staphylococcus aureus* isolates in China. *Diagn Microbiol Infect Dis*, **62**(2)**,** 226-229.

Wang, J., Wang, L., Magal, P., Wang, Y., Zhuo, J., Lu, X., and Ruan, S. (2011). Modelling the transmission dynamics of meticillin-resistant *Staphylococcus aureus* in Beijing Tongren hospital. *J Hosp Infect*, **79** (4), 302-308.

Watanakunakorn, C. (1987). Bacteremic *Staphylococcus aureus* pneumonia. *Scand J Infect Dis,* **19**, 623–627.

Wattal, C., Goel, N., Oberoi, J.K., Raveendran, R., Datta, S., and Prasad, K.J. (2010). Surveillance of multidrug resistant organisms in tertiary care hospital in Delhi, India. *J Assoc Physicians India*, **58**(Suppl), 32–36.

Wisplinghoff, H., Bischoff, T., Tallent, S.M., Seifert, H., Wenzel, R.P., and Edmond M.B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis*, **39**(3), 309-317.

Xiao, Y.H., Giske, C.G., Wei, Z.Q., Shen, P., Heddini, A., and Li, L.J. (2011). Epidemiology and characteristics of antimicrobial resistance in China. *Drug Resist Update*, **14**, 236–250.

Xu, K.D., McFeters, G.A., and Stewart, P.S. (2000). Biofilm resistance to antimicrobial agents. *Microbiology*, **146**, 547-549.

Yamagishi, Y., Togawa, M., and Shiomi, M. (2009). Septic arthritis and acute hematogenous osteomyelitis in childhood at a tertiary hospital in Japan. *Pediatr Int,* **51**(3), 371–376.

APPENDIX

PROFORMA

CLINICAL AND MICROBIOLOGICAL PROFILE OF PATIENTS

Name of the patient……..

Age…..... Sex…..

Address……..

Contact number……..

Occupation…….

Patient's clinical history:

Past history of illness……..

Previous isolate (if any)……..

Past history of hospitalization Yes No

Date and duration of hospitalization…….

Ward/OPD……..

Instrumentations such as urinary catheter, Intravenous cannula, Ventilator, any other…….....

Past history of antibiotic intake……

Duration of antibiotic intake ……

Site of infection……. Underlying disease…….

Clinical diagnosis……..

Risk factors associated (if any)……..