ANTIBIOGRAM OF BIOFILM PRODUCING AND NON-PRODUCING COMMUNITY ACQUIRED-METHICILLIN RESISTANT *Staphylococcus aureus* ISOLATED FROM POTENTIAL RISK POPULATION OF DHARAN, NEPAL

A
Dissertation
Submitted to the **Department of Microbiology,**
Central Campus of Technology, Tribhuvan University, Dharan, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Masters of Science in Microbiology
(Medical)

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RECOMMENDATION

This is to certify that **Mr. Jenish Shakya** has completed this dissertation work entitled **"Antibiogram of Biofilm Producing and Non-Producing Community Acquired–Methicillin Resistant S*taphylococcus aureus* isolated from Potential Risk Population of Dharan, Nepal"** as a partial fulfillment of the requirement of M.Sc. degree in Microbiology (Medical) under my supervision. To my knowledge, this work has not been submitted for any other degree/s.

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CERTIFICATE OF APPROVAL

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ABSTRACT

Staphylococcus aureus is one of the common cause of hospital acquired infection and community acquired infections. Nowadays these organisms gas become resistant towards variety of drugs. MRSA is the emerging antibiotic resistant bacteria that are resistant to methicillin antibiotic and known to be the infectious pathogen causing severe infection and a cause of fatal mortality. Aim: Altogether 200 nasal swabs and 200 hand swabs were taken from and transported to microbiology lab in cold chain. The samples were swabbed in mannitol salt agar containing oxacillin powder of 6mg/L and incubated at 37°C for 24 hrs. *Staphylococcus aureus* colonies were identified based on growth characteristics on MSA plates (golden yellow colonies), Gram stain and positive results for coagulase and catalase test. The pure isolated MRSA were subjected to antibiotic susceptibility tests, biofilm formation assays, and MIC. From our study the overall prevalence of CA-MRSA was 61.5%. Higher frequency of multi-drug resistant MRSA was isolated. The biofilm producing CA-MRSA were 51.2% and rest (48.7%) were non-producers. There was significant association in biofilm production with multi-drug resistance ($p<0.05$). The prevalence of CA-MRSA was found more in barbers followed by beauticians and municipal waste workers in comparison to healthy controls. The 51.2% isolates' were biofilm producing CA-MRSA were which showed significant drug resistance. Ciprofloxacin was most sensitive drug against the isolates which was statistically significant ($p<0.05$). The resistant pattern of biofilm producers reported high ability of multi-drug resistance compared to non-biofilm producers ($p<0.05$). Microtitre plate method was found to be gold standard over tube and congo red agar method for screening biofilm formation. The prevalence of VISA and VRSA among CA-MRSA was found to be 49.5 % and 40.6% respectively among the isolates. Improvement in personal hygiene and formulation of appropriate health policy helps to prevent CA-MRSA infection. This study concludes that CA-MRSA is still emerging with multi-drug resistance. The emergence of VISA and VRSA strains has increased concern in vancomycin treatment failure.

Keywords: CA-MRSA, VISA, VRSA, biofilm, antibiotic susceptibility test, multi-drug resistance

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LIST OF ABBREVIATIONS

AMR	:	Antimicrobial Resistance
ATCC	:	American Type Culture Collection
CA-MRSA	:	Community Acquired Methicillin Resistant *S. aureus*
CDC	:	Centre for Disease Control
CLSI	:	Clinical and Laboratory Standards Institute
CRA	:	Congo Red Agar
DNA	:	Deoxyribonucleic Acid
ELISA	:	Enzyme Linked Immunosorbent Assay
FDA	:	Food and Drug Administration
HA-MRSA	:	Hospital Acquired Methicillin Resistant *S. aureus*
KDa	:	Kilo Dalton
NB	:	Nutrient Broth
MDR	:	Multidrug Resistance
MIC	:	Minimum Inhibitory Concentration
MSA	:	Mannitol Salt Agar
NCCLS	:	National Committee for Clinical Laboratory Standards
OD	:	Optical Density
PBP	:	Penicillin Binding Protein
PBS		Phosphate Buffer Saline
PVL	:	Panton-Valentine leucocidin
TSB	:	Trypticase Soya Broth
TM	:	Tube Method
VISA	:	Vancomycin-intermediate *S. aureus*
VRSA	:	Vancomycin-resistant *S. aureus*
VSSA	:	Vancomycin-susceptible *S. aureus*
WHO	:	World Health Organization

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CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Staphylococcus aureus is one of the most common causes of hospital acquired and community acquired infections worldwide which results in substantial morbidity and mortality. However, in the late 1990s, community-acquired MRSA (CA-MRSA) infections began to appear in otherwise healthy people who had no known risk factors for these infections (Bassetti et al 2009; Gorak et al 1999). Methicillin resistance *Staphylococcus aureus* (MRSA) bacteria is well studied organism of medical significance because of its wide resistance to many antibiotics. This is one of the burning global issues. Methicillin-resistant *Staphylococcus aureus* was first observed in 1960, less than one year after the introduction of this second generation beta-lactam antibiotic into clinical practice. Evolution has allowed bacteria to develop sophisticated methods of survival. One of these methods is biofilm production. Biofilm can be best described as complex bacterial communities embedded in a self-producing slime. Once bacteria form these biofilm communities, they become very difficult to treat with antibiotics. Along with biofilm production, another rising concern is antibiotic resistance to many of our current antibiotics is sharply increasing, thereby creating a critical need to develop novel antimicrobial drugs. Outbreaks of CA-MRSA have occurred among individuals sharing close contact with others in schools, prisons, and locker rooms, but other possible environmental reservoirs of MRSA have yet to be comprehensively explored (Diekema et al 2001).

Since staphylococci are part of the resident microbiological flora of the skin the presence of the biofilm-forming strains among the may be associated with an increased risk of transmission of virulent biofilm-forming strains in the hospital environment. *S. aureus* being the normal flora of the skin is associated with wound infections in addition to it the increasing incidence of multi-drug resistance has created the global concern. *S. aureus* is normal flora of human

anterior nares, nasopharynx, and skin and can colonize various epithelial or mucosal surfaces. They are opportunistic pathogen involved in nosocomial infections and produce large numbers of toxins and exhibit frequent and sometimes multiple resistances to antimicrobials (Gorwitz et al 2008). *S. aureus* is gram-positive cocci, which is about 1µm in diameter. It is a catalase and coagulase positive and is fermentative organism. It grows rapidly under either aerobic or anaerobic. *S. aureus* is gram positive arranged in grape-like cluster, non-sporing, non-motile and usually non-capsulated bacteria. Facultative anaerobic optimum temperature is 37°C where colonies seem to be opaque and maybe golden yellow pigment or creamy catalase positive (Chakraborty et al 2005).

S. aureus has the capacity to produce a wide array of virulence factors, causes various pyogenic infections, food poisoning, and toxic shock syndrome. *S. aureus* possess a large number of cells associated and extracellular factors, which overcome the body‘s defense system and invade, survive and colonize the tissue. The organism possess such virulence factors which oppose destruction by the component of innate immunity i.e., complement and phagocytosis (Bailey and Scott‘s et al 2007).

It is necessary to note that *S. aureus* is naturally susceptible to all antimicrobials, but it is also exceptional efficient in developing resistance. The first strains of antimicrobial resistant *S. aureus* were observed shortly after the introduction of penicillin for clinical use (Kirby 1944). These strains became resistant to penicillin by producing a plasmid-encoded penicillinase, called β-lactamase that can break down the β-lactam ring of penicillin, making it ineffective (Chambers and Deleo, 2009). MRSA are usually multi-drug resistance and it has created many problems in antibiotic therapy since last decade. In 1961, the first methicillin-resistant *Staphylococcus aureus* (MRSA) strains were identified (Barber 1961).

Interestingly, this was only two years after the introduction of methicillin. Today, it is known that MRSA is a subclass of *S. aureus* that developed resistance to

several classes of antibiotics including penicillin, cephalosporin and carbapenems (Chambers and Deleo, 2009). Research has shown that methicillin susceptible *S. aureus* (MSSA) is able to develop resistance to antimicrobials containing a β-lactam ring because of its ability to acquire the resistance encoding *mec*A gene. The *mec*A gene is acquired from a mobile genetic element from other *Staphylococcus* species, which naturally contain this resistance gene (Moellering 2012). The *mec*A gene produces resistance because it encodes the penicillin binding protein 2a (PBP 2a), which improves cell wall synthesis in the presence of β-lactam antimicrobials (Hartman and Tomasz, 1984; Song et al 1987).

Smyth et al (2005) reported that MSA containing 6mg/liter cefoxitin allowed the isolation of all MRSA. CA-MRSA is known to be phenotypically and genotypically different from HA-MRSA. CA-MRSA consists of *SCCmec* type IV or V gene Pentone Valentine Leucocidin (Durenberg et al 2006). Staphylococcal resistance to oxacillin/methicillin occurs when an isolate produces an altered penicillin-binding protein, PBP2a, which is encoded by the *mec*A gene. The variant penicillin-binding protein binds beta-lactams with lower avidity, which results in resistance to this class of antimicrobial agents. Cefoxitin is an even better inducer of the *mec*A gene, and tests using cefoxitin give more reproducible and accurate results than tests with oxacillin. Methicillin resistance is genetically and biochemically complex and mediated by staphylococcal cassette chromosome (*SCCmec*), a mobile genetic element encoding for an altered penicillin-binding protein (PBP2a, *mec*A) with decreased affinity to β-lactams (Gorden et al 2008).

Infection by pathogenic organism is mediated by loss of host immune reaction and even due to trauma. Infection is result of the invasion of pathogen by crushing the host barrier. The human skin harbors many microorganisms like *Staphylococcus aureus, Staphylococcus epidermidis, Candida, Propionibacterium acini,* etc. *S. aureus* is best known organism meant for bacterial infection of wound and tissue. It is even known significant in terms of nosocomial infection. The pathogenicity of *S. aureus* is related with the severity of the tissue, skin, bone, respiratory system, etc. In today‘s era the MRSA is a major global concern because of its wide resistance to different antibiotics. Biofilm producing MRSA

infections are life threatening infection because of its multi-drug resistance and resistance of biofilm on the action of antibiotics. MRSA produces biofilm and are of significance in medical science. Biofilm are aggregated microbial population embedded themselves in extracellular matrix of exopolysaccharide (EPS) which are self-produced. Biofilm formation in *S. aureus* is regulated by expression of polysaccharide intracellular adhesion (PIA) which mediates cell to cell adhesion and is the gene product of *ica* ABDC (Ammendolia 1999).

S. aureus is Gram-positive cocci where the round cells, approximately 1mm in diameter, form grape-like clusters indicative of the ability to divide in more than one plane. They are capable of both aerobic and anaerobic respiration and most strains ferment mannitol anaerobically. They produce catalase, coagulase and an extracellular cell clumping factor, and some strains produce capsules (Bannerman et al 2003).

CA-MRSA is usually susceptible to clindamycin and they are less often multiple resistant to other non β-lactam antibiotics (Naimi et al 2003). The genetic basis of methicillin-resistance in *S. aureus* is associated with carriage of a mobile cassette of genes known as the staphylococcal cassette chromosome *mec* (*SCCmec*) (Katayam 2000). Within this cassette is the *mec*A gene that is responsible for resistance to β-lactams including methicillin. The product of *mec*A is the peptidoglycan synthesis enzyme penicillin binding protein (PBP) 2a involved in cross-linking of peptidoglycan in the bacterial cell wall (Hartman 1984). PBP2a has a lower binding affinity for β-lactam antibiotics than the native PBP proteins encoded in the core genome of *S. aureus*. The subsequent combination of reduced penicillin-binding affinity and increased production of PBP2a accounts for the observed resistance to β-lactam antibiotics. HA-MRSA and CA-MRSA infections can cause severe medical conditions and are resistance to beta-lactam or penicillinase-resistant penicillin such as oxacillin methicillin also is a member of this group (Deleo et al 2010).

In Nepal, Neupane et al (2016) reported higher nasal carriage of MRSA in HIV patients than in non-HIV patients. They reported the major factor associated with

nasal colonization were close personal contact, smoking habit and working or living in a farm. Khanal et al (2015) reported overall nasal carriage of MRSA was 3.4% in health workers of tertiary care hospital of western Nepal.

MIC is the lowest concentration of antimicrobial agent which can inhibit of organism. Vancomycin has been a drug of choice for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infection. The limitation of other antibiotics induces the emergence of VRSA is making the treatment of MRSA difficult (Finks et al 2009). The emergence of VISA strains and VRSA strains has been reported. The higher MIC of Vancomycin resistant of MRSA has been known to be associated with Drug resistance to many other antibiotics (Amatya et al 2014).

The first occurrence of a strain of *S. aureus* with reduced susceptibility to vancomycin (MICs 4 to 16 μg/mL) was reported from Japan in 1997 (Hiramatsu 1991) followed by reports from the United States and France (Fridkin 2001). The exact mechanisms of resistance that result in elevated MICs are unknown, although they likely involve alterations in the cell wall and changes in several metabolic pathways. To date, most vancomycin intermediate *S. aureus* strains appear to have developed from MRSA (Gardete 2014). Resistance in VRSA is conferred by the *van*A gene and operon, which is present on a plasmid (McGuinness 2017).

1.2 Objectives

1.2.1 General objective

- To assess antibiogram of biofilm producing and non-producing community acquired–methicillin resistant *Staphylococcus aureus* (CA-MRSA) isolated from potential risk population of Dharan, Nepal.

1.2.2 Specific objectives

i. To identify the CA-MRSA from body surface of potential risk population of Dharan.
ii. To determine the prevalence of MRSA on the body surface swabs from potential risk population of Dharan, Nepal.
iii. To determine the biofilm production of MRSA.
iv. To assess the antibiotic susceptibility test of MRSA.
v. To determine MIC of vancomycin to MRSA isolates.

