ANTIBIOTIC SUSCEPTIBILITY TEST OF *Staphylococcus aureus* ISOLATED FROM DIFFERENT CLINICAL SAMPLES OF BIRATNAGAR, NEPAL

A Dissertation

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Submitted by:

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RECOMMENDATION

This is to certify that **Sushant Baniya** has completed this dissertation work entitled **ANTIBIOTIC SUSCEPTIBILITY TEST OF *Staphylococcus aureus* ISOLATED FROM DIFFERENT CLINICAL SAMPLES OF BIRATNAGAR, NEPAL** as a part of partial fulfillment of the requirements of M.Sc. degree in Microbiology (Medical) under my supervision. To our knowledge, this work has not been submitted for any other degree.

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ABSTRACT

Staphylococcus aureus is one of the major pathogen both within the hospitals and community. In addition the prevalence of methicillin resistant strains of *S. aureus* has become the major threat in most of the countries. The aim of this study was to determine the prevalence of infections caused by *S. aureus* as well as MRSA strains and determine their antibiotic susceptibility pattern. A constituent and localized study was carried out from August to December 2021 at MEH, Biratnagar, Nepal. 220 *S. aureus* was isolated from 856 clinical specimens. *Staphylococcus* was identified by the biochemical tests and coagulase test was performed as the confirmatory test of the bacterium. Among the isolates Methicillin Resistant *S. aureus* (MRSA) was identified by using the Cefoxitin (30 µg) disc diffusion method followed by Clinical Laboratory Standards Institute (CLSI 2012) guidelines. Among 220 isolates, 56.36% (n=124) were from inpatients and 43.64% (n=96) were from outpatients. Likewise, 62.73% (n=138) were from male patients and 37.28% (n=82) were from female patients. Antibiogram of all 220 *S. aureus* strains showed effectiveness as: chloramphenicol (74.09%) the most effective drug, followed by Clindamycin (73.18%) and meropenem (70.91%), and the least effective drug was found to be erythromycin (35.45%). Frequency of MRSA, using cefoxitin discs, was found to be 48.64% (n=107) whereas, vancomycin was found to be 100% effective. Out of 107 MRSA strains, the maximum number of strains (n=58) were isolated from the inpatients.. High Prevalence of Staphylococcal and MRSA infections in hospital patients manifested the demand of frequent inspection avoiding the random consumption of antibiotic on any such infections.

Key Words: *S. aureus*, MRSA, Antibiogram, Cefoxitin, MEH

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LIST OF ABBREVIATIONS

µM	:	Micrometer
AST	:	Antibiotic Susceptibility Test
CDC	:	Centers for Disease Control and Prevention
CFU	:	Colony Forming Unit
CLSI	:	Clinical and Laboratory Standards Institute
EMB	:	Eosine Methylene Blue
MHA	:	Mueller Hinton Agar
MIC	:	Minimum Inhibitory Concentration
NA	:	Nutrient Agar
NB	:	Nutrient Broth
MSA	:	Mannitol Salt Agar
MRSA	:	Methicillin Resistant *S. aureus*
MSSA	:	Methicillin Susceptible *S. aureus*
HA-MRSA	:	Hospital Acquired Methicillin Resistant *S. aureus*
CA-MRSA	:	Community Acquired Methicillin Resistant *S. aureus*
TSS	:	Toxic Shock Syndrome
TSST	:	Toxic Shock Syndrome Toxin
SSSS	:	Staphylococcal Scalded Skin Syndrome
PBP	:	Penicillin Binding Proteins
(MLSB)	:	Macrolide Lincosamide Streptogramin B
Imlsb	:	Inducible Macrolide Lincosamide Streptogramin B
cMLSB	:	Constitutive Macrolide Lincosamide Streptogramin B

CRF	:	Coagulase reacting Factor
BHI	:	Brain Heart Infusion
BA	:	Blood Agar
BACTEC	:	Automated Blood Culture System

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Staphylococci are Gram positive, spherical shaped bacteria belonging to the family Micrococcaceae. Micrococcaceae cells may occur as irregular clusters or singly (Atlas 1995). *Staphylococcus aureus, Staphylococcus epidermidis and Staphylococcus saprophyticus* are three most frequently encountered species where Staphylococcus aureus is a major pathogen for humans among all three (Rajaduraipandi et al 2006). S. aureus is different from other species of Staphylocci due to its unique characteristic of producing an enzyme that converts fibrinogen to fibrin that results into the clotting of plasma and the enzyme is coagulase (Brooks et al 2004). Staphylococcus aureus is Gram positive coccus appears as grape like clusters, usually non capsulated bacteria, non-motile and non-sporing (Chakraborty et al 2005). Most strains ferment mannitol aerobically and are facultative anaerobes. They are catalase and coagulase positive and produce an extracellular cell clumping factor, and some strains produce capsule (Brown et al 2005).

Staphylococcus aureus has the ability to produce a wide range of virulence factors, causes food poisoning, toxic shock syndrome and pyogenic infections. Staphylococcus aureus generally enters through breaks in the skin and are rarely capable of invading intact normal skin. *Staphylococcus aureus* causes lung abscess, septic arthritis, furuncles, folliculitis, impetigo, pyogenic infections like breast abscess, and post-operative wound infections etc. Disseminated infections by S. aureus includes septicemia often consequent metastatic secondary foci and toxin mediated infections are toxic shock syndrome. Several deaths were caused due to S. aureus before Penicillin; a beta lactam drug was discovered. *Staphylococcal* infections got reduced to minimum after the discovery of penicillin, however after a few years of its discovery, penicillin-resistant strains of *S. aureus* 2 was encountered. These organisms were able to produce plasmid encoded beta-lactamase enzyme, which could disrupt the beta lactam ring.

Therefore no effect of this antibiotic appeared against these organisms. Later, a semisynthetic drug, methicillin was introduced against those beta-lactamase producers and proved to be successful (Chambers 2001).

However, once again in 1961 Methicillin Resistant strains of *S. aureus* was noticed. Since its first report, the strain has been progressively causing increased mortality, morbidity, and health care costs with skin and soft tissue infections, ventilator-associated pneumonia, catheter associated bacteraemia, and many other infections in hospitals and communities (Shanson 1981; Maple et al 1989). Initially MRSA strains were recognized as Healthcare-Associated (HA MRSA) but now MRSA is also recognized in community and also considered as Community Acquired MRSA (CA-MRSA). HA- MRSA are able to develop resistance to various antimicrobial agents hence the treatment of the infections caused due to MRSA strains has become daunting task as the strains not only the penicillin but also some other structurally unrelated antibiotics such as rifampicin. Hospital Acquired infections of MRSA are likely to resist drugs more than Community Acquired infections due to widespread use of antibiotics in the hospital that select for these bacteria. Multi Drug Resistance refers to resistance to two or more antibiotics belonging to different structural classes (CDC 2006). MDR is one the major problems faced by global Public health. As shown by various studies (ASM 2009) the antibiotic resistance is favored by the usage of antibiotic without prescription from clinician or pharmacists.

As the resistance to antimicrobial agents among Staphylococci has become the global challenge which has recommenced the usage of Macrolide Lincosamide Streptogramin B (MLSB) antibiotics to treat the infections of Staphylococcus with clindamycin as the priority agent due to its due to its excellent pharmacokinetic properties (Delialioglu et al 2005; Deotale et al 2010). Target site modification, enzymatic antibiotic inactivation and macrolide efflux pumps are three different mechanisms of resistance by the MLS antibiotics (Jadhav et al 2011). Production of PBP2a, Penicillin Binding Protein production encoded by mecA gene is the principle reason why Staphylococcus species are resistance to oxacillin/methicillin. Penicillin-binding protein binds beta-lactams with lower avidity, which results in resistance to this class of antimicrobial agents. Methicillin resistance is mediated

by staphylococcal cassette chromosome (SCCmec), a mobile genetic element coding for an altered genetically penicillin binding protein (PBP2a,mecA) which decreases the affinity to Beta lactam and hence the resistance is biochemically complex (Gorden et al 2008). Of all the nosocomial infections MRSA infections accounts for 20–80% (Fomda et al 2014; Fluit et al 2001; Krishnamurthy et al 2014). MRSA infections aren't static as the infections may transmit from person to person by physical contact and rarely by air. *S. aureus* are commonly found in the external skin surfaces and upper respiratory tract particularly nasal passages. Mostly Healthy individuals are usually unaware of staphylococcal carriage but they may suffer from minor skin infections such as boils and abscesses as *S. aureus* is an opportunistic pathogen, it can cause more serious infections. The understanding for the mechanism of methicillin resistance has led to the discovery of accessory factors that influences the level and nature of methicllin resistance as MRSA strains are difficult to eradicate as they are multidrug-resistant leaving glycopeptides antibiotics such as vancomycin, as the drugs of choice. Emergence of vancomycin resistance in enterococci and the transmissible property of its resistance gene (Van A and Van B) to other bacterial species including S. aureus, has led the emergence of vancomycin resistance in clinical Staphylococococci of great concern. As the consequence the treatment of suspected S. aureus infections is becoming more complicated and clinical significance of these strains demands further investigation (CDC 2002).

The rise of drug resistant MRSA has become serious problem in the treatment and control of staphylococcal infection. The frequency of MRSA seems to vary from hospital to hospital in various countries. Diverse researches and studies conducted in our country also show the range of percent isolates of MRSA. In a study carried out by Kumari et al (2008), tertiary-care hospital in Eastern Nepal was found to have 26.14% MRSA strains. Lamichhane et al in 1999 reported 31.43% MRSA strains isolated from 35 S. aureus in Kanti Children's Hospital similarly 11.76% MRSA strains were isolated from 17 S. aureus samples collected in TUTH. Likewise, Rajbhandari et al (2002) in 2002 found that 54.9% of S. aureus isolates were resistant to methicillin.

The foremost and principal focus of this study is on the occurrence of infections caused by S. aureus in the patients visiting hospitals as well as to study its antibiotic sensitivity pattern. The study will also demonstrate the current layout of MRSA and the sensitivity pattern of different antibiotics used against it. This study is really useful to plan and formulate policy to combat against the nosocomial infections widely threatened by *S. aureus* and create infection free health centers.

1.2 OBJECTIVES

1.2.1 General objective

- To study antimicrobial susceptibility pattern of Staphylococcus aureus isolated from clinical samples.

1.2.2 Specific objectives

- To isolate and identify Staphylococcus aureus from clinical samples.
- To determine Methicillin resistant Staphylococcus aureus.
- To determine the distribution of Staphylococcus aureus and MRSA according to the age and gender of patients.
- To determine Inducible Clindamycin resistance.