CHAPTER II

LITERATURE REVIEW

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a facultative anaerobic, Gram-positive coccus that normally is arranged in grape-like clusters. Cluster formation is due to successive cell division occurring in asymmetric three planes. The organism has a diameter of 1µm in average and liquid culture shows the arrangement of cocci in single, pairs, tetrads, or short chains of three or four cells. A few strains have capacity to produce capsules in young cultures (Chakraborty et al 2005). They are non-motile and often golden-yellow pigmented cells. The primarily colonization sites are the anterior part of the nares and skin surfaces. The organism is non-spore forming but is resistant to dry conditions and high salt concentrations, which is essential when colonizing the skin surface. *S. aureus* is distinguished from the other species by its ability to clot blood plasma by the action of the enzyme coagulase (Ray et al 2012). Second edition of Bergey‘s manual of systematic bacteriology (2002) classify staphylococcus in family VIII of section XVII belonging to volume 3 which have low G+C content gram positive microorganisms. There are altogether 47 species of staphylococci listed in Bergey‘s manual.

Staphylococci can normally grow in basic media like nutrient agar and nutrient broth. However, in mannitol salt agar they form yellow colony by mannitol fermentation. They are non-motile and range in between 0.5 to 1.5µm in diameter (Murray 2007). *S. aureus* is a commensal commonly found to colonize several animal species, including humans. Around 20-40% of the human populations are carriers and some humans are intermediate carriers whereas others are persistent carries (Williams 1963).

Species of staphylococci found in human skin include *S. epidermidis, S. haemolyticus S. hominis, S. warneri, S. capitis, S. lugdunensis, S. cohnii, S. simulans* and *S. xylosus* (Boldock et al 2018).

S. aureus, a worldwide pathogen with its natural reservoir in human belongs to genus of the micrococcaceae. *S. aureus* cause a variety of infections ranging from minor skin infections to serious conditions such as osteomyelitis, central nervous system infections, bacteremia and infective endocarditis (Tenoverfag 2006). *S. aureus* also produce several different toxins, for example, the toxic shock syndrome toxin, staphylococcal enterotoxin, exfoliatin-toxin, alpha- toxin and leukocidin (Salyersaaw 2002) that causes toxin-induced syndromes such as bullous impetigo, food poisoning, scalded skin syndrome and toxic shock syndrome (Tenoverfag 2006).

S. aureus is one of the most important etiological agents of many hospital-acquired infections as well as community-acquired infections and poses a constant therapeutic problem to clinicians (Klein et al 2007). Methicillin and its derivatives became the drugs of choice for the treatment of infections caused by *S. aureus*. Over time, treatment of serious *S. aureus* infections can be challenging as the widespread use of antibiotics has led some *S. aureus* becoming more resistant to antibiotics (Archer 1998).

The remarkable ability of *S. aureus* to acquire useful genes from various organisms has been revealed through homology alignment and phylogenetic trees. The evidence of repeated lateral and horizontal gene transfers (including plasmids) to and from distantly related organisms includes homologues in vertebrates, other bacterial species and even plants (Kuroda et al 2001). In addition, a large number of mobilizable exogenous DNA stretches, including insertion sequences, transposons, bacteriophages and pathogenicity islands (also referred to as genomic islands) that contain specific determinants responsible for disease and antibiotic resistance have been identified. Overall, the staphylococcal cell wall plays an important role for the bacteria‘s strength and success (Moreillon et al 2005).

Staphylococcus aureus, a gram positive bacterium, is a leading cause of hospital acquired infection (HAI) and community acquired infection (CAI) (Orrett and Land 2006) in both developed and developing countries with infections ranging

from mild infections of the skin and soft tissues to life-threatening sepsis (Duckworth 1993). The emergence of drug resistant strain of *S. aureus* is a major threat in the management of staphylococcal infections (WHO 1997). Nasal carriers of high number of *S. aureus* organisms have a risk of health care-associated infection with the organisms that is three to six times the risk among non-carriers and low level carriers (Luzar et al 1990).

Staphylococcus aureus has the capacity to adhere to catheters and other indwelling medical devices and form biofilm on polymeric surfaces (Cramton et al 1999; Fowler et al 2001). This ability depends on the production of polysaccharide intercellular adhesin (PIA) encoded by the intercellular adhesion gene cluster *ica* ADBC (Cramton et al 1999; Mckenney et al 1999). The polysaccharide intercellular adhesion is composed of linear β-1, 6-linked glucosaminylglycans. It is synthesized in vitro from UDP-*N*-acetylglucosamine by the enzyme *N*-Acetylglucosaminyltranferase, which is encoded by the intercellular adhesion (*ica*) locus, in particular by the *ica*A gene. Sole expression of *ica*A induces only low enzymatic activity, but co-expression of *ica*A with *ica*D leads to a significant increase in activity and is related to phenotypic expression of the capsular polysaccharide (Gerke et al 1998). The initial attachment of staphylococcal cells on a biomaterial is followed by bacterial accumulation and formation of a mature biofilm (Götz 2002). Established biofilm can tolerate antimicrobial agents, and are extraordinarily resistant to phagocytosis, making biofilm extremely difficult to eradicate (Lewis 2001).

To some extent, biofilm-associated infections with *S. aureus* are similar to those with *S. epidermidis*. However, the involvement of *S. aureus* usually requires more intensive care. Often, *S. aureus* biofilm-associated infections are difficult to treat with antibiotics and devices need to be replaced more frequently than those infected with *S. epidermidis*. In addition, they represent a reservoir of dissemination of *S. aureus* infection to other sites in the human body. In this regard, it is critical from a perspective of molecular pathogenesis, whether biofilm-forming *S. aureus* strains are genetically different from those involved in more serious infections, or – alternatively – whether they are in a different

physiological status and might thus develop a more aggressive behavior when spreading within the body (Jones et al 2001).

Staphylococcal Cassette Chromosome mec (SCCmec)

The *mec*A gene of molecular weight 2.1 kb is located on *SCCmec*. Five main types of *SCCmec* have been known. *Staphylococcus aureus* is resistant to methicillin antibiotics due to presence of *mec*A gene that encodes 78 kDa Penicillin Binding Protein (PBP2a) which is the site in the cell wall where β-lactam antibiotics normally bind resulting cell wall disruption and death of bacteria. Since β-lactam antibiotic fails to bind to PBP2a of cell wall the bacteria becomes resistant.

Risk factors of MRSA include the several exposure to antibiotics, prolonged durations of therapy, staying in ICU or burn units, invasive procedures, etc. The risk factors of CA-MRSA include gastrointestinal disease, IV drug use, and indirect contact with contaminated objects (Lu et al 2005).

2.2 Classification of *Staphylococcus*

Staphylococci can be classified in various ways according to their cultural characteristics, colony morphology, biochemical characteristics, pathogenicity and cell wall structure.

2.2.1 Classification on the basis of pigment production

1. *S. aureus* producing golden yellow colonies and are pathogenic.
2. *S. albus* producing white colonies and are non-pathogenic.
3. *S. citreus* producing yellow colonies and are non-pathogenic

(Ananthanarayan and Paniker, 1986).

2.3 Cultural characteristics

S. aureus can usually grow in basic media like nutrient agar within the temperature range of 12-44ºC. The optimum temperature and pH for growth is

37°C and 7.5 respectively. It can be cultured on enriched and selective media like mannitol salt agar (MSA), blood agar and most other media.

i. *S aureus* can produce round, convex, opaque and smooth-glistening surface colony having diameter of 1-3mm in nutrient agar at aerobic incubation of 37°C for 24 hours. Most strain produce golden yellow pigment. Some strain may produce orange or yellow pigment and a few are non-pigment producer. Pigment production is best seen when the cultures are grown aerobically at 22°C (Chakraborty 2005).

ii. Nutrient broth: Most strain give moderate to dense turbidity with powdery deposit at the bottom. No pigmentation is produced in liquid broth (Baired-parker 1997).

iii. In 5% sheep blood agar *S. aureus* show medium to large, smooth, slightly raised, translucent colonies with yellow pigmentation and show beta-haemolysis (Forbes et al 2002).

iv. *S. aureus* is able to grow on MSA media with 7-10% sodium chloride (Cheesbrough 2000). Most bacteria are inhibited on MSA while *S. aureus* is tolerant to sodium chloride incorporated into the media and forms 1mm diameter yellow colonies surrounded by yellow medium due to acid production from mannitol fermentation (Colle et al 1996).

2.3.1 Biochemical characteristics

S. aureus is catalase positive and coagulase positive organism that can ferments sugars namely glucose, lactose, sucrose, maltose, lactose, and mannitol with the production of acid but no gas. The organism can hydrolyzes urea, reduces nitrates to nitrites, liquefy gelatin and is MR/VP positive but indole negative. Urease and esterase production and lactose fermentation are variable characters useful in the differentiation of methicillin resistant strains. *S. aureus* also produces deoxyribonuclease (DNase) and thermonuclease (TNase). Though coagulase test is of diagnostic value in detecting *S. aureus*, some other rare strains also give

positive coagulase test such as *S. intermedius, S. schleiferi sub sp. coagulans* and *S. hyicus* (Forbes et al 2002).

2.4 Virulence factors

2.4.1 Cell-Associated Polymers

2.4.1.1. Capsule

Capsular polysaccharide surrounding the cell wall inhibits opsonization (Kumar 2012).

2.4.1.2. Peptidoglycan

The cell wall polysaccharide peptidoglycan confers rigidity and structural integrity to the bacterial cell. It gives rigidity to the cell and represents 50% of cell wall weight. It has endotoxin like property and septic shock may result from severe infection. The peptidoglycan can stimulate macrophages to produce cytokines and can activate the complement and coagulation cascade (Chakraborty et al 2005). It activates complement and induces release of inflammatory cytokines (Kumar 2012).

2.4.1.3. Teichoic Acids

Teichoic acid, an antigenic component of the cell wall, facilitates adhesion of the cocci to the host cell surface and protects them from complement-mediated opsonisation (Kumar 2012). It confers antigenicity and behaves as surface receptors for Staphylococcal bacteriophage. It mediate adherence of staphylococci to mucosal cells (Chakraborty et al 2005).

2.4.2 Cell Surface Proteins

2.4.2.1. Protein A

Protein A is a group-specific antigen unique to *S. aureus* strains. Ninety percent of protein A is found in the cell wall covalently linked to the peptidoglycan. It is absent in both coagulase negative staphylococci (CNS) and micrococci. Biologic

Properties: Protein A has many biologic properties including chemotactic, anticomplementary, and antiphagocytic and elicits hypersensitivity reactions and platelet injury. It is mitogenic and potentiates natural killer activity of human lymphocytes. The uniqueness of protein A is centered on its ability to interact with normal IgG of most mammalian species. Unlike a specific antigen-antibody reaction, binding involves not the Fab fragment but the Fc portion of the immunoglobulin. Protein A binds IgG molecules, nonspecifically, through Fc region leaving specific Fab sites free to combine with specific antigen. When suspension of such sensitized cells is treated with homologous (test) antigen, the antigen combines with free Fab sites of IgG attached to staphylococcal cells. This is known as co-agglutination. Its ability to bind to the Fc region of IgG has led to numerous applications in immunochemical and cell-surface structural studies (Kumar 2012).

2.4.2.2. Fibrinonectin-binding protein (FBP)

It promotes binding to mucosal cells and tissue matrices (Mongodin et al 2002).

2.4.2.3. Cytoplasmic Membrane

The cytoplasmic membrane is made up of a complex of proteins, lipids, and a small amount of carbohydrates. It serves as an osmotic barrier for the cell and provides an anchorage for the cellular biosynthetic and respiratory enzymes (Mishra et al 2009).

2.4.2.4. Clumping Factor (Bound Coagulase)

The component on the cell wall of *S. aureus* that results in the clumping of whole staphylococci in the presence of plasma is referred to as the clumping factor (also called bound coagulase). It is a surface associated protein also known as bound coagulase, which reacts with fibrinogen (Chakraborty et al 2005). This factor reacts directly with fibrinogen in plasma, converts it to insoluble fibrin, causing the staphylococci to clump or aggregate (Kumar 2012).

2.4.3. Super-antigen exotoxins

These toxins have an affinity for the T-cell receptor-MHC class II antigen complex. They stimulate maximum number of T-lymphocyte, which can cause toxic shock by release of high amount of T cell cytokines such as interleukin-2 (IL-2), interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α) (Chakraborty et al 2005).

2.4.3.1. Enterotoxins

Enterotoxins types A-E, G, H, I and J are commonly produced by 65% of strains of *S. aureus* (Greenwood 2012). When ingested as preformed toxins in contaminated foods, they stimulate the vomiting center in the brain by binding the neural receptors in the upper gastro intestinal tract. These toxic proteins are heat stable and withstand up to 100ºC for several minutes (Chakraborty et al 2005).

2.4.3.2. Toxic shock syndrome toxin (TSST)

TSST is a super antigen and causes toxic shock by stimulating the release of large amounts of IL-1, IL-2 and tumor necrosis factor (TNF). Approximately 5.25% of *S. aureus* isolates carry the gene for TSST (Levinson and Jawetz, 1996). Toxic shock occurs especially in tampon using women or in individuals with wound infections who do not have antibody against TSST (Chakraborty et al 2005).

2.4.3.3. Exofoliatin (Exfoliative toxin)

It causes scalded skin syndrome mainly in young children. After localized infection, the strain produces diffusible exofoliative toxin that exerts distant effects and after the development of painful rash, the epidermis slough off and the skin surface resembles scalding (Chakraborty et al 2005).

2.4.4. Extracellular enzymes

The pathogenicity of *S. aureus* infections is supplemented by its ability to produce wide variety of tissue damaging enzymes (Chakraborty et al 2005).

2.4.4.1. Hyaluronidase

This enzyme breaks down the connective tissue of the host by hydrolyzing the hyaluronic acid, which helps the organism to spread from the localized part to surrounding tissues. This enzyme is also called spreading factor (Chakraborty et al 2005).

2.4.4.2. Lipase

This enzyme degrades lipid of the skins and tissues and helps in its spread. Lipase degradation facilitates *S. aureus* to colonize the sebaceous glands (Chakraborty et al 2005).