CHAPTER II

LITERATURE REVIEW

2.1 General Characteristics of Staphylococci

The term Staphylococcus is derived from the Greek word (staphyle, meaning bunch; kokkus, meaning berry) (Chakraborty et al 2005). These are Gram positive spherical cells, that usually occur in grapelike clusters (Brooks et al 2004). Staphylococci are non-motile, non-spore forming that are occasionally capsulate & catalase positive in nature (Cheesbrough 2008). Staphylococci are mostly found in the skin and mucous membranes of human and bird and are widely spread in nature. S. aureus is the most pathogenic strain that can wide variety of infections like pyogenic infections, superficial infections and toxin mediated illness. Staphylococci are mostly opportunistic pathogen and most commonly found Staphylococci species in skin includes *S. epidermidis, S. haemolyticus, S. hominis, S. warneri, S. capitis, S. lugdunensis and S. simlulans.* These opportunistic pathogens are likely to cause infections on patients with intravascular catheters, implanted prosthetic devices or on immune compromised patients (Colley et al 2006). The pathogenic Staphylococci produce variety of toxins and extracellular enzyme often by causing the hemolysis of blood, coagulating plasma (Brooks et al 2004). Staphylococci are non-static hence gets transmitted from person to person (Forbes et al 2007).

2.2 Classification of Staphylococcus

Depending upon the cultural characteristics, morphology of colony, biochemical properties, pathogenicity and cell wall structure Staphylococci can be classified in various ways.

2.2.1 Classification based on bio-chemical properties

i. Coagulase positive – S. aureus
ii. Coagulase negative – S. epidermidis, S. saprophyticus

2.2.2 Classification based on the production of pigment

1. S. aureu produces golden yellow colonies and are pathogenic.

2. S. albus produces white colonies and are non-pathogenic.

3. S. citreus produces yellow colonies and are non-pathogenic. (Ananthanarayan and panikar, 1986).

2.3 Staphylococcus aureus

Staphylococcus aureus gets it's as "aureus" due to the production of golden colonies in agar media. These are spherical shaped gram positive bacterium that is coagulase and catalase positive. It is the normal micro biota of upper respiratory tract, skin and is opportunistic pathogens.

S. aureus causes wide range of infections from general skin infections like impetigo, boils, cellulitis, folliculitis, scalded skin syndrome and abscesses to life threatening diseases like osteomyelitis, meningitis, endocarditis, toxic shock syndrome and bacteremia (Tenoverfag 2006).

Staphylococcal enterotoxin, exfoliatin toxin, toxic shock syndrome toxin, alpha toxin and leucocidin are the major toxins produced by *Staphylococcus aureus* (Salyersaaw 2002).

2.3.1 Morphological and Cultural Characteristics

Staphylococcus aureus is non- motile, non-sporing, non-capsulated and approximately 1μm in diameter. Although it contain a microcapsule, which can be visualized by electron microscope only but not light microscope (parija 2013).

S. aureus can usually be cultured in basic media like nutrient agar within the temperature range of 12-44°C. The optimum temperature and pH for the growth of it is 37°C and 7.5 respectively.

S. aureus produces round, convex, smooth and opaque colonies of diameter of 1-3mm in nutrient agar at aerobic incubation of 37°C for 24 hours. Most strains produce golden yellow pigment. Some strains may produce orange or

yellow pigment and a few are non-pigment producer. Pigment production can be best seen when the cultures are grown aerobically at 22°C (Chakraborty et al 2005). Most strains give moderate to dense turbidity with powdery deposit at the bottom if cultured on nutrient broth. No pigmentation is produced in liquid broth (Bairedparker 1997).

S. aureus show medium to large, slightly raised, smooth, translucent colonies with yellow pigmentation and show beta-hemolysis in 5% sheep blood agar (Forbes et al 2002). S. aureus is able to grow on MSA media with 8-10% sodium chloride (Ananthanarayan and panikar, 1986).

Where most bacteria inhibit on MSA while S. aureus is tolerant to sodium chloride incorporated into the media, due to acid production during mannitol fermentation S. aureus produce yellow colonies surrounded by yellow medium having 1mm diameter. (Collee et al 1996).

2.3.2 Biochemical Properties

S. aureus is catalase positive, MR/VP positive, indole negative and coagulase positive. The bacterium can ferment sugars like glucose, lactose, sucrose, lactose and mannitol with the production of acid but no gas. It can hydrolyze urea, reduce nitrates to nitrites, liquefy gelatin and produce phosphatase (Parija 2013). S. aureus are hyalurodinase, alkaline phosphatase, lipase positive.

Coagulase test in S. aureus is of diagnostic value however other strains of Staphylococcus are also coagulase positive like *S. intermedius, S. hyicus* (Ananthanarayan and Panikar 1986).

2.4 Virulence factors

Virulence factors of S. aureus are integral to various factors and virulent determinants. The virulence of the bacteria is regulated by the cell wall and extracellular components which is expressed during various stages of infection. The virulence factors allow adhere to surface, invade or avoid the immune system and cause harmful effects to hosts (Bien et al 2013).

2.4.1 Cell Associated Polymers

2.4.1.1 Teichoic Acid

Adhesion of cocci to the cell surface is done by the antigenic component of cell wall i.e. Teichoic acid. It also mediates adherence to mucosal cell (Parija 2013)

2.4.1.2 Capsule

Opsonization is inhibited by the capsular polysaccharide surrounding cell wall (kumar 2012).

2.4.1.3 Peptidoglycan

Structural integrity and rigidity is conferred by the cellular peptidoglycan present in cell wall. Release of inflammatory cytokines is induced activating the complement and production of cytokines stimulating macrophages is done by the Peptidoglycan (Chakraborty et al 2005).

2.4.2 Proteins on Cell Surface

2.4.2.1 Clumping Factor (Bound Coagulase)

The clumping factor reacts with plasma, converts it to insoluble fibrin that causes Staphylococci to clump or aggregate. Hence this surface protein is also considered as bound coagulase as reacts with fibrinogen directly (Kumar 2012).

2.4.2.2 Fibrinonectin Binding Proteins

Adhesion to mucosal cells and tissue matrices are promoted by Fibrinonectin Binding Proteins (FBP) (Mongodin et al 2002).

2.4.2.3 Protein A

Protein A is covalently bonded to Peptidoglycan layer and predominantly found in about 90% of *S. aureus* strains. Protein A is significant in chemotactic, anticomplementary and antiphagocytic also induces platelet damage and hypersensitivity. Protein A binds to Fc region of IgG molecules except IgG3, however Fab regions remain free to combine with specific antigen.

2.4.3 Super-antigen exotoxin

2.4.3.1 Toxic Shock Syndrome Toxin (TSST)

Around 25% of *S. aureus isolates* produce TSST. TSST is a super antigen that acts on vascular system causing inflammation, fever or shock. Maximum volume of this toxin is produced during the post exponential phase of the growth. TSST-1 binds to alpha chain of Class II MHC (Ananthanarayan and Panikar 1986).

2.4.3.2 Exofoliatin (Exfoliative toxin)

Exfoliative toxin is mostly associated with the Staphylococcal scalded skin syndrome. Exfoliative toxins are also known to be epidermolytic toxin. These are glutamate specific serine proteases (Chakraborty et al 2005).

2.4.3.3 Enterotoxins

Staphylococcal enterotoxins have the ability to stimulate the maximum population of T-cells that leads to the production of cytokine bolus. Enterotoxins are generally responsible for the food related infections that causes diarrhea and vomiting. These toxins are remarkably resistant to heat and acid (Chakraborty et al 2005).

2.4.4 Membrane-damaging toxins

2.4.4.1 Leukocidin

Leukocidins are bi component pore forming toxins and kill immune cells. Panton-Valentine leukocidin is the major toxin among various like leukolysins, alpha lysins. PV-Leukocidins are majorly found in CA-MRSA (Parija 2013).

2.4.4.2 Alpha toxin (alpha-hemolysin)

It is the major cytotoxic agent that has lethal effects on majority of cell types. As these toxins can lyse blood hence known as alpha haemolysin. Alpha-Hemolysins are involved in inducing apoptosis. Beta, gamma and delta toxins are some of the toxins that are produced by diverse strains of *S. aureus* (Salyers et al 2002).

2.4.5 Extracellular enzymes

Varieties of extracellular enzymes are produced by *S.aureus* like coagulase, hyaluronidase, staphylokinase, deoxyribonuclease, lipase and phosphatase.

2.4.5.1 Hyaluronidase

Hyaluronidase mainly acts on Hyaluronidic acid at the Beta 1-4 Glycosidic Bond yielding unsaturated disaccharides. This enzyme helps organisms to spread from the localized part to surrounding tissues (Chakraborty et al 2005).

2.4.5.2 Staphylokinase

Staphylokinase (SAK) is also known as Staphylococcal fibrinolysin or Muller's factor. This enzyme activates plasminogen and forms plasmin that results in the digestion of fibrin clots. Staphylokinase cleaves IgG and complement component by inhibiting the phagocytosis (Chakraborty et al 2005).

2.4.5.3 Deoxyribonuclease

DNase hydrolyses the DNA of host cell (Parija 2013).

2.4.5.4 Coagulase

There are two types of coagulase free and bound coagulase produced by the strains of *Staphylococcus aureus*. Coagulase enables the conversion of fibrinogen to fibrin after binding to prothrombin acting along with coagulase reacting factor (CRF) (Maranan et al 1997; Langone 1982).

2.4.5.5 Phosphatase

Every strain of *S. aureus* is phosphatase positive. Phosphatase is responsible for breaking the Phospholipid layer of host cell (Chakraborty et al 2005).

2.4.5.6 Lipase

This enzyme helps in spreading of organism by breaking the lipid layer of skin. In S. aureus this enzyme aids to colonize the sebaceous gland of the host (Chakraborty et al 2005).

2.5 Staphylococcal Diseases

Staphylococcal infections are the most common bacterial infections that originate from both community and hospital. Mostly Neonates, breast feeding mother, hospital patients & Injection drug users are pre-exposed to the *Staphylococcal* infections. *Staphylococci* cause disease by the direct tissue invasion or sometimes destruction of tissues by exotoxin production (Bailey and Scotts et al 2007).

2.5.1 Cutaneous Infection

Impetigo, cellulites, wound infections, and abscesses are the most common *Staphyloccal* cutaneous infections.

2.5.1.1 Cellulitis

It is caused when organism enter through wound or break in skin. This infection is common in lower extremities touch (Bailey and Scotts et al 2007).

2.5.1.2 Impetigo

Impetigo is a contagious infection mainly seen in infants and young children. It appears as red sores on body mainly in face and around feet. If the Impetigo goes severe infection invades deeper layer of skin and forms ecthyma another form of Impetigo (Bailey and Scotts et al 2007).

2.5.1.3 Abscesses

As the organism circulates through blood stream so the infection can be seen in any of the body organ and hence also called as the metastatic Abscesses (Bailey and Scotts et al 2007).

2.5.2 Deep Infections

Osteomyelitis, arthritis, pneumonia, septicemia, meningitis, endocarditis, breast abscess, renal abscesses and abscesses in other organs are the major deep infections caused by *S. aureus* (Bailey and Scotts et al 2007).

2.5.2.1 Pneumonia

Staphylococcal Pneumonia is mostly seen in young infants but can also be seen in immune compromised old patients mainly having respiratory issues. Naturally

associated viral respiratory tract infections on infants promote the *Staphylococcal* Pneumonia. Hospital Acquired *Staphylococcal* pneumonia is often seen in infants (Strohlb et al 2002).

2.5.2.2 Osteomyelitis

Osteomyelitis is the inflammatory bone infection caused due to the invasion of *S. aureus* in the skeleton system. At the severe condition this may also result into the cut off of blood supply to the affected bone region and then to bone loss or destruction (Oryanetal 2014).

2.5.2.3 Acute Endocarditis

S. aureus causes Acute Endocarditis damaging the cardiac valves with the onset of high fever, chills, myalgia, and embolization of vegetation to extra cardiac sites that might result to death (Mohiyiddeen et al 2008).

2.5.2.4 Septic Arthritis

Septic Arthritis is the infection in joints that is due to the presence of *S. aureus* that travels through blood stream. It leads to the joint inflammation, pain in a single joint associated with a decreased ability to move the joint (Horowitz et al 2011).

2.5.3 Toxin Mediated Diseases

2.5.3.1. Toxic Shock Syndrome (TSS)

TSS is the infection caused by the release of toxins from an overgrowth of *S. aureus.* The toxin responsible for the syndrome is referred as TSST-I (Toxic Shock Syndrome Toxin –I). Toxic Shock Syndrome is mainly considered as the disease of menstruating women who use tampons (Shands et al 1980).

2.5.3.2. Staphylococcal scalded skin syndrome (SSSS)

Staphylococcal Scalded Skin Syndrome (SSSS) is the disease that causes skin to blister and peel due to the production of toxin. Symptoms of it include irritability, tiredness, fever, redness of skin. SSSS is predominantly seen in children less than 5 years due to the immature immune system that lacks antibodies against exotoxins (Kumar 2012).

2.5.3.3 Food Poisoning

Staphylococcal Food poisoning is the result due to the consumption of *S. aureus* contaminated food that has multiplied and formed enterotoxin. However the illness isn't fatal and limited to an individual. The gastrointestinal illness is characterized by nausea, vomiting, stomach cramps and diarrhea (Hennekinne et al 2012).

2.6. Methicillin Resistant *Staphylococcus aureus* (MRSA)

Methicillin resistant *Staphylococcus aureus* are one of the strains of *S. aureus* that are resistant to related beta-lactam antibiotics and methicillin. MRSA first emerged as Nosocomial infections in early 1960s. MRSA infections have become the threatening problem around the globe as the infections are found as Hospital Acquired (HA-MRSA), Community Acquired (CA-MRSA) and Livestock Acquired (LA-MRSA).

MRSA virtually have occurred as the strains resisting all the antibiotics except Vancomycin. However it isn't the appropriate to refer but can be considered to be the "Multiple Resistant *S. aureus*"as it is also resistant to macrolids, tetracycline, lineosamides, fluroquinolones, and aminoglycosides (Khanal et al 2015).

2.7 Types of MRSA

2.7.1 HA-MRSA (Health Care Acquired MRSA)
2.7.2 CA-MRSA (Community Acquired MRSA)

2.7.1 HA-MRSA (Health care Acquired MRSA)

HA-MRSA infection generally originates from the hospital patients. Surgical wound infections, Pneumonia are the major sites of HA-MRSA. Prolonged hospitalization, prolonged antimicrobial therapy using broad spectrum antibiotics, surgical procedures, surgical wound and Intravenous line are the major risk factors for Nosocomial Acquisition of MRSA (Boyce et al 2005).

2.7.2. CA-MRSA (Community Acquired MRSA)

CA-MRSA usually manifests skin infections and mostly vigilant in community

people and get transmitted within or across the community via infected people i.e. wound infections, pneumonia.

CA-MRSA has become major threat to Public Health. CA-MRSA infections may lead to fatal conditions if invaded the blood stream that results to endocarditis, arthritis, pneumonia and many of other diseases (Parija 2013).

2.8. Classification of MRSA

Taxonomy	Name
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Staphylococcaceae
Genus	*Staphylococcus*
Species	*Staphylococcus aureus*
Sub Species	Methicillin Resistant *Staphylococcus aureus*

(Source: Bergey′s Manual of Determinative Bacterioloby, 1939)

2.9 Cell wall structure and molecular basis of Methicillin resistance *Staphylococcus aureus*

Short glycan chains of approximately 20 alternating N-acetylmuramic acid and beta-1, 4-N-acetylglucosamine residues that makes up the Peptidoglycan surrounds the Staphylococcal cell. Pentapeptide chain referred to as the stem peptide is attached to the N-acetylmuramic acid. Last glycine residue of a pentaglycine cross-bridge attached to the L-lysine residue (position 3) on one stem peptide and the D-Ala residue helps to form the interlinking of glycan chains.

Cell wall becomes mechanically weak if there is no inter linking and may release cytoplasmic content that might result into the death of cell (Koch et al 2003; Waxman et al 1983).

2.10 Source and Transmission of MRSA

MRSA can be transmitted from one person to another by direct contact with the contaminated person. Sharing of cloths, used equipment, sharing of public washrooms are the major hotspots for the infections of MRSA.

Droplet infection is next type of infections that gets transmitted from pneumonia patients. MRSA mostly prevalent in Hospitals are likely to get transmitted in a quicker manner (Bassim et al 2005).

2.11. Prevalence of MRSA

MRSA infections in community level either originated from the Hospital or community has become the major threat due to its resistance capacity to most of the beta lactam related antibiotics.

In the study carried out at the department of microbiology, B.P. Koirala Institute of health Science, Dharan, 78 out of 300 strains of *S. aureus* were found to be MRSA using disc diffusion method (Baral et al 2011).

In contest of India, various degree of methicillin resistance strains of *S. aureus* has been obtained. In a study, out of 13975 isolates of *S. aureus*, 5864 were MRSA and out of 12335 isolates, 5133 were MRSA in 2008 and 2009 respectively (Joshi et al 2013).

In the Study carried out on the Prevalence of MRSA in a tertiary hospital in Nepal during 2018-2020 by P. Pradhan, P. Rajbhandari, S. B. Nagaraja, P. Shrestha, R. Grigoryan, S. Satyanarayana, and H. Davtyan 1027 patients were found with MRSA infections out of 1804 Patients.

2.12 TREATMENT

The numbers of effective antibiotics that are used to treat the MRSA infections are getting reduced. However the most effective antibiotic seen currently across is vancomycin but the treatment options vary depending upon the nature of infections

and other factors (Gould et al 2012).

2.13 D-Test

D-Zone test is for the detection inducible Clindamycin resistance. The test is performed by two antibiotic disc clindamycin and erythromycin kept 20mm apart.

Strains with inducible Clindamycin resistance is difficult to determine in routine laboratory as they appear erythromycin resistant and clindamycin sensitive in vitro if not placed adjacently. Hence D-test helps to determine if clindamycin can be used as therapeutic option or not.

A positive D-test signifies the presence of Macrolide-Inducible to Clindamycin produced by an inducible methylase that alters common ribosomal binding sites for macrolides.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All the equipment, media, reagents, media, antibiotics and other materials used in this study are listed in

3.2 Methods

A paradigm study was done from August to December 2021 at MEH, Biratnagar, Nepal. 220 *Staphylococcus aureus* were isolated from various 856 clinical specimens. Prevalence of MRSA was found using cefoxitin disc and antibiotic sensitivity test was done by using Kirby-Bauer disc diffusion method.

3.2.1 Sample Size

Sample size for the study was calculated using the general formula.

$$\text{Sample size (N)} = \frac{Z^2 pq}{r^2}$$

Frequency of patient visiting the hospital, population size until the sampling time period was estimated to be 5000, considering 3% margin error and 5% confidence limit sample size is calculated as 880.

3.2.2 Sample Collection

Pus/Swab from wound, throat as well as blood was major clinical samples that were collected aseptically by the experienced nurses or laboratory technicians whereas urine sample was collected by the patients themselves. Collected samples were appropriately labeled with Patient's identification number.

1ml (neonates) and 5ml (children) of blood was collected then inoculated into Brain Heart Infusion (BHI) broth at the ratio 1:10 (blood:broth).Samples were processed immediately and stored at refrigerated temperature in case of delay.

3.2.3 Sample Processing

After receiving the labeled samples, specimens were processed for Gram staining, microbial culture and microscopic observation in the microbiology laboratory within 2 hours of collection.

Blood specimens were transferred in BACTEC^{TM} blood bottles aseptically and incubated for 3 consecutive days at 37^0 C. Urine, pus/wound samples were directly inoculated into blood agar and incubated for 24^0C for 24hrs. Growth produced in Blood Agar Plate was further in Mannitol Salt Agar (MSA) Plate. Only cocci obtained after the gram staining of the colonies obtained from MSA was subjected for the identification of *S. aureus*. Catalase, Coagulase (Slide and tube) test was performed to confirm *S. aureus*.

3.2.4 Bacteriological Characterization of *S.aureus*

S. aureus was confirmed by the nature of colonies formed, gram staining and catalase, coagulase test. Gram-positive cocci in grape-like cluster in Gram staining, golden yellow pigmented colonies in MSA and Beta Hemolytic in blood agar, catalase and coagulase test positive results made to identify the organism as *S.aureus*.

Appendix IV includes the procedure of Gram Staining, Catalase test, Coagulase test for the confirmatory identification of *Staphylococcus aureus*.

3.2.5 Antibiotic susceptibility testing of *S.aureus*

S. aureus identified were subjected for antibiotic susceptibility testing. In-vitro Antibiotic susceptibility test was performed using Kirby-Bauer disc diffusion method as per the CLSI guidelines (2012). Turbidity equivalent to 0.5 Mcfarland barium sulfate standards (1.5x108 CFU/ml) was obtained after transferring the fresh colonies to NB. MHA plates were inoculated aseptically by cotton swab then

antibiotics disc was placed using sterile forceps and allowed for Pre-diffusion for around 15 minutes and then incubated at 37^{o}C for 18-24 hrs.

Following antibiotics were used in the study: chloramphenicol (30µg), co-trimoxazole (25µg), clindamycin (2µg), erythromycin (15µg), gentamicin (10µg), meropenem (10µg), tetracycline (30µg), vancomycin (30µg), amikacin (30µg), cefoxitin (30µg), 25 ciprofloxacin (5µg) and clindamycin (2µg) discs (Hi-media-India), erythromycin (15µg)at 15mm apart were also used in the same plate for the detection of inducible Clindamycin resistance.

Clindamycin Resistance was detected as:

i. *Staphylococcal* isolate showing resistance to erythromycin (Zone size ≤13 mm) and being sensitive to clindamycin (zone size ≥21 mm) and giving D-shaped zone are considered to be Inducible resistance phenotypes (iMLSB)

ii. *Staphylococcal* isolates resistance to both erythromycin and clindamycin are considered to be Constitutive resistance phenotypes (cMLSB)

iii. *Staphylococcal* isolate showing resistance to erythromycin (Zone size ≤13 mm) and being sensitive to clindamycin (zone size ≥21 mm) and giving circular zone of inhibition around clindamycin was referred as MS phenotype.