2.4.4.3. Staphylokinase

It is also called fibrinolysin, which lyses fibrin by activating plasminogen. It forms a complex and causes dissolution of fibrin clots by proteolytic activity (Chakraborty et al 2005).

2.4.4.4. Deoxyribonuclease

This enzyme degrades host's DNA (Chakraborty et al 2005).

2.4.4.5. Coagulase

It is an enzyme, which causes plasma to clot by activating prothrombin, which in turn converts fibrinogen to fibrin. It is of two types; free coagulase and bound coagulase. About 97% of *S. aureus* produce both forms of coagulase (Maranan et al 1997; Langone 1982).

2.4.4.6. Phosphatase

This enzyme breaks down phospholipid of the host cell (Chakraborty et al 2005).

2.5 Staphylococcal Diseases

Staphylococcal infections are the common bacterial infections that range from acute to Chronic. Staphylococcal infections are characteristically localized pyogenic lesions, in contrast to the spreading nature of streptococcal infections. *S.*

aureus causes disease through the direct invasion and destruction of tissue or through the production of toxin (Bailey and Scott‘s et al 2007).

2.5.1 Cutaneous Infections

These include: wound and burn infection, pustules (small cutaneous abscesses), furuncles or boils (large cutaneous abscesses), carbuncles, styes, impetigo and pemphigus neonatorum (Bailey and Scott‘s et al 2007).

2.5.1.1. Impetigo

This contagious infection usually occurs on the face, especially around the mouth. Small vesicles lead to pustules, which crust over to become honey coloured, wet and flaky (Bailey and Scott‘s et al 2007).

2.5.1.2. Cellulites

This is a deeper infection of the cells. The tissue becomes hot, red, shiny and swollen (Bailey and Scott‘s et al 2007).

2.5.1.3. Wound infections

Any skin wound can be infected with *Staphylococcus aureus*, resulting in an abscess, cellulites or both. When a sutured post-surgical wound becomes infected, it must be reported and treated (Bailey and Scott‘s et al 2007).

2.5.1.4. Abscesses

These can occur in any organ when the organism circulates in the bloodstream. These abscesses are often called metastatic abscesses because they occur by the spread of bacteria from the original site (Bailey and Scott‘s et al 2007).

2.5.2 Deep Infections

These include: osteomyelitis, periostitis, tonsillitis, pharyngitis, sinusitis, bronchopneumonia, empyema, septicemia, meningitis, endocarditis, breast

abscess, renal abscess and abscesses in other organs (Bailey and Scott‘s et al 2007).

2.5.2.1. Pneumonia

S. aureus is a rare but severe cause of community acquired bacterial pneumonia. Pneumonia is more common in hospitalized patients (Strohl et al 2002). Pneumonia is more commonly seen in postoperative patients or following viral respiratory infection especially by influenza virus. The violent, destructive, necrotizing pneumonia frequently causes effusions and emphysema. In community, pneumonia cases primarily occurred in children, but older age groups may be affected (Francis et al 2005).

2.5.2.2. Osteomyelitis

This bone infection usually occurs in boys under 12 years of age. The infection spreads to the bone hematogenously, presenting locally with warm, swollen tissue over the bone and with systematic fever and shakes (Fridkin et al 2005).

2.5.2.3. Septic arthritis

Invasion of the synovial membrane by *S. aureus* results in a closed infection of the joint cavity. Septic arthritis should be treated immediately because collected pus can rapidly cause irreparable cartilage damage. Therapy requires drainage of the joint and antimicrobial therapy (Fridkin et al 2005).

2.5.2.4. Acute Endocarditis

It is generally associated with intravenous drug abuse and is caused by injection of contaminated preparation or by needles that are contaminated with *S. aureus*. It causes destructive infection of heart valves with the sudden onset of high fever, chills and myalgia. Intravenous drug users usually develop a right-sided tricuspid valve endocarditis (Vandenesch et al 2003).

2.5.2.5. Meningitis, Cerebritis and brain abscess

Patient with these disease show symptoms like high fever, stiff neck, headache, coma and focal neurological signs (Levinson and Jawetz, 1996).

2.5.2.6. Septicemia

It can be originate from any localized lesion, especially wound infection or as a result of intravenous drug abuse (Gonzalez et al 2005; Lowy 1998).

2.5.3 Toxin-Mediated Diseases

2.5.3.1. Food Poisoning

Staphylococcal food poisoning (nausea, vomiting and diarrhea) may follow 2-6 hours after the ingestion of food in which *S. aureus* has multiplied and formed enterotoxin. The types of food usually responsible are meat, fish, milk and milk products. The illness is usually self-limited, with recovery in a day or so. The illness is rarely fatal (Hennekinne et al 2012).

2.5.3.2. Toxic Shock Syndrome (TSS)

Toxin-producing strains of *S. aureus* have been implicated in most cases of TSS, a multisystem disease that primarily afflicts young women. Most cases occur in menstruating women who use tampons. However, non-menstruating women, children, and men with boils or staphylococcal infections of wounds can also have TSS. The disease is initiated with the localized growth of toxin-producing strains of *S. aureus* in the vagina or a wound, followed by release of the toxin into the blood stream. Though tampon-related TSS is now rare, the syndrome occurs in other infections of the skin, mucosa and other sites and also in some surgical wounds (Jamart et al 2005).

2.5.3.3. Exfoliative Diseases

These lesions are produced by the strains of *S. aureus* which produce epidermolytic toxins. This toxin is responsible for the staphylococcal scalded skin syndrome‘ (SSSS), exfoliative skin diseases in which the outer layer of

epidermis gets separated from the underlying tissues. SSSS is seen mostly in young children and only rarely in older children and adults (Kumar 2012).

2.6 Classification of MRSA

Taxonomy	**Name**
Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Firmicutes
Class:	Baccilli
Order:	Bacillales
Family:	Staphylococcaceae
Genus:	*Staphylococcus*
Species:	*Staphylococcus aureus*
Subspecies:	Methicillin-resistant *Staphylococcus aureus*

(Source: Bergey's Manual of Determinative Bacteriology, 1939)

2.7 Types of MRSA

Hospital-acquired MRSA (HA-MRSA): It is an infection picked up by a patient who is admitted to the hospital for an unrelated problem and subsequently becomes infected by methicillin resistant *Staphylococcus aureus* from a hospital source secondary to their initial health care issue (David et al 2010).

Community-acquired MRSA (CA-MRSA): It is infections develop from exposure to a carrier or contaminated surface in the greater community (outside the hospital).

Livestock associated MRSA (LA-MRSA): Farm animals have been identified as an emerging reservoir for transmission of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) to humans.

2.7.1 Community-acquired MRSA (CA-MRSA)

Cases of MRSA documented in healthy community dwelling persons without established risk factors for MRSA acquisition is referred to as community

acquired (CA-MRSA). In the 1990s, the first case of MRSA were seen in people who were not hospitalized in Australia, followed by USA and is now highly prevalent worldwide (Ottor et al 2010). The CA-MRSA infects healthy individuals without any health care contact, harbors smaller and more mobile *SCCmec* types (IV and V), is usually Panton-Valentine leucocidin (PVL) positive, susceptible to non-β-lactam drugs and typically manifests as skin and soft tissue infections. Community acquired MRSA isolates have been associated with many of the same clinical presentations known to occur with traditional *S. aureus* infection. However, several outbreaks of epidemic furunculosis and severe invasive pediatric infections have been particularly noteworthy (Eguia et al 2003). Recent investigations have revealed several characteristics that differentiate CA-MRSA from HA-MRSA strains. The antimicrobial patterns of community isolates are unique and differ from HA-MRSA. Community isolates tend to be susceptible to a variety of non-β lactam antibiotics, whereas HA-MRSA is 21 typically resistant to multiple antibiotics. Most of them are susceptible to trimethoprim-sulphamethoxazole, rifampicin, doxycycline, minocycline and fusidic acid. Reports based on genotype evidence have suggested that CA-MRSA is likely spreading within hospital as well as blurring the line between CA-MRSA and HA-MRSA infections (Popovinch et al 2008). It is hypothesized that the evolution of CA-MRSA is a recent event due to the acquisition of *mec* DNA by previously Methicillin susceptible strains that circulated in the community. Skin infections caused by *S. aureus* are generally believed to follow colonization of the skin and nares of the host.

2.7.2 Hospital- associated MRSA (HA-MRSA)

HA-MRSA has been regarded as a nosocomial pathogen which characteristically colonizes or infects hospitalized individuals with predisposing risk factors (Deleo et al 2010). The strains usually harbor *SCCmec* type I, II and III and are multidrug resistant (Gorden and Lowly, 2008). In health facilities, there is selective advantage for MRSA survival as a result of antibiotic use (Lee et al 2011). In 2005, the proportion of MRSA among *S. aureus* hospital associated infections in

the US was estimated at 53% with large local variations (Styres et al 2006) and now it is more. MRSA is highly transmissible among hospitalized patients and the infected or colonized patients as well as colonized employee are the main reservoir of the bacteria in the hospital worldwide (Tambic et al 1997). A number of factors have been found to associated with a higher risk for nosocomial acquisition of MRSA: prolonged hospitalization, care in an intensive care unit, prolonged antimicrobial therapy using broad spectrum antibiotics, surgical procedures, having a surgical wound and intravenous (IV) line, severe underlying illness and close proximity to other ill patients who infected or colonized with MRSA (Boyce et al 2005). Certain patient population with complicating medical conditions such as diabetes mellitus, HIV infection, chronic dermatological diseases, indwelling catheters haemodialysis, intravenous drug use and post-surgical wound infections are at 20 increased risks of serious MRSA infections (Lowy 1998; Boyce et al 2005). These patients also have increased rate of MRSA carriage. Some strains associated with outbreaks are called epidemic MRSA (EMRSA) because once introduced they have a remarkable ability to spread within a hospital (Reboli et al 1989). Many studies have characterized EMRSA appear to be well adapted to the hospital environment, are established in several hospitals within a country or have spread internationally. ICU nursing staffs are found to harbor MRSA in the nose, nails and uniforms. Some studies have suggested that contaminated environmental surfaces may serve as a reservoir for MRSA in hospitals. Currently in the US, 50% of nosocomial *S. aureus* infections in ICU are due to MRSA (NNIS 2002).

2.8 Sources and transmission of MRSA

MRSA is primarily transmitted from person to person by direct contact, usually from the hands of an infected or colonized individual. It can also be transmitted by sharing towels, personal hygiene items, athletic equipment, clothes, and public used bath and used equipment. Droplet-borne transmission is less common but may be important in patients with tracheostomies who are unable to control their secretions. Person with pneumonia in close contact with others can transmit

MRSA by coughing up large droplets of infectious particles. MRSA can colonize the skin, nose, blood and urine. Colonized domestic pets such as horses, pigs can also act as source of transmission (Voss et al 2005).

2.9 Mechanism of resistance

Unlike penicillin resistance that results from a plasmid encoded enzyme (β-lactamase), methicillin resistance is genetically and biochemically complex and mediated by staphylococcal cassette chromosome (SCC*mec*), a mobile genetic element encoding for an altered penicillin-binding protein (PBP2a, *mec*A) with decreased affinity to β-lactams (Gordon et al 2008). The *mec*A gene is a component of a large DNA fragment designated *mec* DNA, which is located at the specific site of the *S. aureus* chromosome and has been suggested to be transmitted from other bacterial species (Hiramastu et al 1996). Hence, it is hypothesized that removal of the repressor function for *mec*A is a pre-requisite for constitutive expression of methicillin resistance in *S. aureus* with *mec* DNA. Indeed, the deletion of *mec*I or point mutation in the *mec*I gene has been found in a number of methicillin resistant staphylococci isolates. In some strains, point mutations were detected in *mec*A promoter 19 region corresponding to a presumptive operator of *mec*A, i.e. the binding site of the repressor protein. Some strains of *S. aureus* over express β– lactamase and appear to be resistant to oxacillin and, rarely methicillin despite being *mec*A negative. They have slightly raised minimum inhibitory concentrations (MICs) and may thus be described as ―minimally resistant". Other strains express modified PBPs (not PBP2a) and exhibit varying degrees of β– lactam antibiotic resistance (Chambers 1997). *mec*A is the primary determinant of intrinsic methicillin resistance but additional genes are required for a high level resistance phenotypes, besides other environmental factors (Petinaki 2001).

2.10 Resistance and treatment

Most β-lactam antibiotics are ineffective against both HA and CA-MRSA. The CA-MRSA strains are often susceptible to non β-lactam drugs. Cutaneous abscesses need surgical incision and drainage irrespective of the antibiotic susceptibility pattern and in most cases antibiotics provide little or no benefit, and are not recommended except for patients of advanced age or those with severe disease symptoms of systemic illness, immunosuppression or abscess in an area that is difficult to drain (Deleo et al 2010). Linezoid exhibits an excellent anti staphylococcal activity, comparable to that of daptomycin and tigecycline have been approved by FDA for MRSA management (Chamber and Deleo, 2009).

2.11 Prevention

Preventing CA-MRSA

- **Wash your hands.** Careful hand washing remains your best defense against germs. Scrub hands briskly for at least 15 seconds, then dry them with a disposable towel and use another towel to turn off the faucet. Carry a small bottle of hand sanitizer containing at least 62 percent alcohol for times when you don't have access to soap and water.

- **Keep wounds covered.** Keep cuts and abrasions clean and covered with sterile, dry bandages until they heal. The pus from infected sores may contain MRSA, and keeping wounds covered will help prevent the bacteria from spreading.

- **Keep personal items personal.** Avoid sharing personal items such as towels, sheets, razors, clothing and athletic equipment. MRSA spreads on contaminated objects as well as through direct contact.

- **Shower after athletic games or practices.** Shower immediately after each game or practice. Use soap and water. Don't share towels.

- **Sanitize linens.** If you have a cut or sore, wash towels and bed linens in a washing machine set to the hottest water setting (with added bleach, if possible) and dry them in a hot dryer. Wash gym and athletic clothes after each wearing.