Zone-size interpretative chart to confirm organism as resistant, intermediate and susceptible is given in Appendix 5

3.2.6 Quality control for test

Quality control and calibration of all the equipment is the most important factor to conclude the study with better findings. Therefore to maintain quality control of chemical reagents, antibiotics and media, they were prepared and stored as per instructions provided by the respective companies. Hence Quality control was maintained throughout the study.

3.2.7 Data Analysis

All the unprocessed data were collected and documented in a microbiology laboratory. After the processing of the samples and raw data findings were compared with the various other previous researches to calibrate the results of the study.

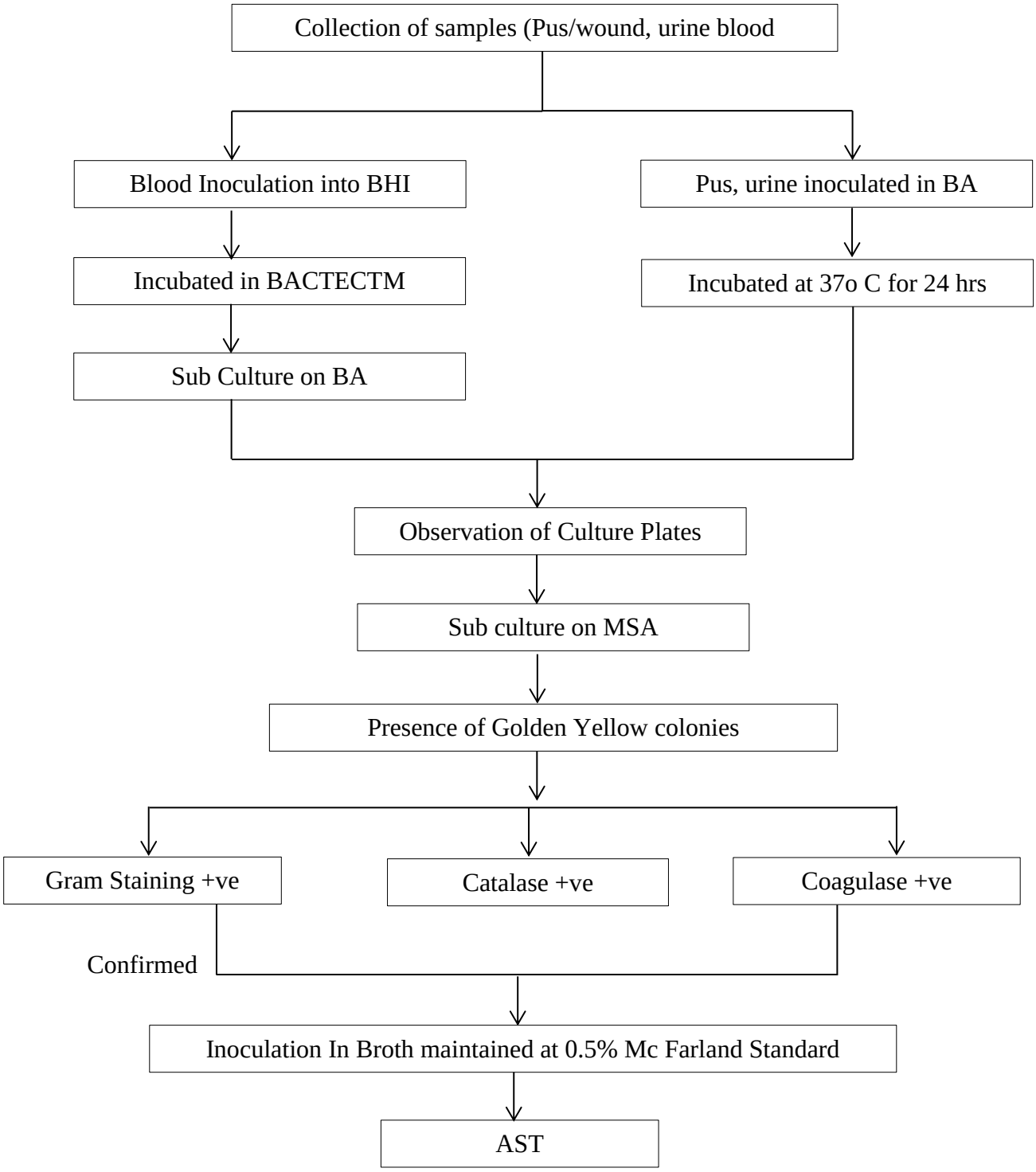


Fig: flow chart of isolation and identification of Staphylococcus aureus from clinical specimens.

CHAPTER IV

RESULTS

4.1 Study population

This study was done in Makalu Everest Hospital, Biratnagar, Nepal from August 2021 to December 2021. Among 856 different clinical specimens 532 (62.1%) samples were from male and 332 (37.9%) were from female.

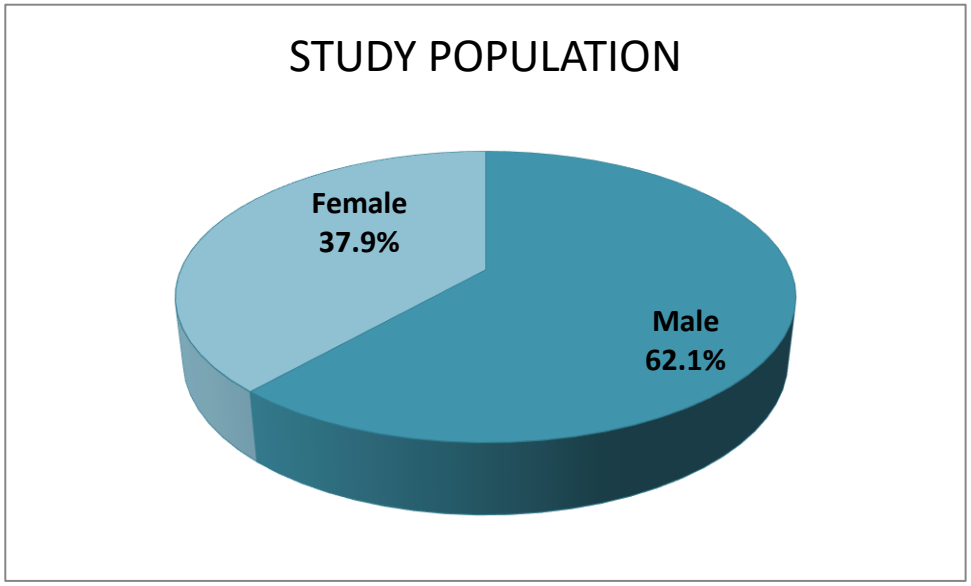


Figure1. Study Population

4.2 Number of *S.aureus* isolated from different clinical specimen

Out of 220 *S. aureus* isolated from various clinical specimens, highest isolates were from pus/wound 161 (73.18%), followed by blood specimens 43 (19.5%) and least from urine samples 16 (7.3%).

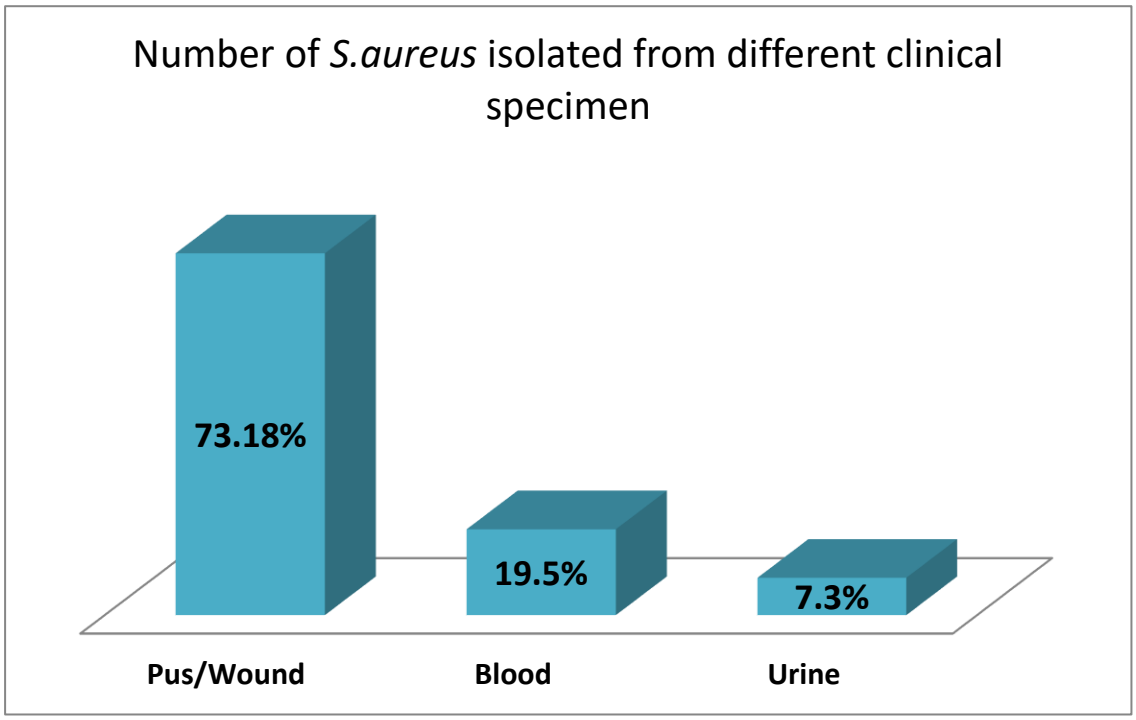


Fig: Number of *S.aureus* isolated from different clinical specimens

4.3 Distribution of S. aureus in different types of patients

Out of 856 clinical specimens, bacterial growth was seen in 542(63.32%) samples. Among all the bacterial isolates *S. aureus* were isolated in 220 samples that included 56.36% (n=124) from inpatients and 43.64% (n=96) from outpatients.

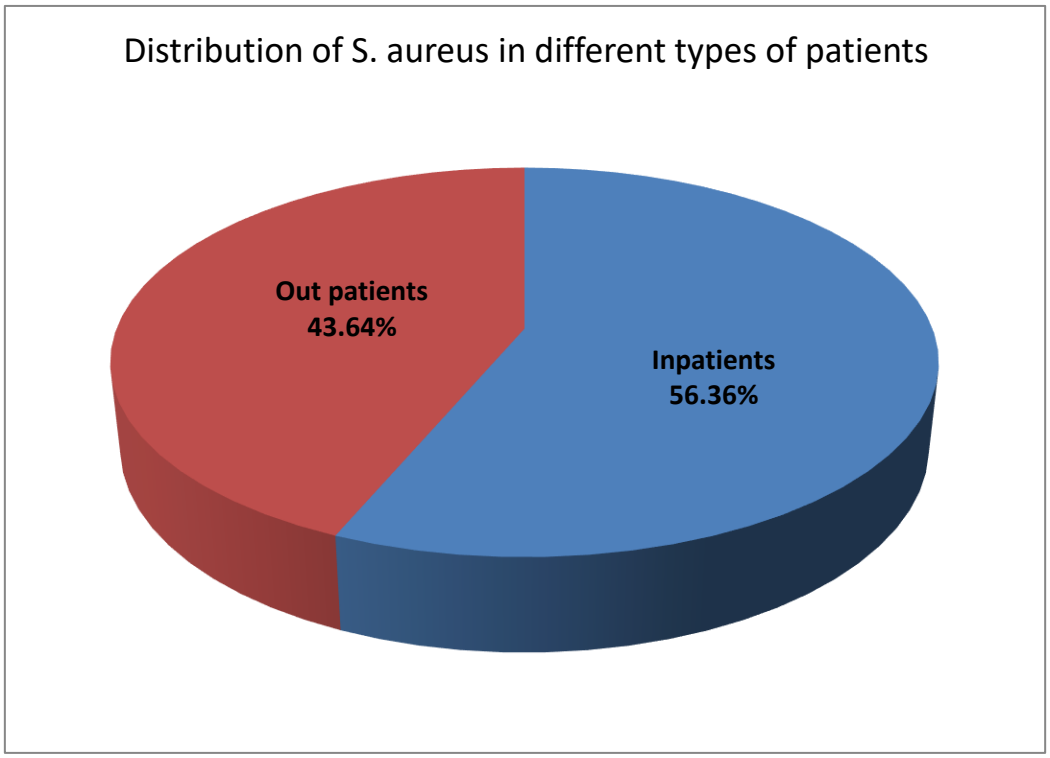


Fig: Distribution of S. aureus in different types of patients

4.4 Distribution of S. aureus according to gender of patients

Out of 856 clinical specimens, bacterial growth was seen in 542(63.32%) samples. Among all the bacterial isolates *S. aureus* were isolated in 220 samples that included 62.73% (n=138) from male patients and 37.28% (n=82) from female patients.

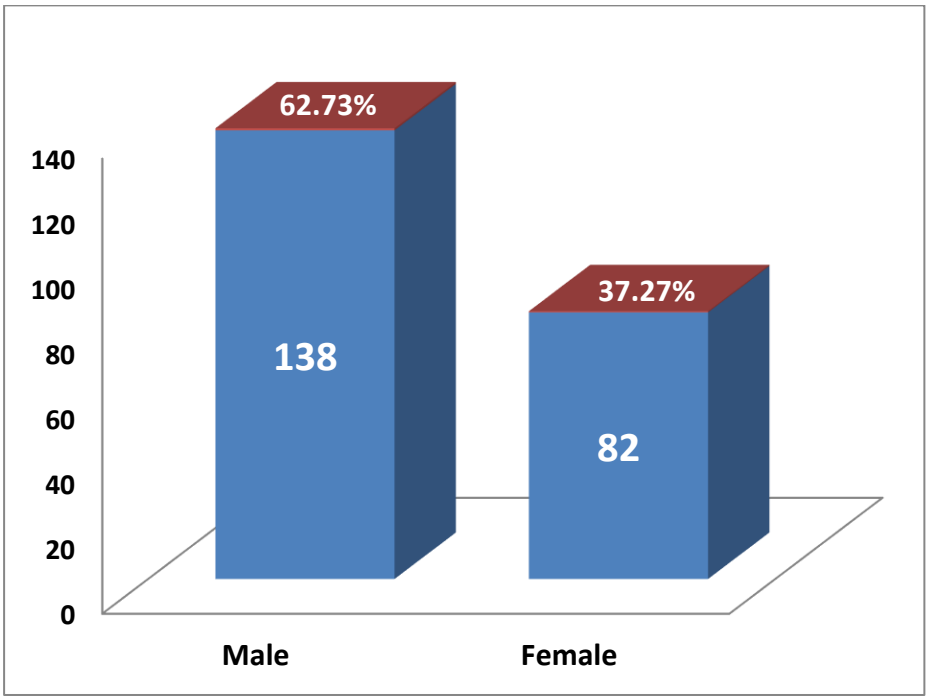


Fig: Distribution of S. aureus according to gender of patients

4.5 Distribution of MRSA in clinical specimens

The table below represents the data of distribution of MRSA in clinical specimens:

Samples	MRSA		MSSA		Total clinical samples	
	Number	Percentage	Number	Percentage	Number	Percentage
Pus/Wound swab	91	85.046729	70	61.94690265	161	73.18
Blood	11	10.280374	32	28.31858407	43	19.55
urine	5	4.6728972	11	9.734513274	16	7.23

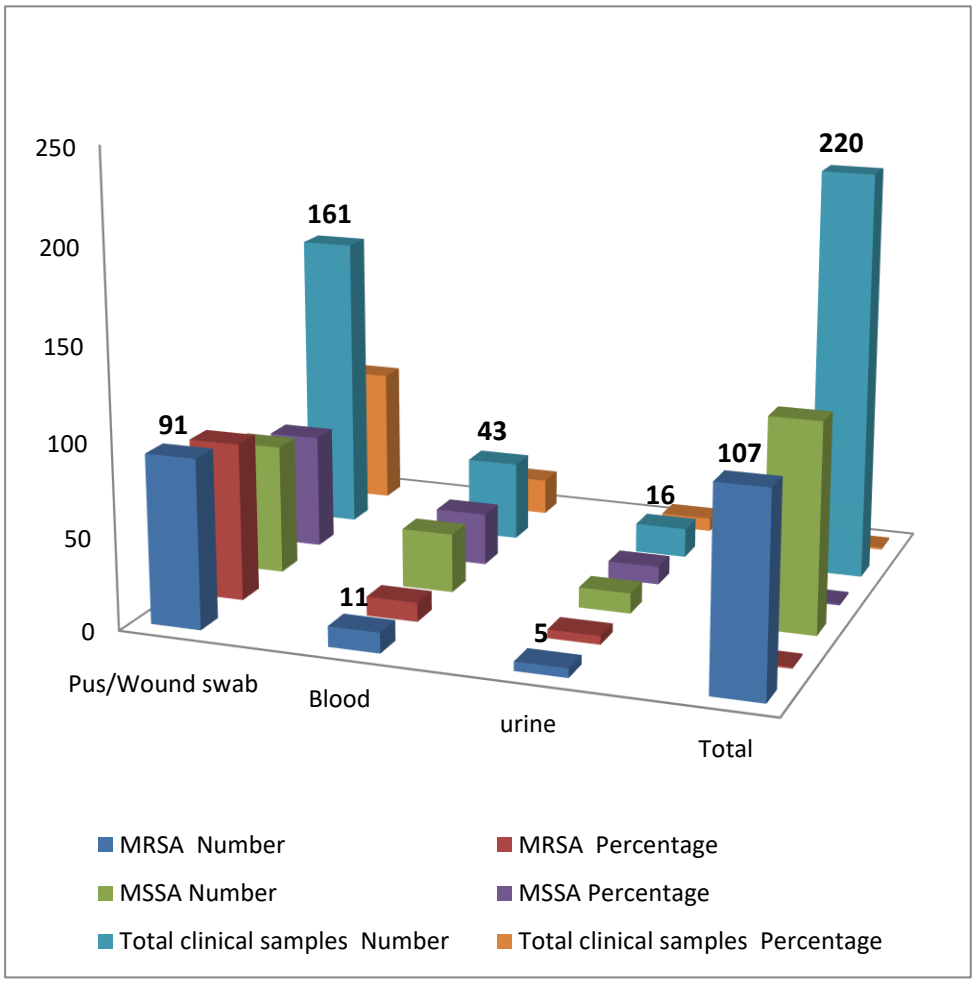


Fig: Distribution of MRSA in clinical specimens

4.6 Ward- wise distribution of MRSA

The table below represents the data of distribution of MRSA ward wise:

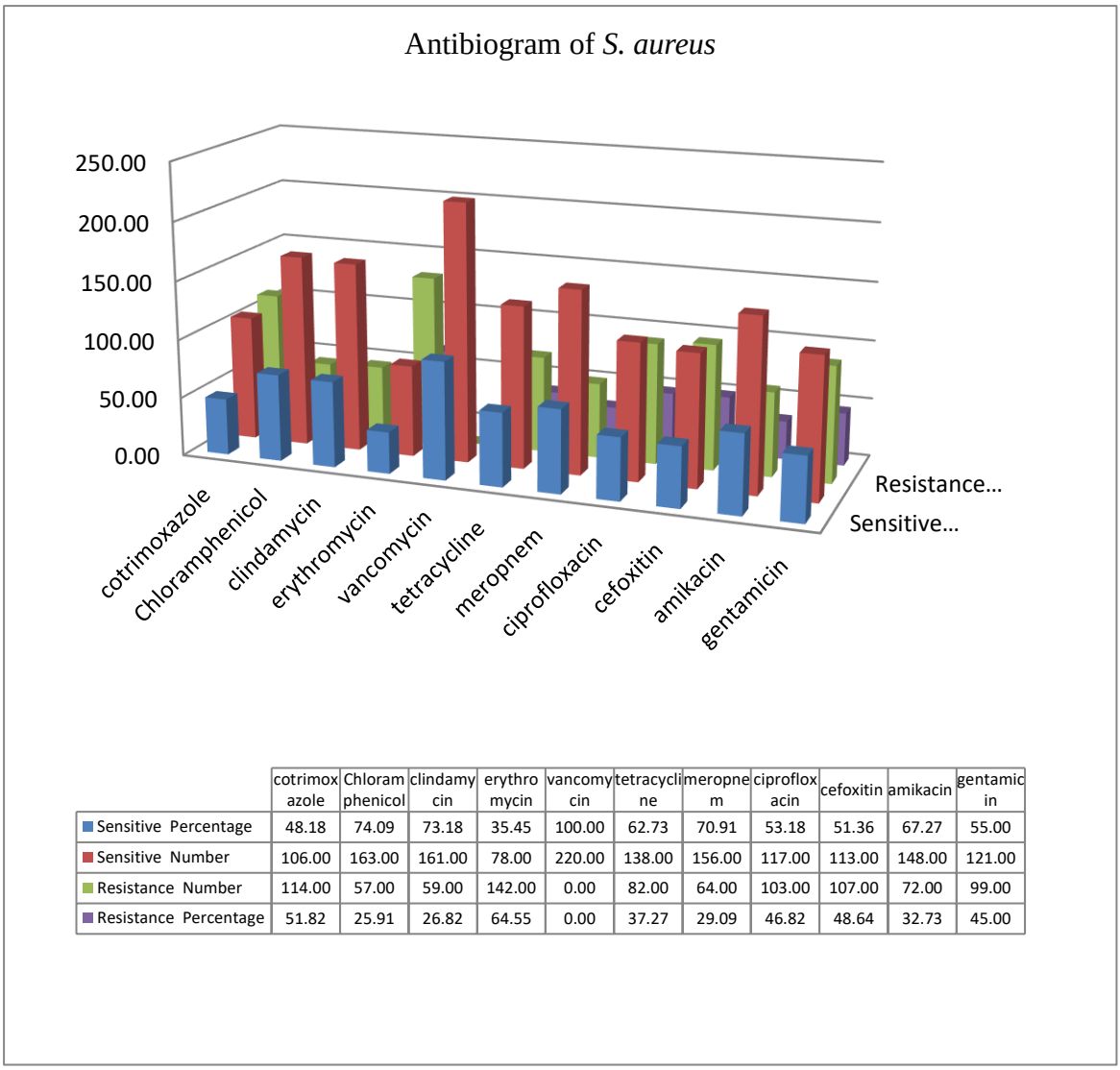
Methicillin Resistant	Ward		Total
	In patients	Out Patients	
MRSA	58	49	107
MSSA	66	47	113
Total	124	96	220

4.7 Antibiogram of *S. aureus*

220 isolates were tested with various antibiotics by using Kirby-Bauer disc diffusion method. The pattern of antibiotic susceptibility test was obtained as chloramphenicol (74.09%) the most effective drug, followed by clindamycin (73.18%) and meropenem (70.91%) and resistant to erythromycin (64.55%) followed by co-trimoxazole (51.82%).