- **Don't inject illicit drugs.** Intravenous drug users are at risk of many types of dangerous infections, including MRSA, human immunodeficiency virus (HIV) and hepatitis C.

SOURCE: Centers for Disease Control and Prevention. Prevention of MRSA. National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Healthcare Quality Promotion (DHQP).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipment, media and reagents used in this study are listed in Appendix II.

3.2 Methods

3.2.1 Study design

The study was conducted from June to November 2018 after receiving Ethical approval (Reg. No -297/2018) from Nepal Health Research Council (NHRC) on 20 June 2018. This study was a laboratory based cross-sectional study. Potential risk population‘s municipal waste workers, barbers, beauticians and physically healthy (controls) were participated in the research. All the work concerning this research was carried out in microbiology laboratory of Central Campus of Technology, Dharan.

3.2.2 Sample size and types

In the study, 200 populations were taken. The different clinical samples analyzed were from different potential risk population (50-barbers, 50-beauticians, 50-municipal waste workers and 50-healthy individuals). Sample from nasal and skin was taken from each individual.

3.2.3 Laboratory set up

Laboratory setting was done in microbiology laboratory, Central Campus of Technology, Dharan.

3.2.4 Data collection

Each sample population was asked for their medical history. The information of patient including name, sex, age, skin infection, antibiotic intake and clinical history were collected through Schedule.

3.3 Collection of samples

Samples were collected from potential risk population of Dharan. Samples taken for this study were skin and nasal swabs. Nasal and skin swabs were taken by using sterile swabs in 10ml nutrient broth. Nasal swab and skin swab samples were collected with sterile disposable cotton swabs and was stored in a sterile vial and transported in cold chain to microbiology Lab.

3.4 Processing of samples

The MRSA isolates were isolated and identified according to Smyth et al (2005). The samples were swabbed in MSA containing oxacillin powder of 6 mg/L and incubated at 37°C for 24 hours. *Staphylococcus aureus* colonies were identified based on growth characteristics on MSA plates (yellow colonies), Gram's stain and positive results for coagulase tube test and catalase test.

3.5 Identification of MRSA

The isolated colonies were identified as MRSA based on evidence from growth on MSA media containing oxacillin powder, Gram's staining, catalase test, and coagulase test. Further the isolates were confirmed to be MRSA by cefoxitin disk diffusion method. Detection of MRSA was performed by using cefoxitin disc (30µg). For this lawn culture was performed on Mueller-Hinton agar plate, using the broth culture of *S. aureus* with turbidity adjusted to 0.5 McFarland standards. Then a cefoxitin disc (30µg) was kept on the lawn culture after it had been left to dry for about 5 minutes. Finally, the agar plate was incubated aerobically at 35ºC for 18 hrs. The strains showing diameter of zone of inhibition of ≤21mm were considered as methicillin resistant *S. aureus* (MRSA) as recommended by CLSI guidelines (2012).

3.6 Antibiotic susceptibility testing

All MRSA isolated from clinical samples were subjected to in-vitro antibiotic susceptibility test by Kirby-Bauer disc diffusion techniques using Mueller Hinton agar (HiMedia) containing 4% NaCl as recommended by CLSI guidelines (2012).

Fresh colonies were selected and transferred into NB to obtain turbidity equivalent to 0.5 McFarland barium sulfate standards ($1.5x10^8$ CFU/ml). MHA plates were inoculated with sterile cotton swabs then antibiotics were placed with sterile forceps and allowed to stand at room temperature for 15 minutes for pre-diffusion then incubated at 37ºC for 16-18 hours. The zone of inhibition was interpreted as susceptible, intermediate and resistant according to CLSI –Diffusion Supplemental Table" (2012). In this study the antibiotics used Amoxicillin (AMX, 10mcg), Ampicillin (AMP, 10mcg), Cefoxitin (CX, 30mcg), Cefotaxime (CTX, 30mcg), Chloramphenicol (C, 30mcg), Ciprofloxacin (CIP, 5mcg), Co-Trimoxazole (COT, 25mcg), Erythromycin (E, 15mcg), Gentamicin (GEN, 10mcg), Norfloxacin (NX, 10mcg), Ofloxacin (OF, 5mcg), Teicoplanin (TEI, 30mcg), Trimethoprim (TR, 5mcg), Tetracycline (TE, 30mcg) (Himedia, Mumbai, India).

3.7 Biofilm formation test

3.7.1 Microtitre plate method

The quantification of biofilm by microtitre plate was performed according to Christensen et al (1985). In this method, 5 ml of overnight culture of MRSA was prepared. Then 100 microliter of diluted culture was inoculated in a microtitre well containing TSB with glucose. The plate was incubated at 37ºC for 24 hours for biofilm production. The unbound cell was discarded and washes several times by PBS (pH-7.2). 125µl of 0.1% crystal-violet solution was added and left for 10-15 minutes incubation. The plate was washed and left inverted for dry. The quantitative determination was performed by solubilizing the biofilm by adding 125µl of 30% acetic acid to each well and incubated the plate for 10-15 minutes at room temperature and transfer to another microtitre plates and reading the absorbance at 405nm by spectrophotometer. Interpretation is made on optical density (OD) by subtracting OD of control wells from OD of test wells.

The optical density (OD_s) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (OD_{nc}). The following classification was used for the determination of biofilm formation: no biofilm production ($OD_s \leq OD_{nc}$), weak biofilm production ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm production ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$) and strong biofilm production ($4.OD_{nc} < OD_s$) (Stepanovic et al 2007).

3.7.2 Tube method

A qualitative assessment of biofilm formation was done as described by Christensen et al (1985). The TSB glu (10 mL) was inoculated with a loop full of MRSA from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.2) and dried. Then the tubes were stained by 0.1% crystal violet. Stain was removed by deionized water. Tubes were then dried in inverted position for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Experiments was performed in triplicate and repeated for three times.

3.7.3 Congo red agar method (CRA)

The MRSA culture was streaked on surface of congo red agar and incubated at 37ºC for 24-48 hours (Freeman et al 1989). Black coloured colonies with dry crystalline consistency interpreted as positive biofilm producing strains. Red coloured colonies- interpreted as negative for biofilm production.

3.7.4 Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration of vancomycin (HiMedia, India) to MRSA isolates were screened by Microdilution method as suggested by CLSI (2012) guidelines. The vancomycin powder was accurately weighed and stock solution of 128µg/ml was prepared. The known volume of 0.5 McFarland suspension of bacterial culture was added in each well containing the TSB broth. From the stock solution the different concentrations of drug ranging from 64µg/ml to 0.125µg/ml

was made in in round bottom microtitre plates by serial dilution. The wells for positive and negative controls were even maintained in the plates. The microtitre plates were incubated at 37°C for 24 hours. The well with concentration of drug in which the growth of bacteria was inhibited was known to be the MIC.

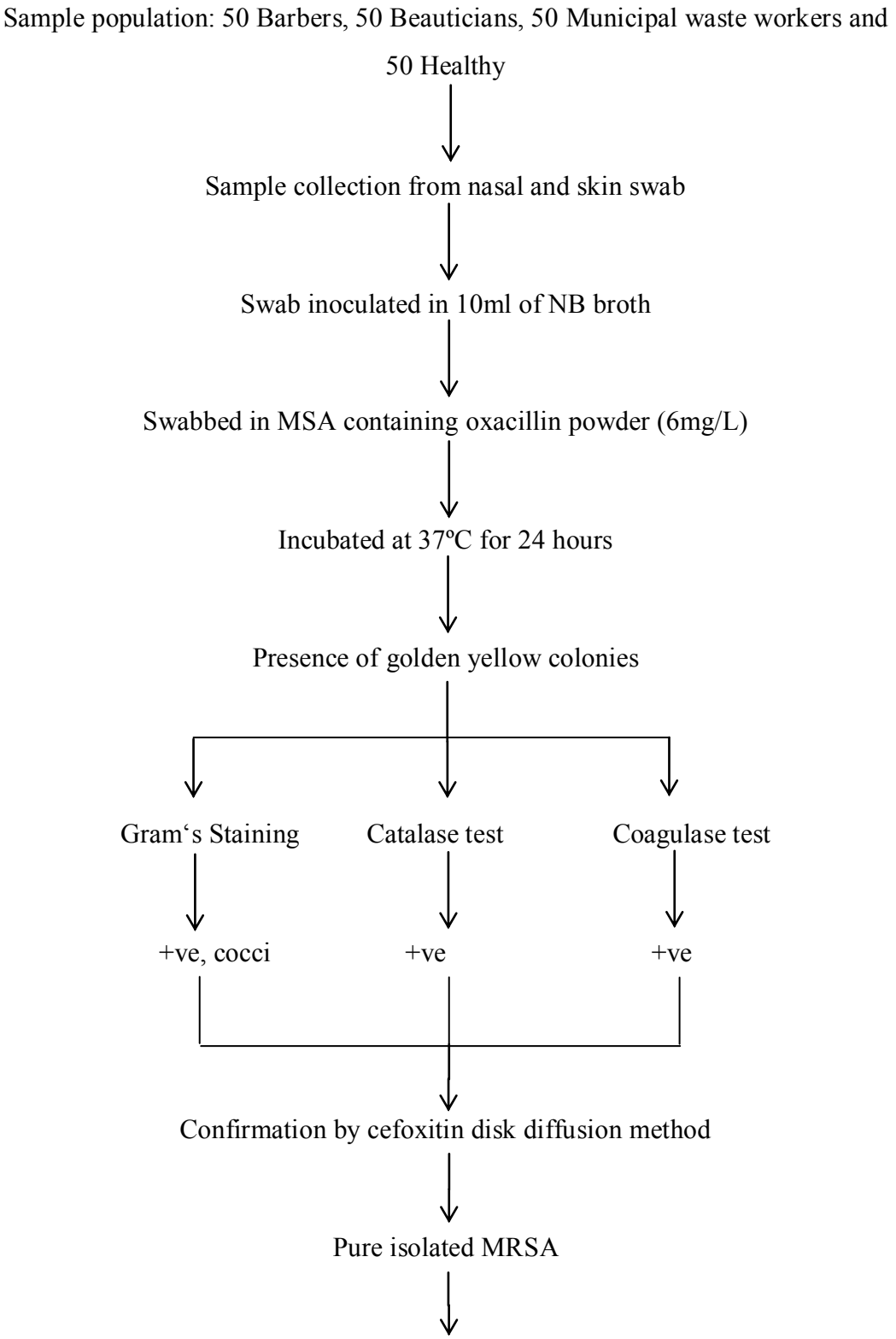
3.8 Quality control for tests

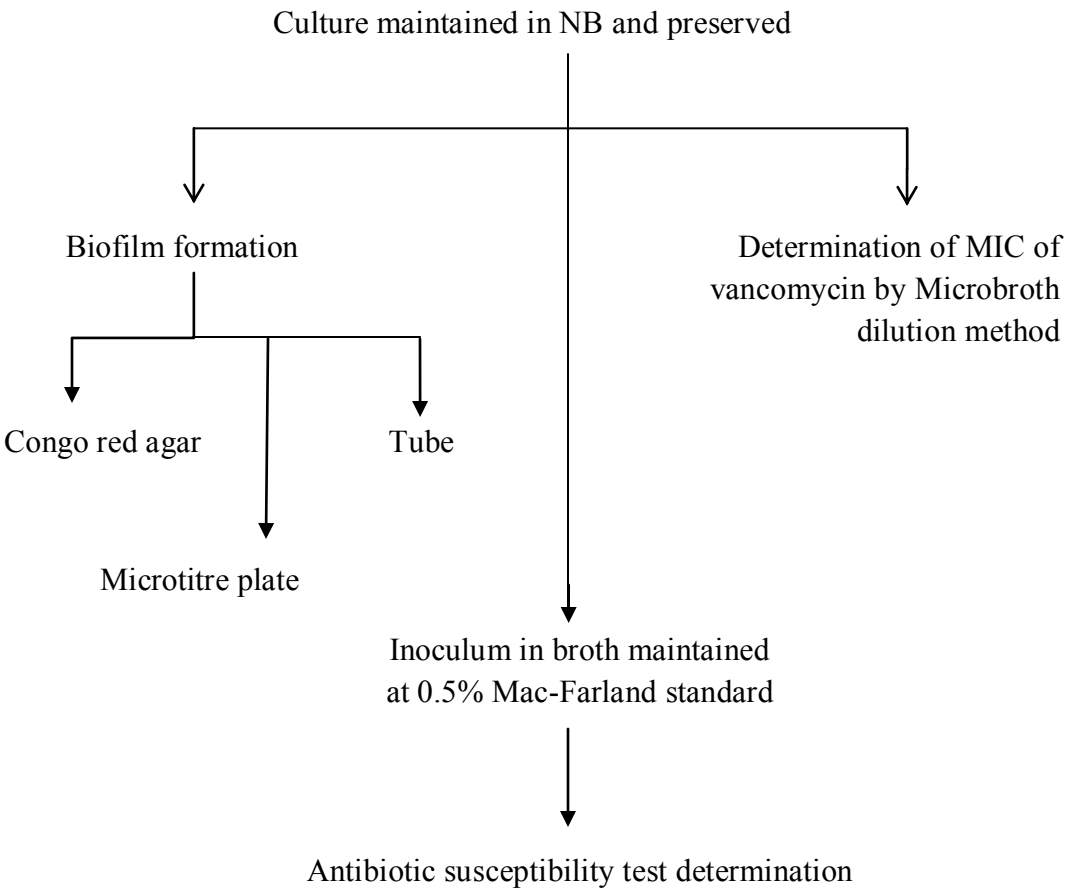
In this study, quality and accuracy of all test was maintained by following standard procedures of collection, isolation and identification. For quality control, media, antibiotics and reagents were prepared, stored and utilized as recommended by the manufacturing company. Antibiotic discs were stored at refrigerator temperature.