Table: Showing Antibiotic Susceptibility pattern of *S. aureus*

Antibiotics	Sensitive		Resistance	
	Percentage	Number	Number	Percentage
cotrimoxazole	48.18	106.00	114.00	51.82
Chloramphenicol	74.09	163.00	57.00	25.91
clindamycin	73.18	161.00	59.00	26.82
erythromycin	35.45	78.00	142.00	64.55
vancomycin	100.00	220.00	0.00	0.00
tetracycline	62.73	138.00	82.00	37.27
meropnem	70.91	156.00	64.00	29.09
ciprofloxacin	53.18	117.00	103.00	46.82
cefoxitin	51.36	113.00	107.00	48.64
amikacin	67.27	148.00	72.00	32.73
gentamicin	55.00	121.00	99.00	45.00



	cotrimoxazole	Chloramphenicol	clindamycin	erythromycin	vancomycin	tetracycline	meropnem	ciprofloxacin	cefoxitin	amikacin	gentamicin
Sensitive Percentage	48.18	74.09	73.18	35.45	100.00	62.73	70.91	53.18	51.36	67.27	55.00
Sensitive Number	106.00	163.00	161.00	78.00	220.00	138.00	156.00	117.00	113.00	148.00	121.00
Resistance Number	114.00	57.00	59.00	142.00	0.00	82.00	64.00	103.00	107.00	72.00	99.00
Resistance Percentage	51.82	25.91	26.82	64.55	0.00	37.27	29.09	46.82	48.64	32.73	45.00

Fig: Antibiogram of *S. aureus*

4.8 Antibiogram of MRSA & MSSA

Antibiogram of MRSA (107) and MSSA (113) is summarized in the table given below:

Table: Antibiogram of MRSA & MSSA

Antibiotics Used	MRSA				MSSA				
	Sensitive		Resistance		Sensitive		Resistance		
	No.	%	No.	%	No.	%	No.	%	P -Value
Cotrimoxazole	48	44.86	59	55.14	72	63.72	61	53.98	0.040
Chloramphenicol	83	77.57	24	22.43	91	80.53	22	19.47	0.864
Clindamycin	67	62.62	40	37.38	86	76.11	27	23.89	0.003
Erythromycin	26	24.30	81	75.70	49	43.36	64	56.64	0.007
Vancomycin	107	100.00	0	0.00	113	100.00	0	0.00	--
Tetracycline	56	52.34	51	47.66	76	67.26	37	32.74	0.025
Meropnem	71	66.36	36	33.64	96	84.96	17	15.04	0.005
Ciprofloxacin	42	39.25	65	60.75	86	76.11	27	23.89	0.00
Cefoxitin	0	0.00	107	100.00	113	100.00	0	0.00	0.00
Amikacin	85	79.44	22	20.56	72	63.72	61	53.98	0.01
Gentamicin	53	49.53	54	50.47	75	66.37	38	33.63	0.034

All the isolates were found sensitive to Vancomycin. Similarly all the MRSA isolates were resistant to cefoxitin whereas all the MSSA isolates were found sensitive to cefoxitin.

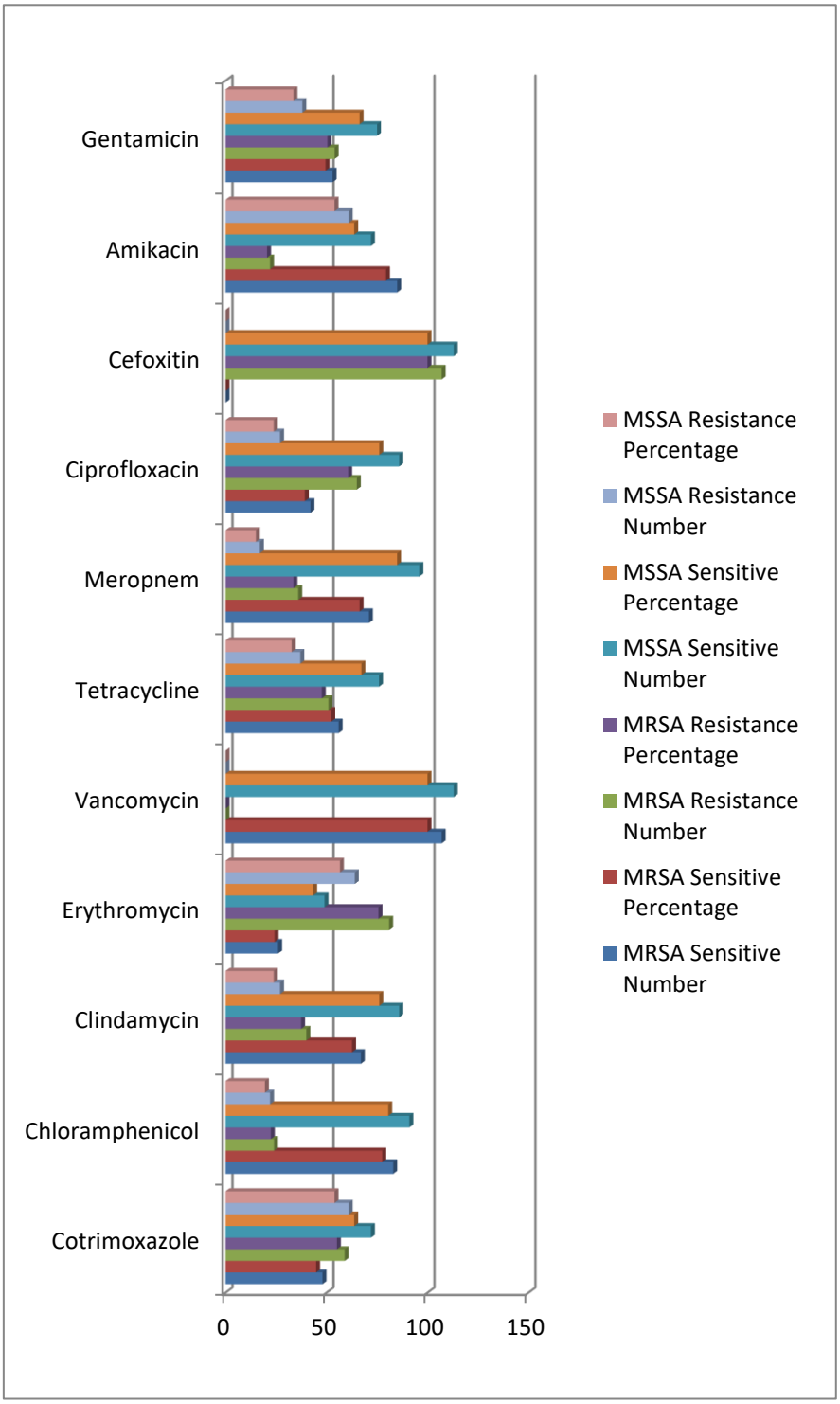


Fig: Bar representation of Antibiogram of MRSA & MSSA

4.9 Inducible clindamycin resistance

Out of 220 *S. aureus* inducible Macrolide-Lincosamide-Streptogramin B (iMLSB) resistance was found to be 26, constitutive (cMLSB) was found to be 38 and MS was found to be 40. Among which out of 107 MRSA strains iMLSB and CMLSB were found dominant over MS whereas MS was found dominant in MSSA strains.

Phenotype	Erythromycin	Clindamycin	D-Test	MRSA		MSSA		P-Value
				No.	%	No.	%	0.004
iMLSB	R	S	Positive	18	16.82	8	7.08	
cMLSB	R	R	Negative	26	24.30	12	10.62	
MS	R	S	Negative	13	12.15	27	23.89	

Table: Inducible Clindamycin Resistance Test

CHAPTER V

DISCUSSION

Staphylococcus aureus is the most common micro biota of human body found in upper respiratory tract, skin and intact with the wounds or skin ruptures. These are the opportunistic pathogens that reside in our body and likely to cause variety of diseases ranging from skin infections to deep infections. Varieties of Toxin mediated infections are caused by the *S. aureus* like TSS (Toxic Shock Syndrome), SSSS (Staphylococcal Scalded Skin Syndrome), and food poisoning and so on. *S. aureus* is the most common organisms that account for the most of the nosocomial infections that generally invades the host through the wounds or skin break or might be the droplet infections too. Since the infection caused by the bacteria ranges from cutaneous to invasive so the study its antibiotic resistivity pattern is of great concern. Moreover the developing resistivity towards the once effective drug has made the study more essential for the medical treatment of such infections. Staphylococcus aureus very commonly causes infections in humans: virtually every person will have one or more Staphylococcus aureus infections in his or her lifetime.

An achievement most microbes do not have on their resume. Most infections occur after an abrasion or cut of the skin due to (non-) accidental trauma, like a child that falls on the street. A lesion of the skin, especially when it has not been cleansed thoroughly, can eventually become painful, red, swollen, and warm, after a day or two. These signs are usually accompanied by a creamy discharge from the wound, known as purulence. This describes the symptoms of an ordinary S. aureus wound infection. If such a wound infection occurs, and is cleaned and kept clean, the infection usually subsides and antibiotics are not necessary. One of the reasons that S. aureus is a frequent cause of infections is that it can survive for months on any type of surface.1 S. aureus cells also possess a wide armamentarium of virulence factors. These virulence factors include factors for adherence, for cell internalization, for evasion of host defense mechanisms, and for invasion of host tissue with the help of these virulence factors.