3.9 Data analysis

The information collected from schedule was documented and tabulated. The data were statistically analyzed at 5% level of significance by SPSS. A p-value of less than or equal to 0.05 was considered to be statistically significant ($p \leq 0.05$).

Flow chart for Laboratory Procedure




Culture maintained in NB and preserved
Biofilm formation
Determination of MIC of vancomycin by Microbroth dilution method
Congo red agar
Tube
Microtitre plate
Inoculum in broth maintained at 0.5% Mac-Farland standard
Antibiotic susceptibility test determination

CHAPTER IV

RESULTS

4.1 Study population of sample population

From each study population the two different samples were taken; one from hand‘s skin and next from nasal cavity. Thus, out of 400 samples taken from 200 study population, 210 samples were from males (53%) and 190 samples were from females (47%).

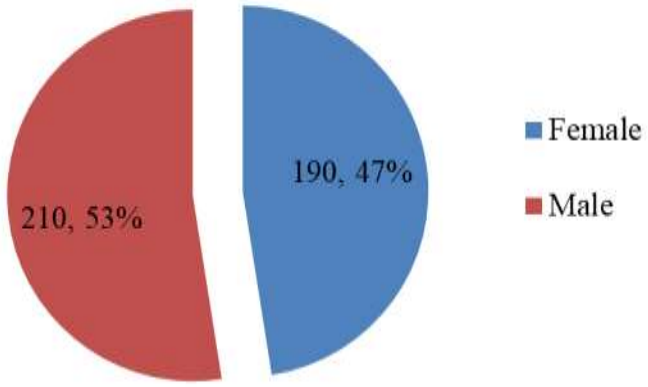


Figure 4.1: Study population of sample population

4.1.1 Population type of sample population

Among 200 sample population, 50 males were from barber‘s population whereas 50 females were from beautician‘s population. In municipal waste worker‘s population 36 samples were from males and 14 samples were from females. In healthy population 19 samples were from males whereas 31 were from females.

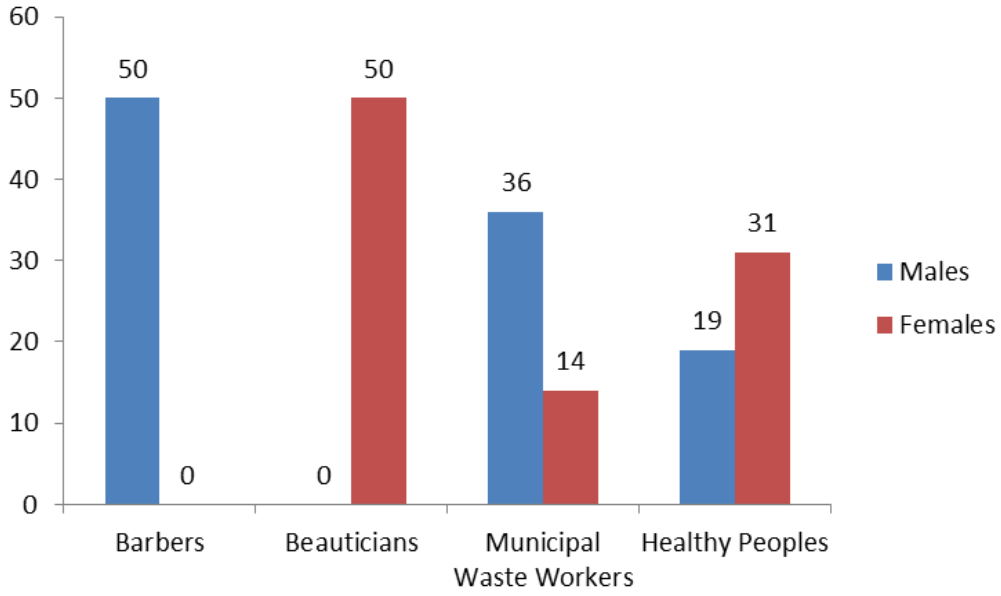


Figure 4.2: Population type of sample population

4.2 Gender-wise distribution of MRSA in sample population

Out of 400 samples taken, 123 were MRSA positive isolates out of which 67 (54.4%) were from males and 56 (45.5%) were from females.

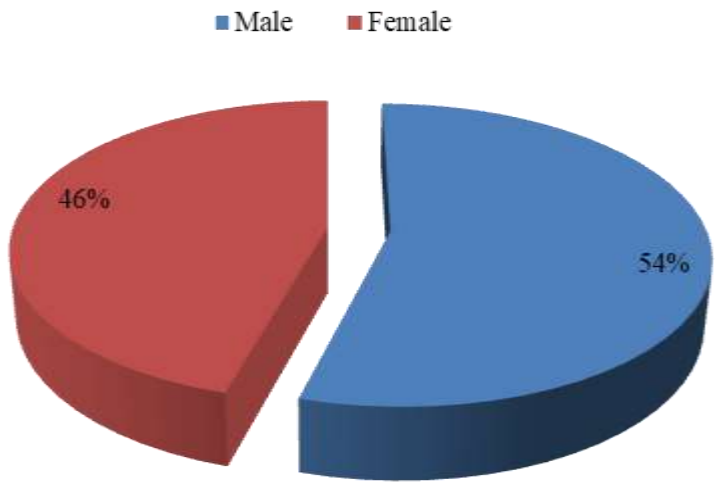


Figure 4.3: Gender-wise distribution of MRSA in sample population

4.2.1 Gender-wise distribution of MRSA positive in sample population

Out of 400 samples taken, 123 were MRSA positive isolates among which 34 MRSA were isolated from barber‘s populations. Similarly, 32 MRSA were isolated from beautician‘s populations. In municipal waste worker‘s population, 19 MRSA isolates were obtained from males whereas 10 were isolated from female‘s population whereas in healthy population, 14 MRSA isolates were obtained from males and 14 were from female‘s population.

Table 4.1: Gender wise distribution of MRSA positive in sample population

S.N.	Sample population	Male	Female	p-value
1.	Barbers	34	-	-
2.	Beauticians	-	32	-
3.	Municipal waste workers	19	10	0.064
4.	Healthy	14	14	0.062
	Total	67	56	

4.3 Comparative study of MRSA isolated from total samples

In this study, all samples were analyzed for MRSA. Higher number of MRSA was isolated from hand (36.5%). The prevalence of MRSA isolates from nasal was 25% and from hand was 36.5%.

Table 4.2: Comparative study of MRSA isolated from total samples

Samples	Total sample	No. of MRSA	MRSA (%)	p-value
Hand	200	73	36.5	
Nasal	200	50	25	0.013
Total	400	123	61.5	

4.4 Comparative study of MRSA isolated from different samples

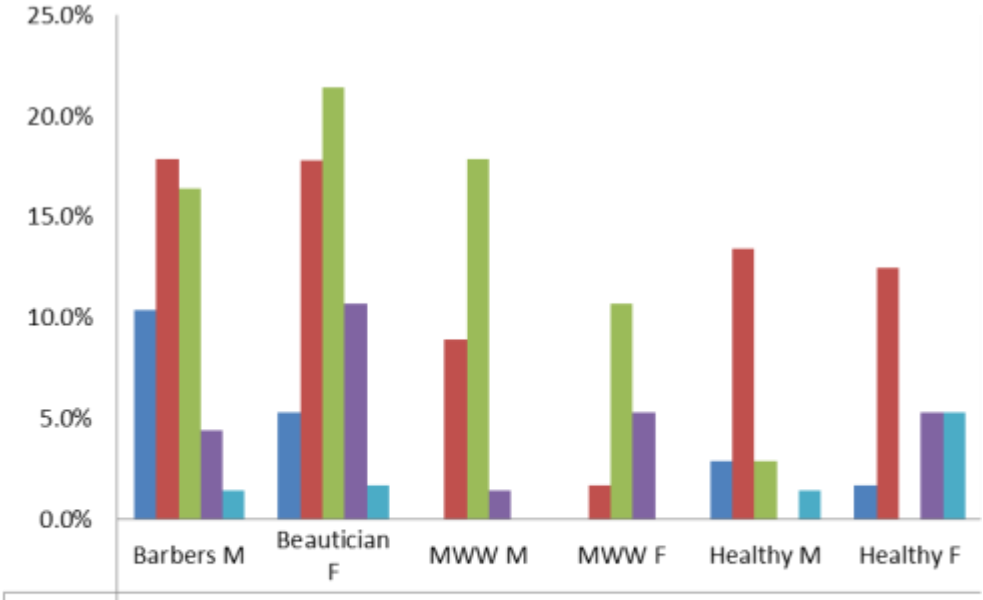
The MRSA isolated from hand were 73 and from nasal were 50. In 24 individuals the MRSA was isolated from both the hand and nasal. The higher frequency of MRSA was isolated from hand than in nasal. Maximum MRSA isolates were obtained from skin surface of barber‘s hand.

Table 4.3: Comparative study of MRSA isolated from different samples

Sample population	Source			p-value
	Hand (Only)	Nasal (Only)	Both	
Barber	23	11	8	0.011
Beauticians	19	13	4	0.086
Municipal waste workers	16	13	4	0.509
Healthy	15	13	8	1.00
Total	73	50	24	

4.5 MRSA isolated from different age groups of male and female

Among 123 MRSA isolated, MRSA from male were 67 (54.4%) and MRSA were from female 56 (45.5%). In male the highest number of MRSA, 27 (40.2%) was from the age group of 20-29 years followed by 25 (37.3%) from 30-39 years of age. In female the highest number of MRSA, 18 (32.1%) was isolated from age group of 20-29 years and 30-39 years of age.



	Barbers M	Beautician F	MWW M	MWW F	Healthy M	Healthy F
Below 20	10.4%	5.3%	0.0%	0.0%	2.9%	1.7%
20-29	17.9%	17.8%	8.9%	1.7%	13.4%	12.5%
30-39	16.4%	21.4%	17.9%	10.7%	2.9%	0.0%
40-49	4.4%	10.7%	1.4%	5.3%	0.0%	5.3%
Above 50	1.4%	1.7%	0.0%	0.0%	1.4%	5.3%

Figure 4.4: MRSA isolated from different age groups of male and female

4.6 Prevalence of MRSA in sample population

Out of total 200 population samples, there were 123 (61.5%) positive MRSA in sample populations obtained from 400 samples of 200 study population.

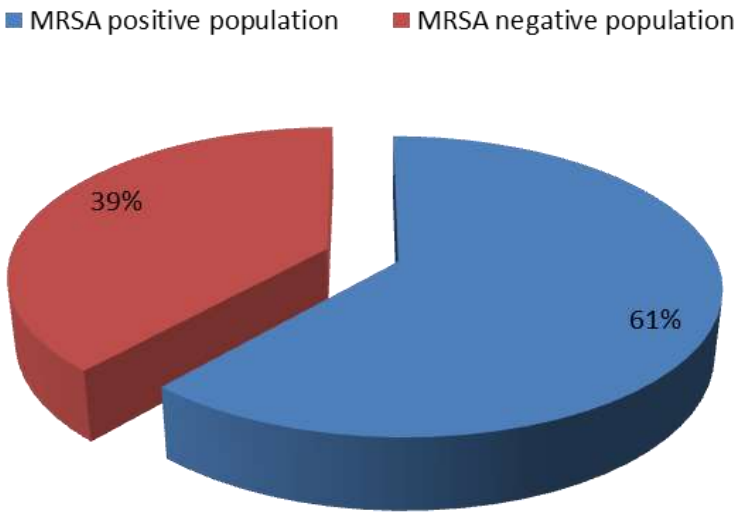


Figure 4.5: Prevalence of MRSA in sample population

4.7 Comparative study of biofilm formation by MRSA in potential risk population

The biofilm formation assay showed that maximum isolates were biofilm producer. The biofilm producing MRSA was found maximum in beautician (56.2%), followed by barber (52.9%). Similarly, in municipal waste workers and healthy population biofilm producing MRSA were 48.2% and 46.4% respectively.

Table 4.4: Comparative study of biofilm formation by MRSA in potential risk population

Biofilm	Barber	Beautician	Municipal waste workers	Healthy	p-value
Strong	7 (20.5%)	5 (15.6%)	2 (6.8%)	5 (17.8%)	0.032
Moderate	11 (32.3%)	13 (40.6%)	12 (41.3%)	8 (28.5%)	0.033
Weak	16 (47%)	14 (43.7%)	15 (51.7%)	15 (53.5%)	0.045
Total	34	32	29	28	

4.8 Biofilm formation assay

The biofilm forming ability of isolated MRSA was performed by three methods: microtitre plate method, tube method and congo red agar method.

Table 4.5: Biofilm formation by MRSA by three methods

Biofilm formation	Microtitre plate method	Tube method	Congo red agar method	p-value
High	19 (15.4%)	14 (11.3%)	11 (8.9%)	
Moderate	44 (35.7%)	37 (30%)	31 (25.2%	0.000
Weak/None	60 (48.7%)	72 (58.5%)	81 (65.8%)	
Total Isolates	123	123	123	

4.9 Sensitivity and specificity of biofilm screening methods

The microtitre plate method was found to be most efficient standard method for studying biofilm formation as compared to tube method and congo red agar method. The parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated. True positives were biofilm producers by microtitre, tube and congo red agar method. False positive were biofilm producers by TM and CRA method and not by microtitre method. False negative were the isolates which were non-biofilm producers by microtitre plate and CRA but were biofilm producer by microtitre method. True negatives are those which were non biofilm producers by all three methods (Hassan et al 2011).

Table 4.6: Sensitivity and specificity of biofilm screening methods

Biofilm screening method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Tube method	75	74.6	58.8	86.1	64.2
Congo red agar method	54.8	85.2	79	65	21.4

Note: PPV - Positive Predictive Value, NPV - Negative Predictive Value

4.10 Antibiotic susceptibility pattern of MRSA isolates

The provided table shows the resistance and sensitivity pattern of isolated MRSA strains towards different antibiotics. It was found that MRSA was most sensitive towards ciprofloxacin (93.4%), chloramphenicol (80.4%) and co-trimoxazole (74.7%) which was statistically significant (P<0.05).The isolated MRSA were resistant to ampicillin (100%), amoxicillin (100%) and trimethoprim (23.5%).

Table 4.7: Antibiotic susceptibility pattern of MRSA isolates

	Resistant		Sensitive		p value
Antibiotics	MRSA	%	MRSA	%	
Ampicillin	123	100	0	0	-
Amoxicillin	123	100	0	0	-
Cefotaxime	65	52.8	58	47.1	0.00
Chloramphenicol	25	20.3	98	80.4	0.00
Ciprofloxacin	8	6.5	115	93.4	0.00
Co-trimoxazole	31	25.2	92	74.7	0.00
Erythromycin	90	73.1	33	26.8	0.01
Gentamicin	42	34.1	81	65.8	0.04
Norfloxacin	76	61.7	47	38.2	0.00
Ofloxacin	38	30.8	85	69.1	0.00
Teicoplanin	55	44.7	68	55.2	0.82
Tetracycline	59	47.9	64	52	0.62
Trimethoprim	94	76.4	29	23.5	0.00

4.11 Resistance pattern of biofilm producing MRSA

The biofilm producing MRSA showed resistance to cefotaxime, chloramphenicol, teicoplanin, co-trimoxazole, erythromycin, norfloxacin, trimethoprim. The non-biofilm producing MRSA showed resistance to tetracycline, ofloxacin, gentamicin, and ciprofloxacin. Ampicillin and amoxicillin were resisted by both biofilm producers and non-biofilm producers.

Table 4.8: Resistance pattern of biofilm producing MRSA

Antibiotics	% of biofilm producing resistant	% of non-biofilm producing resistant	p-value
Ampicillin	100%	100%	0.00
Amoxicillin	100%	100%	
Cefotaxime	56.6%	38%	
Chloramphenicol	20.6%	15%	
Ciprofloxacin	1.5%	10%	
Co-trimoxazole	25.3%	25%	
Erythromycin	73%	65%	
Gentamicin	33.3%	38.3%	
Norfloxacin	66.6%	60.3%	
Ofloxacin	25.3%	36.6%	
Teicoplanin	48.3%	34.9%	
Tetracycline	30.1%	55%	
Trimethoprim	78.3%	77.7%	

4.12 Multidrug resistant (MDR) CA-MRSA

Multidrug resistant methicillin resistant *S. aureus* (MRSA) were identified by their antibiotic resistivity pattern on, three or more than three commonly prescribed antibiotics of different classes. The prevalence of MDR CA-MRSA was 91%.

Table 4.9: Multidrug resistant (MDR) CA-MRSA

	CA-MRSA
Total isolates	123
Multi-drug resistant	112 (91%)

4.13 MIC of vancomycin to total MRSA isolates

The MIC of vancomycin to the MRSA isolates was screened by microbroth dilution assay performed in microtitre wells. In our study, out of 123 MRSA samples the VSSA isolates were 9.7%, VISA isolates were 49.5% and VRSA isolates were 40.6%. In our study the high prevalence of VISA and VRSA isolates were screened.

Table 4.10: MIC of vancomycin to total MRSA isolates

Sample	VSSA (≤2μg/ml)	VISA (4-8μg/ml)	VRSA (≥16μg/ml)	Total	p-value
MRSA (n=123)	12 (9.7%)	61 (49.5%)	50 (40.6%)	123	0.00

4.14 MIC of vancomycin to MRSA isolates from different sample population

In our study the VSSA, VISA and VRSA strains of MRSA were screened from all sample population under study. The highest prevalence of VISA strain was found in healthy population (15.4%). The highest prevalence of VRSA was found in barbers (15.4%).

Table 4.11: MIC of vancomycin to MRSA isolates from different sample population

Sample population	MIC Vancomycin			Total	p-value
	VSSA (≤2μg/ml)	VISA (4-8μg/ml)	VRSA (≥16μg/ml)		
MWW	2 (1.6%)	18 (14.6%)	9 (7.3%)	29 (23.5%)	0.00
Barbers	3 (2.4%)	12 (9.7%)	19 (15.4%)	34 (27.6%)	0.00
Beauticians	4 (3.2%)	12 (9.7%)	16 (13%)	32 (26%)	0.00
Healthy	3 (2.4%)	19 (15.4%)	7 (5.6%)	28 (22.7%)	0.00
	12	61	50	123	

4.15 MIC of vancomycin to MRSA isolates from different gender population

The highest prevalence of VISA and VRSA were found to be in male population than in female population. However, the sample populations of female were less than that of male in our study.

Table 4.12: MIC of vancomycin to MRSA isolates from different gender population

MIC vancomycin	Male	Female	Total	p-value
VSSA (≤2μg/ml)	5 (4%)	7 (5.6%)	12	0.348
VISA (4-8μg/ml)	33 (26.8%)	28 (22.7%)	61	0.113
VRSA (≥16μg/ml)	29 (23.5%)	21 (17%)	50	0.515
Total	67	56	123	

4.18 MIC of vancomycin to MRSA isolates from hand and nasal sample

In this study, the highest prevalence of VISA was found to be in hand (31.7%) than in nasal (17.8%). Similarly, the highest prevalence of VRSA was found to be in hand (21.9%) than in nasal (18.6%). However, VSSA isolates were found more in hand (5.6%) sample than in nasal (4%) sample.

Table 4.15: MIC of vancomycin to MRSA isolates from hand and nasal sample

MIC vancomycin	Hand	Nasal	Total	p-value
VSSA (≤2μg/ml)	7 (5.6%)	5 (4%)	12	0.940
VISA (4-8μg/ml)	39 (31.7%)	22 (17.8%)	61	0.304
VRSA (≥16μg/ml)	27 (21.9%)	23 (18.6%)	50	0.317
Total	73	50	123	

4.19 MIC of vancomycin to MRSA isolates from different age groups of sample population

Among 123 MRSA isolates, the highest VISA isolates were screened from the age group 20-29 years (21.1%). Even the highest percentage of VRSA was screened from the age group of 20-29 years (14.6%).

Table 4.16: MIC of vancomycin to MRSA isolates from different age groups of sample population

Age group	VSSA (≤2μg/ml)	VISA (4-8μg/ml)	VRSA (≥16μg/ml)	Total	p-value
Below 20 Years	0	10 (8.1%)	10 (8.1%)	20 (16.2%)	
20-29 Years	3 (2.4%)	26 (21.1%)	18 (14.6%)	47 (38.2%)	
30-39 Years	2 (1.6%)	15 (12.1%)	15 (12.1%)	32 (26%)	0.86
40-49 Years	2 (1.6%)	8 (6.5%)	6 (4.8%)	16 (13%)	
50 and Above	5 (4%)	2 (1.6%)	1 (0.8%)	8 (6.5%)	
	12	61	50	123	

CHAPTER-V

DISCUSSIONS

Staphylococcus aureus is one of the common pathogens isolated in most microbiological laboratories (Ansari et al 2014). It is responsible for a wide range of infections including superficial skin infections, food poisoning, osteomyelitis and septicemia (Forbes et al 2007). Treatment of infections caused by MRSA is challenging as these organisms are resistant to currently available antibiotics.

Methicillin-resistant strains of staphylococci emerged by late 1970s and now have stood as prevalent as nosocomial pathogens (Edmond 1996). Methicillin-resistant *Staphylococcus aureus* (MRSA) has spread worldwide and is responsible for significant morbidity, mortality, and health care costs. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a specific strain of *Staphylococcus aureus* that has developed antibiotic resistance first to penicillin since 1947 and later to methicillin in 1961 and since that time it has spread rapidly worldwide, becoming a leading cause of hospital and community acquired infections. It is because MRSA are often resistant to other antibiotics too so it is difficult to treat such infections. The medical community was comforted by the fact that vancomycin, provided effective therapy for all strains of MRSA. Nevertheless, the emergence of vancomycin resistant strains of coagulase negative staphylococci, enterococci and now among *S. aureus* has caused concern that nothing will be left in the antimicrobial arsenal to treat patients infected by these strains of staphylococci (Rai et al 1990).

Our study was carried out at microbiology laboratory of Central Campus of Technology, Hattisar, Dharan, Sub-metropolitan city. During the study period, two types of samples were collected from single individual under study and processed for the isolation and identification of CA-MRSA. The sample included nasal swab and hand swab from each person. In our study 123 community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) was isolated from 400 samples in which 200 were nasal swab sample and 200 were hand swab

sample. In this study the total identified MRSA was 123 (30.7%) out of 400 samples. Out of 123 MRSA isolates the highest percentage of MRSA was isolated from hand 73 (59.3%) and in nasal it was found to be 50 (40.6%). In our study the prevalence of MRSA in 200 sample population was found to be 61.5%, The prevalence of MRSA in Nepal ranges from 39% to 69% (Kumari et al 2008: Rijal et al 2008; Tiwari et al 2009; Khanal et al 2010; Mukhiya et al 2012) which shows similar result to our study.

In our study higher frequency of MRSA was isolated from males than in females, 67 (54.4%) from males and 56 (45.5%) from females. Prevalence of MRSA in male was higher than in female according to previous study done by Khanal et al (2010). Hence, prevalence of MRSA was seen more in males as compared with females. Similar findings were obtained even in our study although the prevalence of MRSA in two gender was not found statistically significant, p=0.599.

In male, the prevalence of MRSA was highest with 27 (40.2%) in age group 20-29 years followed by 25 (37.3%) from 30-39 years of age. In female, the prevalence of MRSA was highest in age group of 20-29 years of age with 18 (32.1%) positive samples. However, the prevalence of MRSA among different age group was not statistically significant, p=0.535.

In our study the highest percentage of MRSA was isolated from hand 73 (59.3%) and in nasal it was found to be 50 (40.6%). Lower frequency of nasal carriage of MRSA was found in the studies performed by Khatri et al (2017) that reported 7.5% nasal carriage. The prevalence of CA-MRSA in hand and nasal was statistically significant, p=0.013.

From 50 barber‘s population the total samples obtained were 100 (50 hand and 50-nasal). Out of total barbers samples 34 MRSA were isolated; 23 (46%) from hand and 11 (22%) from nasal. In beauticians, 32 MRSA were isolated; 19 (38%) from hand and 13 (26%) from nasal. In municipal waste workers the isolated MRSA were 29 out of which 16 (32%) were from hand swab and 13 (26%) were from nasal swab. In healthy population isolated MRSA were 28 out of which 14

(28%) were from hand swab and 14 (28%) were from nasal swab. The frequency of isolated MRSA was reported more from the barbers followed by beauticians and municipal waste workers. This study reported the carriage of CA-MRSA higher in potential risk population. The prevalence of MRSA in hand and nasal of Barber‘s population was statistically significant ($p<0.05$).

Presence of MRSA in waste workers may be due to the direct contact with contaminated clinical wastes. There was statistical significant relation between presence of MRSA and workers exposure to clinical wastes (p-value=0.007). Municipal waste workers were under regular exposure to clinical and domestic wastes which could be the reason behind higher prevalence of MRSA in municipal waste workers. Prevalence of MRSA with the previous history of skin infection in the population was found to be statistically significant (p-value=0.001).

Risk factors of CA-MRSA in skin infections include exposure to clinical wastes, occupations, skin to skin contact, irrational use of antibiotics, old age, etc. For the treatment of MRSA infection the most often used drug include vancomycin but the emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) has become a great issue as it leads to failure of clinical therapy in treating infections.

CDC estimated that about 12% of MRSA infections are now community associated but this percentage can vary by community and patient population due to the recent antibiotic use, sharing contaminating items, having active skin diseases or injuries, poor hygiene and living in crowded settings. The transmission of MRSA is largely from people with active MRSA skin infections and spread always by four routes of transmission of MRSA. They are direct contact with infected or colonized individuals, indirect contact through contaminated hands of health care workers, contaminated air and contaminated environmental surfaces or equipment.

In our study, the antibiotic susceptibility test of CA-MRSA reported that the most effective antibiotic was ciprofloxacin, chloramphenicol and co-trimaxole. Out of

123 CA-MRSA positive samples, ciprofloxacin was 93.4% sensitive, chloramphenicol was 80.4% sensitive and co-trimoxazole was 74.7% sensitive. The sensitivity of the MRSA against those drugs was statistically significant ($p<0.05$). Ampicillin and amoxicillin were 100% resistant. Other antibiotics such as trimethoprim (23.5%), erythromycin (26.8%), norfloxacin (38.2%) and cefotaxime (47.1%) showed less than 50% sensitivity whereas; tetracycline (52%), teicoplanin (55.2%), gentamicin (65.8%), and ofloxacin (69.1%) showed more than 50% sensitivity. In a study conducted by Wylie et al (2005) erythromycin susceptibility was 40% to CA-MRSA. Erythromycin susceptibility of CA-MRSA strains was 51.7% as done by Mandelia et al (2012). Around 73.1% of CA-MRSA isolates were resistant to erythromycin in our study. Our result supports the previous studies conducted in Nepal which reported increasing resistance of bacteria towards erythromycin and co-trimoxazole due to excessive use of their drugs to treat infections (Ansari et al 2014, Neopane et al 2018). Even in our study the isolated superbug showed reduced sensitivity to erythromycin.

The susceptibility of ciprofloxacin was 88% observed in study conducted by Wylie et al (2005). In our study the susceptibility to ciprofloxacin was 93.4% which was found high in comparison to other studies. Isolates MRSA were most sensitive to ciprofloxacin drug which was statistically significant with $p <0.05$. In study conducted by Mandelia et al (2012) the susceptibility to ciprofloxacin was only 18.3%. Chloramphenicol, co-trimoxazole and ciprofloxacin were much sensitive drugs in our study with statistical significance ($p<0.05$). Khatri et al (2017) reported that higher MRSA sensitive to vancomycin followed by co-trimoxazole (84.2%).

A two-way ANOVA of the susceptibility of the isolates to the antibiotics drugs shows that the type of antibiotic used adversely affected the microbial susceptibility with $p<0.05$. It explains that the type of antibiotic affects the microbial susceptibility to drugs.