S. aureus ranks second as the cause of nosocomial blood stream infections, that leads to increased morbidity, mortality, hospital stay, and costs.4-7 Patients admitted to the hospital are, in general, at increased risk for infection. They are ill and, therefore, moderately to severely immune compromised. Hospital treatment usually requires that first line barriers for pathogens, of which the skin is an important one, are intentionally breached, as occurs during surgery or placing of indwelling devices, such as bladder and intravascular catheters. Surgery can result in postoperative wound infections, urine catheterization in urinary tract infections, and intravascular catheters in blood stream infections. Therefore, prevention of these infections is important.

MRSA is an important group of multi drug resistant organisms which are responsible for increasing the rate of morbidity and mortality (Wolk et al 2009). *S. aureus* infections are a significant clinical problem in medical practice as they show resistance to the commonly used first line drugs. In this study, the occurrence of *S. aureus* was studied among the pediatric patients visiting Makalu Everest Hospital, using various types of sample. The study here focuses onto the analysis of the prevalence of *S. aureus* and MRSA in the clinical specimens or can be referred as to identify whether *S. aureus* is the principal cause of Hospital infections or not. All the samples with clinically detected *S. aureus* may serve as a reservoir of MRSA, which may transmit the infection in a community. Thus, there is a chance of a rapid increase in the development of community-acquired MRSA infection. As MRSA depending upon the epidemiology of the infections are termed as HA-MRSA and CA-MRSA. Here hence the study was carried out in the Hospital and the samples collected were from the hospital visitors or patients. Therefore the study focuses more into the study of prevalence of infections caused by HA-MRSA.

The study also focuses onto the route of infection or host-pathogen interaction as the sample collection is done through various routes blood, sample, pus sample and urine, however the positive result would be less in the urine sample as it is the rare route for the host interaction but there could be the significant positive samples

from the blood sample and pus sample as it is the prompt and most liked route of the bacterium.

In present study 220 *S. aureus* were isolated from 512 culture positive samples i.e. 42.95% of the positive culture samples and 25.78% of total samples collected. The results somewhat resembles to those reported by Regmi et al (2020), with growth positivity of 33.8%. Kumari et al (2008), in their report, it is mentioned that out of total 98-gram positive isolates, *S. aureus* occupied 83.67%. Results obtained in a study by Karkee *et al* (2008) are in resemblance too. With growth positivity of 17.4% the results are accordance to those reported by Mukhiya et al (2012).

In the study out of 220 *S. aureus* isolate highest percentage of it was isolated from Pus/Wound samples (73.18%), followed by Blood sample (19.5%) and then urine sample (7.3%). Antibiogram of 220 *S. aureus* strains 107 (48.64%) showed methicillin resistant whereas 113 (51.36%) strains showed methicillin sensitive and it was determined by cefoxitin disc diffusion method.

Among 107 identified MRSA 91, 11 & 5 from Pus/Wound, Blood & Urine samples respectively. Similarly out of 113 MSSA 70, 32 & 11 isolates from Pus/Wound, Blood & Urine samples respectively. The findings of the study correlate with the study of Kumari et al (2008) in a tertiary-care hospital in Eastern Nepal i.e. 64% *S. aureus* isolates from the pus samples. Meanwhile the data also resembles with the findings of Sapkota et al (2006) who reported 53.4% of *S. aureus* isolates were from pus swab from wound. Here the results shows that *S. aureus* are more prone to pyogenic infections i.e. skin and soft tissue (SST) was the most common site for the infection as the organisms are general inhabitants of skin. The findings also correlate with the study of Baral et al (2011) i.e. higher percentage of S. aureus isolate from pus (74%) followed by blood (14%) and urine (2.66%).

In our study out of total 220 *S. aureus* 138 (62.73%) were from male and 82 (37.27%) from female. Similarly 56.36% isolates from the inpatient and 43.34% from outpatients which is in accordance to the study of Baral et al (2011) in Nepal

which reports prevalence of *S. aureus* (75%) among the inpatient and 23% among outpatients. This result signifies infections caused by *S. aureus* are more prevalent in hospital i.e. *S. aureus* might be the probable cause of nosocomial infections. The study suggests making the hospitals or health care centers contamination free with the proper sanitization, proper disposal of used equipment and other safety measures.

Likewise the study also found out the prevalence of MRSA among different types of patient. And it was found to be 58 (54.2%) MRSA from the samples of Inpatient and 49 (45.8%) MRSA from the samples of outpatient. This result resembles with the findings of Sanjana et al (2010) that reports prevalence of MRSA among the inpatient was recorded to be 86 (62.3%) as compared with with 52 (37.7%) in outpatients. The result signifying the highest occurrence of MRSA among Inpatients suggests the hospital acquired infections of MRSA i.e. HA-MRSA. This findings could be the result of prolong stay in hospital, instrument and invasion procedures, immune compromised condition, ill hospital environment, antibiotic therapy which pre-disposes patients to MRSA acquisition. Unlike methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA) has been associated with increased antibiotic exposure, prolonged hospital stays, and admission to the pediatric intensive care unit. In preventing S. aureus infections, it is essential to keep the prevalence of methicillin-resistant S. aureus (MRSA) strains low. Infections with MRSA can only be treated with usually less effective and generally more expensive antibiotics. Furthermore, MRSA infections have a worse prognosis than infections with susceptible strains.

As the use of clindamycin for treatment of Staphylococcal infection may failure hence clindamycin should not be used for treatment of such infection instead r it should be used only for treatment of the infections caused by bacteria which are negative for inducible resistance (D-test positive) and it has also been suggested that inducible clindamycin resistant strains should be reported as clindamycin resistant (Prabhu et al 2011; CLSI 2013). Avoiding the use of clindamycin for the treatment of infections caused by erythromycin resistant strains also omits the chance of treatment failure (Fiebelkorn et al 2003).

The present study report of prevalence of MRSA by 48.64% is also in accordance to the results of Rajbhandari et al 54.9% MRSA isolates at Bir Hospital. As per the reports from Kumari et al 2008: Rijal et al 2008; Tiwari et al 2009; Khanal et al 2010; Mukhiya et al 2012 prevalence of MRSA in Nepal ranges from 35% to 70% which is in resemblance to the results of our study. Subedi and Brahmadathan (15.4%) and Baral et al (26%) reported the lower prevalence of MRSA as 15.4% and 26% respectively. Differences in ranges of prevalence of MRSA might be due to the different techniques, hospital environment, and efficacy of infection and control practices, usage of antibiotics. Uncontrolled and unscientific consumption of antibiotics accounts for increasing prevalence of MRSA in health care centers and community. *S. aureus* has been able to develop the resistance among wide range of antibiotics over the passage of time.

In this study, among 220 *S. aureus* isolates 26, 38 & 40 was found to be inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance, constitutive MLS_B, and MS respectively. Similarly out of 107 MRSA isolates 18, 26 & 13 was found to be inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance, constitutive MLS_B, and MS respectively and 8, 12 and 27 of MSSA isolates were reported to be inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance, constitutive MLS_B, and MS respectively. This result resembles with the study of Ansari et al (2014) where an inducible clindamycin resistance was observed in 12.4% of the isolates which is higher than Adhikari et al (2017) where the inducible clindamycin resistance was found in 10% of *S. aureus* isolates. In the present study overall iMLSB is recorded to be 23.9%, cMLSB is recorded to be 34.92% and MS is recorded to be 36.04%. Clindamycin is one of the major drugs used to eradicate most of skin and soft tissues infections caused by *S. aureus* and also taken as alternative to Penicillin allergic Patients. However the use of clindamycin must be limited to only the cases of negative induced clindamycin resistant case i.e. Positive D tests.

In the present study, Antibiogram study of 220 *S. aureus* isolates were examined with eleven varieties of antibiotic disc and similarly MRSA isolates were

distinguished using cefoxitin disc diffusion method. It was recorded that all the isolates were sensitive to Vancomycin (100%).This result exactly resembles with the reports of Boncompion et al (2017) in Argentina and Khanal et al (2015) in Western Nepal and Khatri et al (2017). In Overall Chloramphenicol and Meropnem considered being the effective drug with less than 30% resistant to it and can be used against Staphylococcal infections after microbial diagnosis. Most of the studies suggest cefoxitin to be reliable antibiotic against the bacterium as cefoxitin is a potent inducer of the mecA regulatory system.

Adequate awareness on the *Staphylococcal* colonization or its understanding is required to avoid the infection. To combat the ability of bacteria to cause infection understanding the diverse factors of host body interaction and its possible ways are to be understood as it involves host, pathogen and environment. In the study only the routes of infection like through blood or pus has been considered, however the possible host-pathogen and environment interactions, immune responses by the host on the interaction or colonization has to be studied.

The study also records MRSA strains being mores resistant to most of the antibiotics than MSSA. Antibiotic Susceptibility pattern of MRSA were recorded as Vancomycin 100% sensitive followed by Amikacin 79.44% and Chloramphenicol 77.57%. MRSA isolates were recorded with high resistance against erythromycin 75.70% followed by ciprofloxacin 60.75% and Cotrimoxazole 55.14%. The empirical and indiscriminate use of these drugs is probably the reason of emergence of MRSA and highly prevalent in the Health Centers.

CHAPTER VI

CONCLUSION & RECOMMENDATIONS

6.1 CONCLUSION

The prevalence of MRSA was found significant as 48.64% (107) isolates were determined as MRSA out of 220 *S. aureus* isolates. Highest number of MRSA was found in Inpatients than out patients. Similarly highest MRSA infections were observed from the wound/pus samples.

This study clearly suggests *S. aureus* and MRSA as the most common agent for Nosocomial infections as MRSA prevailed mostly in the Pediatric patients of the hospital. This is the significant health issue for the area and most probably country as well. As the findings and results correlates with the findings of other researches produced by the researchers.

Adherence of health care workers to infection control guidelines is an important consideration. The degree to which health care workers follow standard and contact precautions influences the result. An intervention will be less effective in a unit where standard precautions are scrupulously followed than in a unit where lapses are common

MRSA was found to be resistant to all the antibiotics except vancomycin, hence the study suggests alerting against the probable nosocomial infections where *S. aureus* and MRSA shall be the Principal cause. Empirical therapy should be given less importance and prescription of drugs must be after the proper microbiological report. Vancomycin mustn't be the first choice of drug against MRSA infections. To keep the value of the vancomycin drug and inhibit the probable mutation of the strains, use of vancomycin should be limited only to the cases required.