In Nepal, due to unnecessary use of antibiotic without doctor‘s prescription, the emergence of antibiotic resistance has been increasing. People can purchase antibiotic directly from pharmaceuticals, which has led to antibiotic resistant strains of microorganisms. However, the emergence of antibiotic resistant bacteria continues to threaten the ability to treat infections. Recently, antibiotic resistant pathogens have been emerging in community, which may increase the impact they have on populations.

Antimicrobial resistance is an innate feature of bacterial biofilms and biofilm formation is higher in MRSA (CharanKaur and Khare, 2013). The biofilm forming ability of isolated MRSA was performed by microtitre plate method, tube method and congo red agar method. The biofilm producing isolated MRSA by microtitre plate method was 51.2% and non-biofilm producer were 48.7%. On the other hand 41.4% of isolated MRSA were shown biofilm producer by tube method. Congo red agar method reported 34.1% MRSA isolate as biofilm producer.

In our study the strong, moderate and weak biofilm producers by microtitre plate methods were 15.4%, 35.7% and 48.7% respectively. The strong, moderate and weak biofilm producer by tube method was 11.3%, 30% and 58.5% respectively. The strong, moderate and weak biofilm producers by congo red agar method were 8.9%, 25.2% and 65.8% respectively. In overall the biofilm producing CA-MRSA were 51.2% and rest (48.7%) were non-producers. Number of false positive and false negative was reported in the comparison. It was difficult to discriminate strong, moderate and weak biofilm producers in tube method and congo red agar method due to phenotypic variations. The sensitivity and specificity of microtitre plate method was found 91.8% and 74.3% respectively. Sensitivity and specificity of tube method was found to be 75% and 74.6% respectively. For congo red agar method the sensitivity and specificity was found to be 54.8 % and 85.2%. The statistical analysis of screening were similar even in our findings which supports different other similar findings done before. Hassan et al (2011) concluded microtitre plate method as gold standard technique for screening biofilm as

compared to tube method and congo red agar method. In our study, the microtitre plate method was found to be most sensitive and efficient method for quantitative screening of biofilm as compared to tube and congo red agar method. The screening of biofilm by microtitre method, tube method and congo red agar method was statistically significant ($p<0.05$). The study conducted by Rezaei et al (2013) reported that 15.4% of CA-MRSA was strong biofilm producer and rest 19.2% and 65.4% were medium and weak biofilm producer respectively.

The biofilm producing MRSA showed high resistance to cefotaxime, chloramphenicol, teicoplanin, co-trimoxazole, erythromycin, norfloxacin, trimethoprim. The non-biofilm producing MRSA showed resistance to tetracycline, ofloxacin, gentamicin, and ciprofloxacin. The resistivity pattern of biofilm producer and non-biofilm producer was statistically significant ($p<0.05$). Ampicillin and amoxicillin were resisted by both biofilm producers and non-biofilm producers. The resistant pattern of biofilm producing reported the ability of biofilm formation in drug resistance. However, non-biofilm producing antibiotic resistant was even found in our study. It can be explained that other than biofilm formation many other contributory role of bacteria is responsible for drug resistance.

Ciprofloxacin is known to be most effective against biofilm forming bacteria. In our study ciprofloxacin showed effectiveness to both biofilm producing and non-producing MRSA strains. In our study, only 1.5% biofilm producers were resistant to ciprofloxacin and rests were all sensitive. In our study the sensitivity of MRSA to ciprofloxacin was statistically significant ($p<0.05$). The study conducted by El Shekh et al (2010) reported that 99.9% of MRSA isolates were susceptible to ciprofloxacin and vancomycin. Similar finding was seen even in our study. Most of the biofilm producing MRSA isolates showed higher resistance to trimethoprim and erythromycin. In overall 76.4% CA-MRSA were resistant to trimethoprim. Pate et al (2009) reported that trimethoprim/sulfamethoxazole resistance associated with MRSA infections. In our study the Trimethoprim resistance MRSA was 76.4% which shows increasing resistance pattern of CA-

MRSA. The antimicrobial resistance seen in our study was higher among biofilm producing MRSA. The study indicates that biofilm producing ability might be one of the crucial factors for resistance towards the antibiotics.

In our study, 91% of CA-MRSA isolates were known to be MDR. Multidrug resistant bacteria were higher among biofilm producers which was also statistically significant ($p<0.05$). The emergence of multi-drug resistant MRSA has further increased the need for search of alternative antimicrobial therapeutic agents in order to tackle with the infections caused by MDR CA-MRSA. The study strongly suggests adding up proper policy on sanitation, precaution and awareness to prevent the infections of CA-MRSA. Multidrug resistance pattern may limit option for clinical therapy.

Our study reported a significant prevalence of VISA in nasal swab as well as in hand swab from all potential and healthy population. However, the prevalence of VISA in hand and nasal was not statistically significant ($p>0.05$). In overall study population the 49.5 % of MRSA were identified as VISA strains (4-8µg/ml). Similarly, the study performed by Mendez et al (2013) reported the nasal and hand surface colonized by VISA strains but showed lower prevalence of VISA (20%).

VRSA and VISA were detected in all potential risk population as well as in healthy controls. In the study, 40.65 % of MRSA were identified as VRSA and the overall prevalence of VRSA in population was 25%. The prevalence of VRSA in nasal cavity of study population was 11.5 %. The prevalence of VRSA was high in potential risk populations. In our Study 38% of MRSA isolates from barber population were identified as VRSA. The similar prevalence of VRSA strains were identified in beauticians (32%). In our study the prevalence of VRSA and VISA strains in nasal cavity were 11.5%. However, the study conducted by Elsayed et al (2018) reported only one strain of VISA from nasal cavity of health care worker. Gohniem et al (2014) reported higher resistance rate 20.68% VISA and 20.68% VRSA from clinical samples which indicates the rising prevalence of

VRSA and VISA strains. Although vancomycin has been used as a drug of choice for treating MRSA, recently the VISA has been reported in CA-MRSA clones (Graber et al 2007).

Detection of vancomycin resistance is essential not only for an optimal therapy, but also for infection control measures and epidemiological purposes. As recommended by the Clinical & Laboratory Standards Institute (CLSI), the broth dilution method was used for MIC determination for the detection of VISA and VRSA strains in this study. Most of the microbiological laboratories in Nepal depend upon disk diffusion method to identify susceptibility of *S. aureus* to vancomycin, which however cannot differentiate VISA and VRSA strains because of similar zone of inhibition. Detection of VRSA raises the concern and issue not only in clinical therapy but even in epidemiological settings.

In our study significant rise of VISA and VRSA were reported from CA-MRSA. The pathogen is spreading in community, hospitals and in wide areas. It can be warned that lack of hygiene, exposure to wastes and unauthorized use of drugs lead in transmission of the MRSA. These results concludes that emergence of VISA and VRSA in community setting requires immediate collaborative effort to search for measures to fight with the superbug.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The prevalence of CA-MRSA was found to be 61.5%. This study concludes that CA-MRSA is emerging with multi-drug resistance. The CA-MRSA was prevalent more in potential risk population then in healthy population controls. MIC of vancomycin against isolated CA-MRSA showed significant prevalence of VISA and VRSA from all potential risk population which raises serious concern in clinical failure of vancomycin. This study reported the biofilm formation by CA-MRSA was associated with multi-drug resistance. Isolates MRSA were most sensitive to drug ciprofloxacin which was statistically significant. The microtitre plate method was most efficient standard method of screening biofilm than the tube and congo red method. Improvement in personal hygiene and formulation of appropriate antimicrobial policy will help to prevent CA-MRSA infection.

6.2 Recommendations

1. Strict personal hygiene should be maintained.
2. The use of antibiotics without doctor‘s prescription should be avoided.
3. Management of clinical wastes should be performed properly using protective equipment by the municipal waste workers.
4. Barbers and beauticians must wear personal protective equipment (gowns, gloves, etc.) to prevent the spread and transmission through contacts.
5. For confirmatory screening of biofilm, RFLP is recommended
6. Confirmation of MRSA must be done through detection of *mec*A gene.
7. MRSA are transmitted under poor domestic and hospital settings by direct skin contact exposure to waste stuff. The regular surveillance in MRSA transmission is required to prevent MRSA infection.
8. The study must be conducted in other potential risk populations like in cancer patients, HIV-patients, sex workers etc.
9. Reliable clinical and epidemiological tool must be developed to differentiate CA-MRSA from HA-MRSA.
10. It is recommended to follow Microbroth dilution method to screen VISA and VRSA strains.

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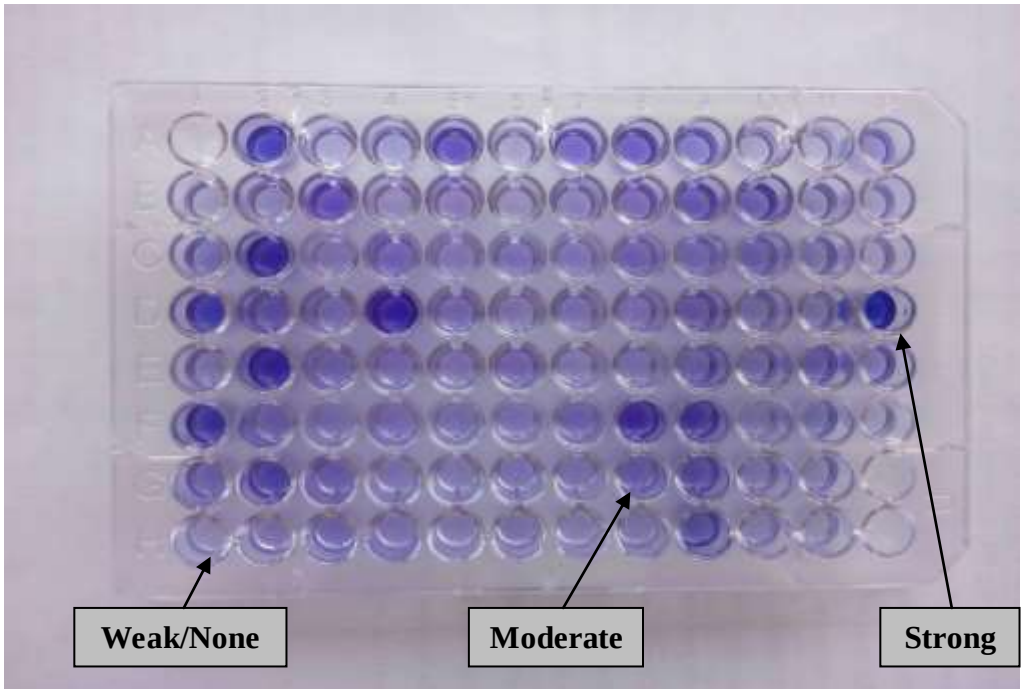
Photograph 1: Sample collection

Photograph 2: Biofilm screening by congo red agar positive (black colonies)



Photograph 3: Biofilm production of CA-MRSA in microtitre wells

Photograph 4: Operating ELISA reader for determining biofilm OD

Photograph 5: Antibiotic susceptibility test of CA-MRSA

Photograph 6: MIC of vancomycin by Microbroth dilution method

APPENDIX: I

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 3127

20 June 2018

Mr. Jenish Shakya
Principal Investigator
Central Campus of Technology

Ref: **Approval of thesis proposal** entitled **Antibiogram of biofilm producing and non-producing CA-MRSA isolated from potential risk population of Dharan, Nepal**

Dear Mr. Shakya,

It is my pleasure to inform you that the above-mentioned proposal submitted on **28 May 2018** (**Reg. no. 297/2018**) has been approved by Nepal Health Research Council (NHRC) National Ethical Guidelines for Health Research in Nepal, Standard Operating Procedures Section 'C' point no. 6.3 through Expedited Review Procedures.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol. Expiration date of this proposal is **September 2018.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and **submit progress report in between and full or summary report upon completion.**

As per your thesis proposal, the total research budget is **NRs 17,000** and accordingly the processing fee amounts to **NRs 1,000.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

Prof. Dr. Anjani Kumar Jha
Executive Chairperson

APPENDIX: II

Materials and Equipments

A. List of Materials

1. Glass wares

Beaker
Conical flask
Petri plates
Measuring cylinder
Test tubes
Glass rod
Micropipette
Micropipette tips
Glass slides
Dolly rods

2. Miscellaneous

Bunsen burner
Gloves
Hi-media sterile cotton swabs
Bacteriological loop
Forceps
Permanent marker
Soaps
Labeling tags
96-well ELISA plates
Ice box

3. Equipments

Autoclave
Hot air oven
Incubator
Refrigerator
Compound Microscope
ELISA Reader
Weight Balance

4. Chemical and Reagents

Crystal Violet (CV) solution 0.1%
30% acetic acid
Methylene blue
PBS
Ethanol 1N
Lysol
Plasma
Microscope oil
Sodium Chloride
3% H_2O_2

5. Microbiological Media (HiMedia)

Mannitol Salt Agar (MSA)
Muller Hinton Agar (MHA)
Trypticase Soya Broth (TSB)
Nutrient Broth (NB)
Nutrient Agar (NA)
Agar

6. Antibiotics Discs

HiMedia

Ampicillin (AMP, 10mcg)
Amoxicillin (AMX, 10mcg)
Cefoxitin (CX, 30mcg)
Cefotaxime (CTX, 30mcg)
Chloramphenicol (C, 30mcg)
Ciprofloxacin (CIP, 5mcg)
Co-Trimoxazole (COT, 25mcg)
Erythromycin (E, 15mcg)
Gentamicin (GEN, 10mcg)
Norfloxacin (NX, 10mcg)
Ofloxacin (OF, 5mcg)
Teicoplanin (TEI, 30mcg)
Tetracycline (TE, 30mcg)
Trimethoprim (TR, 5mcg)
Vancomycin powder (HiMedia, India)

APPENDIX: III

Bacteriological media

Composition and preparation of different types of media

1. Mannitol Salt Agar (MSA)

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	5.000
Pancreatic digest of casein	5.000
Beef extract	1.000
Sodium chloride	75.000
D-Mannitol	10.000
Phenol red	0.025
Agar	15.000
pH after sterilization (at 25°C)	7.4±0.2

2. Nutrient Broth (NB)

Peptone	5.0g
Sodium chloride	5.0g
Beef Extract	1.5g
Yeast Extract	1.5g
Final p^H	7.4±0.2

1.3 gm of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121°C for 15 minutes.

3. Muller Hinton Agar (MHA)

Beef infusion Broth	300.0g
Casein Acid Hydrolysate	17.0g
Starch	1.0g
Agar	17.0g
Final $_p$H	7.0±0.2

3.8 gm of media was suspended in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^{o}C for 15 minutes. It was then poured while at 45-48^{o}C into sterile petriplates in 25 ml quantity.

4. Phosphate Buffer Solution (PBS)

For 1 liter of 1X PBS: Add 8gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na2HPO4, 0.2 gm of KH2PO4, Adjust the pH to 7.4 with HCl. Add distill water to total volume of 1Liter and sterilize. Store at room temperature.

5. Trypticase Soya Broth (TSB)

Pancreatic Digest of casein- 17.0 gm, Sodium Chloride- 5.0 gm, Papaic Digest of Soyabean meal -3.0 gm, Dextrose -2.5 gm, Dipotassium phosphate-2.5 gm
Final pH-7.3 ±0.2 at 25 ºC

6. Congo Red Agar Media

Brain heart infusion broth (HiMedia, Mumbai, India) - 37 g/L
Sucrose - 50 g/L
Agar No. 1 (HiMedia, Mumbai, India) - 10 g/L
Congo Red indicator (HiMedia, Mumbai, India) - 8 g/L

APPENDIX: IV

Composition and preparation of different reagents

1. Gram staining reagents

i. Crystal violet Gram stain

Crystal violet	20g
Ammonium oxalate	9g
Ethanol or methanol, absolute	95ml
Distilled water	1 litre

Preparation:

Crystal violet is weighed and transferred to a clean bottle and absolute ethanol is added and mixed until dye is completely dissolved.

Ammonium oxalate is weighed and dissolved in about 200 ml of distilled water. Then it was added to the stain and total volume is made 1 litre by adding distilled water and mixed well.

ii. Iodine Solution

Potassium iodide	1.5g
Iodine	1.0g
Distilled water	150ml

Preparation:

Potassium iodide is weighed and transferred to a clean bottle 30-40 ml of distilled water is added to Potassium iodide and mixed until it is fully dissolved.

Iodine is weighed and added to potassium iodide solution and mixed well.

Final volume is made 150ml by adding distilled water and mixed well.

iii. Acetone-alcohol decolorizer

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25ml

To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then 500 ml acetone was added and mixed well.

iv. Counterstain solution

Safranine	10gm
Distilled water	1 lit

In a piece of clean paper, 10 gm of safranine was weighed and transferred to a clean bottle. Then after, 1 liter distilled water was added to the bottle and mixed well until safranine dissolves completely.

v. Catalase reagent (To make 100 ml)

Hydrogen peroxide solution	3ml
Distilled water	97ml

Preparation:

To 97 ml distilled water, 3 ml of hydrogen peroxide solution was added and mixed well.

APPENDIX: V

Procedure of different biochemical tests

1. Gram's strain: (Mackie and McCartney Vol.2., 14th edition).

Isolated colony selected for staining:

1. Smear was made from pure culture by emulsifying a colony in normal saline and heat fixed.
2. Smear flooded with crystal violet for 1 mint.
3. Wash with water
4. Add Gram's Iodine for 1minute.
5. Wash with water.
6. Decolorize with absolute alcohol for 10-15secs.
7. Wash with water
8. Flood with saffranin for 1minute.
9. Wash with water, blot dry and examine under oil immersion objective of the microscope.

2. Catalase test

1. A small amount of isolated colony from pure culture was transferred to the surface of clean dry glass slide.
2. A drop of 3% H_2O_2 was placed onto the inoculum.
3. The evolution of oxygen bubbles was recorded immediately.
4. The slide was then discarded into a disinfectant.

3. Coagulase test

I. Slide test (to detect bound coagulase)

1. A drop of physiological saline was placed on end of a slide and colony of test organism was emulsified in each of the drops to make two thick suspensions.
2. A drop of plasma was added to one of the suspensions and mixed gently. It was looked for clumping of the organism within 10 seconds. But no plasma was added to second suspension. This is used to differentiate any granular appearance of the organism from the coagulase clumping.

II. Tube test (to detect free coagulase)

1. The plasma was diluted 1 in 10 physiological saline (mixing 0.2 ml of plasma with 1.8 ml of saline)
2. 3 tubes were taken and labeled as:

T = test organism (18-24 hour broth culture),
P = Positive control (*S. aureus* broth culture),
N = Negative control (sterile broth).

3. 0.5 ml of diluted plasma was pipetted into each tube.
4. About 5-5 drops each of test organism, *S. aureus* culture, and sterile broth was added to the tubes labeled 'T', 'P' and 'N' respectively.
5. After mixing gently, 3 tubes were incubated at 37^{o}C. It was examined for clotting after 1 hour. If no clotting occurs tubes were examined at 30 minutes intervals for up to 6 hours.

APPENDIX: VI

Antibiotic Susceptibility Test (Kirby-Bauer's Disc Diffusion Method)

In vitro susceptibility of the pure bacterial species to fifteen different antibiotics was determined using Kirby- Bauer disk diffusion technique using Muller-Hinton agar and antibiotic discs as described by the National Committee for Clinical Laboratory Standards (CLSI, 2006). One ml of each bacterial isolates prepared directly from an overnight agar plates adjusted to 0.5 McFarland Standard was inoculated using sterile swab into each of the Petri-dishes containing Mueller-Hinton agar and were allowed to stand for 30 minutes for pre-diffusion of the inoculated organisms.

Antibiotic discs were seeded into the petri dishes containing Mueller-Hinton agar (MHA) for each bacterial isolates. The AST of the isolates towards various antimicrobial discs was done by modified Kirby-Bauer M2-A9 disc diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI) using MHA as follows:

1. MHA was prepared and sterilized as instructed by the manufacturer.
2. The pH of the medium was adjusted to 7.2-7.4 and the depth of the medium at 4mm (about 25 ml per plate) was maintained in petri dish.
3. Using a sterile wire loop, a single isolated colony whose susceptibility pattern is to be determined was touched and inoculated into MHB tube and was incubated at 37oC for 2-4 hrs.
4. After incubation, the turbidity of the suspension was matched with the McFarland standard tube number 0.5 (which is equivalent to 10 to power 4 organisms).
5. Using a sterile swab, an MHA plate was inoculated with the matched suspension using a carpet culture technique.
6. The plate was then allowed to stand for 20-30 minutes for the pre-diffusion of the inoculated organisms.
7. Using clean and sterile forceps, the above mentioned antibiotic discs (6 mm) were placed on the MHA. The discs were placed at the considerable distance

apart from each other on a 90 mm Petri-dish. Then the plate was incubated at 37 ºC for 24 hrs.

8. After incubation, the plates were observed for zone of inhibition and the diameters of inhibition zones were measured in millimeters (mm). The measurement was interpreted as sensitive and resistant according to the manufacture's standard zone size interpretative manual of CLSI (2006).

The percentage resistance was calculated using the formula *PR=a/b×100, where 'PR'* was percentage resistance, '*a'* was the number of resistant isolates and '*b'* was the number of isolates tested with the antibiotic. The percentage sensitivity was calculated using the formula *PS=c/d×100, where 'PS'* was percentage sensitivity, '*c'* was the number of sensitive isolates and '*d'* was the number of isolates tested with the antibiotic.

B. Antibiotic disc used and procedure of susceptibility test

1. Antibiotic disc used

Antibiotics used	Symbol	Disc Content (mcg)	Diameter of Zone of inhibition (mm)		
			Resistant	Intermediate	Sensitive
Ampicillin	AMP	10	28	-	29
Amoxicillin	AMX	10	19	-	20
Cefoxitin	CX	30	21	-	22
Cefotaxime	CTX	30	14	15-22	23
Chloramphenicol	C	30	12	13-17	18
Ciprofloxacin	CIP	5	15	16-20	21
Cotrimoxazole	COT	25	10	11-15	16
Erythromycin	E	15	13	14-22	23
Gentamicin	GEN	10	12	13-14	15
Norfloxacin	NX	10	12	13-16	17
Ofloxacin	OF	5	12	13-15	16
Teicoplanin	TEI	30	10	11-13	14
Tetracycline	TE	30	14	15-18	19
Trimethoprim	TR	5	14	14-16	17

Minimum Inhibitory Concentration Interpretative Criteria

The interpretive criteria for vancomycin and *S. aureus* were lowered to ≤ 2 μg/mL for susceptible, 4 to 8 μg/mL for intermediate, and ≥ 16 μg/mL for resistant. (M07-A9-2012)

1) Susceptible – The isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

2) Intermediate – a category that includes isolates with antimicrobial agent minimal inhibitory concentrations that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates.

NOTE: The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g., quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g., β-lactams). This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

3) Resistant – The isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate minimal inhibitory concentrations that fall in the range in which specific microbial resistance mechanisms (e.g., βlactamases) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

(Source: CLSI M07-A9, 2012).

APPENDIX: VII

1. Biofilm formation and MDR pattern of MRSA isolates

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	409.839[a]	4	.000
Likelihood Ratio	497.853	4	.000
N of Valid Cases	400		

a. 3 cells (33.3%) have expected count less than 5. The minimum expected count is .46.

$H_{0:}$ Biofilm formation has no significant effect on Multi Drug Resistance of MRSA.
H_1: Biofilm formation has significant effect on Multi Drug Resistance of MRSA.
Since , $p < 0.01$ we fail to accept Null Hypothesis
Conclusion: Test is statistically significant. Biofilm formation has significant effect on Multi Drug Resistance of MRSA.

2. Age and Prevalence of MRSA

ANOVA(ONE WAY)

Age_yrs

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.612	1	5.612	.055	.816
Within Groups	40773.162	396	102.963		
Total	40778.774	397			

Test is not statistically significant.

3. Gender vs MRSA Prevalence

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.277[a]	1	.599		
Continuity Correction[b]	.174	1	.676		
Likelihood Ratio	.277	1	.599		
Fisher's Exact Test				.664	.338
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 58.43.
b. Computed only for a 2x2 table

Test is not statistically significant.

4. Work duration vs prevalence of MRSA

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	34.336[a]	32	.356
Likelihood Ratio	40.193	32	.152
N of Valid Cases	400		

a. 41 cells (62.1%) have expected count less than 5. The minimum expected count is .62.

Test is not statistically significant

5. Biofilm and Ampicillin Drug Resistance

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	400.000[a]	3	.000
Likelihood Ratio	493.669	3	.000
N of Valid Cases	400		

a. 0 cells (0.0%) have expected count less than 5.

Test is statistically significant

6. Skin infection history vs MRSA prevalence

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	11.849[a]	1	.001		
Continuity Correction[b]	10.611	1	.001		
Likelihood Ratio	10.910	1	.001		
Fisher's Exact Test				.001	.001
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 11.69.

b. Computed only for a 2x2 table

Test is statistically significant

7. Nasal and Hand Source and MRSA Prevalence

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.211[a]	1	.013		
Continuity Correction[b]	5.682	1	.017		
Likelihood Ratio	6.238	1	.013		
Fisher's Exact Test				.017	.008
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 61.50.

b. Computed only for a 2x2 table

Test is statistically significant

8. Biofilm formation with Ciprofloxacin Drug

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.052[a]	1	.044		
Continuity Correction[b]	2.637	1	.104		
Likelihood Ratio	4.441	1	.035		
Fisher's Exact Test				.058	.050
N of Valid Cases	123				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.41.

b. Computed only for a 2x2 table

9. Prevalence of MRSA and regular exposure to waste

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7.349[a]	1	.007		
Continuity Correction[b]	6.743	1	.009		
Likelihood Ratio	7.214	1	.007		
Fisher's Exact Test				.009	.005
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 42.13.

Test is statistically significant

10. Prevalence of MRSA with Marital Status

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.070[a]	1	.791		
Continuity Correction[b]	.022	1	.883		
Likelihood Ratio	.070	1	.791		
Fisher's Exact Test				.816	.444
N of Valid Cases	400				

Test is not statistically significant

11. MIC of vancomycin to MRSA isolates from hand and nasal sample

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.225[a]	1	.635		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.228	1	.633		
Fisher's Exact Test				1.000	.595
N of Valid Cases	9				

a. 4 cells (100.0%) have expected count less than 5. The minimum expected count is 1.33.

Test is not statistically significant

12. MIC of Vancomycin to total MRSA isolates

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	101.000[a]	4	.000
Likelihood Ratio	11.220	4	.024
N of Valid Cases	101		

a. 5 cells (55.6%) have expected count less than 5.

13. ANOVA (Two Way)

Tests of Between-Subjects Effects

Dependent Variable: Sensitivity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26427.942[a]	13	2032.919	35.577	.000
Intercept	59921.503	1	59921.503	1048.646	.000
Antibiotics	26427.942	13	2032.919	35.577	.000
Error	799.985	14	57.142		
Total	87149.430	28			
Corrected Total	27227.927	27			

a. R Squared = .971 (Adjusted R Squared = .943)

Result: Test is statistically significant

APPENDIX: VIII

Formulas:

The sensitivity, specificity, PPV and NPV values were calculated by using the following formula:

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100\% \qquad \text{Specificity} = \frac{TN}{TN+FP} \times 100\%$$

$$\text{PPV} = \frac{TP}{TP+FP} \times 100\% \qquad \text{NPV} = \frac{TP}{TP+FP} \times 100\%$$

PPV = positive predictive value

NPV = negative predictive value

TP = true positive

TN = true negative

FP = false positive

FN = false negative