6.2 Recommendations

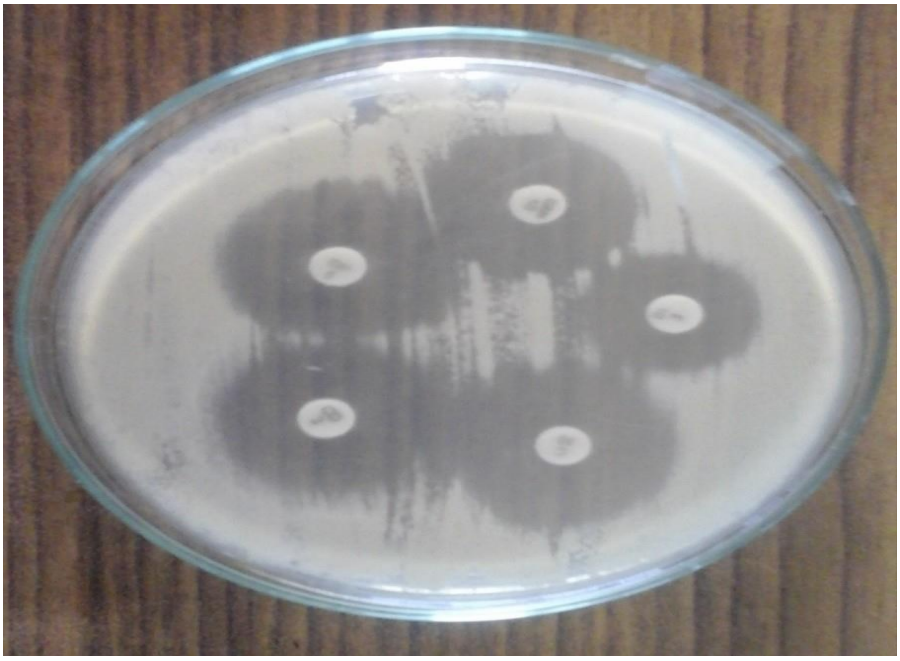
- Irrational use of antibiotics without prescription and unauthorized distribution must be banned.
- In order to make the treatment effective drugs must be given or prescribed on the basis of culture and antibiotic susceptibility report.
- Regular surveillance is required to reduce the risk of nosocomial infections that might occur due to MRSA infections.
- Determination of MIC of methicillin antibiotics could aid to the study which wasn't performed due to lack of resources.
- Research on molecular level using various molecular techniques like RFLP or PCR shall help to unmask the epidemiology of the MRSA strains isolated.

PHOTOGRAPHS

Photograph 1: Blood Sample Collection

Photograph 2: Sample Processing in Microbiology lab

Photograph 3: *Staphylococcus aureus* in Mannitol Salt Agar (MSA)



Photograph 4: Antibiotic susceptibility test of MRSA

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APPENDICES

APPENDIX 1

मिती :२०७८/०४/३१

यो जो सँग सम्बन्धित छ।

टी यु रजिष्ट्रेशन नं. ५२३३२१८-२०११ केन्द्रीय प्रविधि क्याम्पसका ८५९/०७३ रोल भएका विराटनगर-१२ निवासी **सुशान्त बानिया** लाई स्नात्तकोत्तर पूरा गर्नका निम्ति विश्वविधालय को नियमा अनुरुप पर्ने थीसिस पुरा गर्न कार्य क्षेत्र अस्पतालको सुक्ष्म जीवविज्ञान प्रयोगशाला हुने गरी यही मिती **२०७८/०५/०१** (**अगस्त १७ २०२१**) देखि अस्पताल प्रशासनले आवश्यक काम वा खोज गर्न स्वीकृति दिएको छ।

थीसिस अवधी : **६ महिना**

मो. सलिम

अस्पताल प्रशासन

APPENDIX 2

Materials and Equipment's

List of Materials

Glass wares Beaker

Conical flask Test tubes

Glass rod Slides

Pipettes Measuring cylinder

Micropipette Micropipette tips

Miscellaneous

Bacteriological loop labeling stickers

Bunsen burner sterile cotton swabs

Spirit lamp Tube holder

Forceps Gloves

Marker Soaps

Tissue paper

Equipment's

Autoclave Incubator

Water bath Refrigerator

Hot air oven Compound Microscope Water distillation plant

Chemical and Reagents

Crystal violet

Gram's iodine

Ethanol 1N Hydrochloride acid

Safranin Distilled water

3% Hydrogen peroxide

Physiological saline

Plasma

40% Potassium Hydroxide

MacFarland's Nephelometer Standard (0.5)

Microscope oil Lysol

Antibiotics (HiMedia Company) Media (Hi Media Company)

Amikacin (30mcg)

Cefoxitin (30mcg)

Chloramphenicol (30mcg)

Ciprofloxacillin (5mcg)

Co-trimoxazole (25mcg)

Clindamycin (2mcg)

Gentamicin (10mcg)

Meropenem (10mcg)

Tetracycline (30mcg)

Vancomycin (30mcg)

Blood Agar

Brain-Heart Infusion Broth

Nutrient Broth

Muller Hinton Agar

Peptone

Mannitol Salt Agar Erythromycin (15mcg)

APPENDIX 3

Bacteriological media

Composition and preparation of different types of media

1. Blood agar (BA)

Blood agar base (infusion agar) +5-10% sheep blood

Ingredients	**Gram/litre**
Beef heart infusion	500.0
Tryptose	10.0
Sodium Chloride	5.0
Agar	15.0
Final pH (at 25ºC)	7.3±0.2

About 42.5 gram of the blood agar base medium was suspended in 1000ml distilled water and sterilized by autoclaving at 121ºC (15lbs pressure for15 minutes. After cooling to 40-50ºC, 50ml sterile defibrinated sheep blood was added aseptically and mixed well before pouring.

2. Brain Heart Infusion Broth (BHI)

Ingredients	Gram/litre
HM infusion powder	12.5
BHI powder	5.0
Proteose peptone	10.0
Dextrose (Glucose)	2.0
Sodium chloride	5.0
Disodium hydrogen phosphate	2.5
Final pH (at 25℃)	7.4±0.2

Suspend 37.0 grams in 1000 ml purified/distilled water. Dispense into bottles or tubes and sterilize by autoclaving at15 lbs pressure (121°C) for 15 minutes.

3. Mannitol Salt Agar (MSA)

IngredientsGram/litre

Protease peptone 10.0

Sodium chloride 75.0

D-Mannitol 10.0

Phenol red 0.025

Agar 15.0

pH (at 25^0 C) *7.4±0.2*

111 grams of the medium was suspended in 1000 ml distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs at 121^0C for 15 minutes.

4. Muller-Hinton Agar (MHA)

Ingredients	**Gram/litre**
Beef extract	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
pH (at 25^0 C)	*7.4±0.2*

38 grams of the medium was suspended in 1000ml water and boiled to dissolve completely. The media was then autoclaved at 15 lbs at 121^0C for 15 minutes.

5. Nutrient Broth (NB)

Ingredients	**Gram/litre**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
pH at 25^0C	*7.4±0.2*

13 gram of medium was dissolved in 1000 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs at 121^0C for 15 minutes.

APPENDIX 4

Composition and Preparation of different Reagents

1. Gram's Stain Reagent

I Crystal Violet Solution

Solution A

Crystal violet	2.0 gm
95% ethyl alcohol	20.0 ml

Solution B

Ammonium oxalate	0.8 gm
Distilled water	30.0 ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate was dissolved in distilled water. Then, solution A and solution B were mixed

II Gram's Iodine solution

Iodine Potassium iodide	gm
Distilled water	gm
	30.0 ml

III Ethyl Alcohol (95%)

Absolute alcohol	9 5.0 ml
Distilled water	5.0 ml

IV Safranin

Safranine (99% dye content)	10 gm

Distilled water	1000 ml

2 Catalase Reagent

3% Hydrogen peroxide solution (100ml)

Hydrogen peroxide	3 ml
Distilled water	97 ml

1 N Hydrochloric acid (1 mol/litre)

Concentrated hydrochloric acid	8.6 ml
Distilled water	100 ml

3 MacFarland Nephelometer Standards (0.5)

1% V/V solution of Sulphuric acid was prepared by adding 1ml of concentrated Sulphuric acid to 99 ml of distilled water. 1% W/V solution of barium chloride was prepared by dissolving 0.5 gram of dehydrate barium chloride in 50 ml of distilled water. Then to the

99.5ml of 1% Sulphuric acid solution, 0.5 ml of barium chloride solution was mixed and stirred continuously. Then the solution was transferred in to the clean screw capped tube and stored at dark place until use. The test tube for the broth preparation should be of same size as of McFarland tube. The tubes can be stored and used for six months.

APPENDIX 5

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 minute.
3 Wash with water.
4 Add Gram′s iodine for 1 minute.
5 Wash with water.
6 Decolorize with absolute alcohol for 10-15 seconds.
7 Wash with water.
8 Flood with saffron for 1 minute.
9 Wash with water, blot dry and examine under oil immersion objective of the microscope.

Gram positive cocci seen in grape-like clusters were an indicative of Staphylococci.

2. Catalase test

1. A small amount of isolated colony form pure culture was transferred to the surface of clean and grease free 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. Coagulation test

I slide test (to detect bound coagulase)

1. A drop of physiological saline was placed on on each end of a slide and colony of test organism was emulsified in eah of the drop to make two thick suspensions.
2. Add a drop of plasma to one of the suspensions, and mix gently.
3. It was looked for clumping of the organisms within 10 seconds.
4. No plasma is added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

II. Tube test (to detect free coagulase)

1. The plasma was diluted 1 in 10 physiological salines (mixing 0.2ml 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. 0.5ml 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 37ºC. 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 6

A. 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 disc 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 discs containing Muellar-Hinton Agar and were allowed to stand for 30 minute for pre-diffusion of the inoculated organisms.

Antibiotic disc were seeded into the petri dishes containing Mueller-Hinton agar (MHA) for each bacterial isolates. The AST of the isolates towards various antimicrobial disc was done by modifide Kirby-Bauer 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 25ml per plate) was maintained in petri dish.
3. A single isolated colony whose susceptibility pattern is to be determined was touched and inoculated into nutrient broth with the help of sterile wire loop and incubated at 37ºC for 24 hours.
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. Sterile cotton swab was then dipped into the tube and excess inoculum was removed by pressing and rotating the swab firmly against the wall of the tube.
6. Swabbing was done evenly over the surface of the MHA plate by rotating the plates.
7. The petri dish was closed with its lid and then kept at room temperature for 3-5 minutes for the surface of agar to dry.

8. Appropriate antibiotic discs were taken out of the respective vials with the help of sterile forceps and placed carefully on the agar surface. The discs were placed at the considerable distance apart from each other on a 90mm petri-dish. Then the plate was incubated at 37ºc for 24 hours.
9. After incubation, the plates were observed for the 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).
10. 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 antibiotics.

Antibiotics used	Symbol	Strength (mcg)	Resistant	Intermediate	Sensitive
Amikacin	AK	30	≤14	15-16	≥17
Cefoxitin	CX	30	≤21	-	≥22
Chloramphenicol	C	30	≤12	13-17	≥18
Ciprofloxacin	CIP	5	≤15	16-20	≥21
Clindamycin	CD	2	≤14	15-20	≥21
Co-trimoxazole	COT	25	≤10	11-15	≥16
Erythromycin	E	15	≤13	14-22	≥23
Gentamicin	GEN	10	≤12	13-14	≥15
Meropenem	MRP	10	≤13	14-15	≥16
Nitrofurantoin	NIT	300	≤14	15-16	≥17
Tetracycline	TE	30	≤14	15-18	≥19
Vancomycin	VA	30	≤13	14-16	≥17

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