CHAPTER I

1. INTRODUCTION

Staphylococci, gram positive cocci, are one of the common causes of human infections. *Staphylococcus aureus,* in particular, is a leading cause of diseases ranging from skin infections, food poisoning to life-threatening post surgical infections. Usually, the skin infections appear as localized lesions, such as boils, furuncles, impetigo & sties. There is, however, one skin condition caused by *S. aureus* that has a more generalized distribution-scalded skin syndrome, which occurs primarily in infants & gives infant the appearance of having been scalded by hot water (Salyers & Whitt, 2002).

S. aureus is also found to be responsible for an impressive variety of diseases, including infection of heart (endocarditis), infection of bone (osteomyelitis), central nervous system infections such as brain abscesses & pneumonia. Besides, it is also a common cause of toxin-mediated food poisoning. Toxin-mediated another dangerous condition associated with *S. aureus* is toxic-shock syndrome.

S. aureus is found to be inhabited in many areas of the body of a healthy individual but is present in highest numbers in the nose (anterior nares). About one-third of all adults are colonized with *S. aureus* at any point in time; about 25% are colonized persistently. Sooner or later, however, virtually everyone is colonized with *S. aureus*. Thus, it is said that *S. aureus* is the fact of life (Salyers & Whitt, 2002).

S. aureus can be differentiated from other species of staphylococci by a unique characteristic of it to produce coagulase, an enzyme that converts fibrinogen to fibrin and clots the plasma. Among all other species of staphylococci, coagulase positive *S. aureus* is the most pathogenic one. Different virulence factors help these organisms to establish them at the particular locations of the body as well as help them in spreading from one location to another in the progress of disease. Others help them to overcome

the host defense and protect themselves. However, all the virulence factors may not be present in a single strain.

Infection due to *S. aureus* caused several deaths before the discovery of penicillin, a beta-lactam drug. After the discovery of this antibiotic, the frequency of staphylococcal infections reduced to a minimum level, however, soon after a few years of its discovery, penicillin-resistant strains of *S. aureus* developed. These organisms produced an enzyme called beta-lactamase, which is plasmid encoded, and caused the disruption of the beta-lactam ring, hence, no effect of this antibiotic appeared against these organisms. Later, methicillin, a semi-synthetic drug was introduced against those beta-lactamase producers and proved to be successful. However, once again, soon after its discovery, methicillin resistant strains of *S. aureus* appeared in 1961. 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. When MRSA strains first appeared, they occurred predominantly in the healthcare setting. However, methicillin resistance is now increasingly recognized in the community (Chambers, 2001). Healthcare-associated MRSA (HA-MRSA) is particularly efficient at developing resistance to antimicrobial agents.

Infections caused by *Staphylococcus aureus* have a poorer prognosis when the infecting strain is MRSA (Cosgrove *et al*., 2003). Treatment of the infections caused by these strains became more difficult since *S. aureus* became resistant not only to usual penicillin related (beta-lactam) antibiotics but also most other structurally unrelated antibiotics such as rifampicin, chloramphenicol. Some authors used the term ‘multiple resistant *S. aureus*’ for these organisms.

In addition to the awful consequences of infections, MRSA strains are important for their resistance to many other commonly used antibiotics and the emergence of resistance to vancomycin, the drug that has been used to treat MRSA infection for more

than three decades. Thus, it is immensely important to study the antibiogram of S. aureus in a given hospital so that the results of such studies can be exploited to minimize the irrational use of antibiotics.

Methicillin resistance among staphylococci has steadily increased worldwide, especially among cases acquired in hospitals. More people in the US now die from MRSA infection than from AIDS. About 40% of *S. aureus* infections acquired in large US hospitals (500 or more beds) are methicillin-resistant (Red Book, 2003). Methicillin-resistant *S. aureus* was responsible for an estimated 94,000 life-threatening infections and 18,650 deaths in 2005, as reported by CDC in the Oct 17, 2007 issue of The Journal of the American Medical Association. The prevalence of MRSA has varied from hospital to hospital in various countries. Several researches and studies conducted in our country also present the range of percent isolates. In a study carried out by Lamichhane *et al*. in 1999, 11.76% MRSA strains were isolated from 17 *S. aureus* samples collected in TUTH whereas 31.43% MRSA strains were isolated from 35 *S. aureus* in Kanti Children's Hospital. Rajbhandari *et al.* in 2002 reported 54.9% strains of MRSA. Likewise, Kumari *et al.* in 2008 reported 26.14% MRSA strains in a study carried out in a tertiary-care hospital in Eastern Nepal. These studies clearly show the situation of infection caused by MRSA. Common reasons for the rapid increase in frequency of MRSA strains as well as MDR-MRSA may be the overuse or misuse of antibiotics. Similarly, another probable reason may be the lack of hygienic practices of the medical personnel in hospitals or health care centers; lack of isolation room for the known patients already infected with MRSA strains in health care centers.

The prime focus of the study is on the frequency of infections caused by *S. aureus* in the patients visiting hospitals as well as on its antibiotic sensitivity pattern. The study will also demonstrate the present scenario of MRSA and the sensitivity pattern of different antibiotics used against it. This is really useful for the future planning and policy making in healthcare centers and hospitals in order to combat with the spreading dangerous infectious diseases.

CHAPTER II

2. OBJECTIVES

2.1 General objective

To describe the antibiogram pattern of *S. aureus* isolates from different clinical samples collected in the Microbiology Laboratory of Bir Hospital.

2.2 Specific objectives

i. To determine the frequency of staphylococcal infection in different clinical samples and its antibiogram pattern.
ii. To determine the methicillin resistant *Staphylococcus aureus*.

CHAPTER III

3. LITERATURE REVIEW

3.1 Staphylococci

Staphylococci are spherical shaped, gram positive, cluster-forming bacteria belonging to the family Micrococcaceae. According to Bergey's Manual of Systemic Bacteriology, 2nd edition (2002), *Staphylococcus* is classified in family VIII of section XVII belonging to volume 3 which have low G+C content (Madigan *et al.*, 2003). They form a well defined taxonomic group whose cell walls contain peptidoglycan (mucopeptide) and teichoic acids, important cell-adherence factors. Moreover, their peptidoglycan chains are linked by pentaglycine bridges. The organism was first isolated by Pasteur in 1880 from pus and produced abscesses in rabbit by inoculation of the organism. In the same year, Sir Alexander Ogston, a surgeon from Scotland, established conclusively the pathogenic role of staphylococci in abscesses and suppurative lesions. He gave the name *Staphylococcus* (staphyle, meaning bunch; kokkus, meaning berry) (Chakraborty, 2005).

Staphylococci are non-motile, non-spore forming, occasionally capsulate. Most are catalase positive. With the exception of one species, *S. saccharolyticus*, which is a true anaerobe, Staphylococci ferment glucose (Hugh & Leifson O/F test) and are facultative anaerobes (Collee *et al*., 2006). They require no specific growth medium and can grow well on basal media like nutrient agar and nutrient broth.

Staphylococci are widespread in nature, their normal habitats being the skin and mucous membranes of mammals and birds. Human skin is densely colonized with several of the coagulase negative species, and to a lesser extent with *S. aureus*, a coagulase positive organism. However, only a few of them are found with any frequency in clinical specimens, and fewer still are primary human pathogen. Most important are *S. aureus*, which can cause both superficial and deep pyogenic infections as well as a number of toxin-mediated illnesses, and the coagulase negative *Staphylococcus saprophyticus*, an important cause of urinary tract infections in women

of child-bearing age. Common species of staphylococci that are found on human skin include *S. epidermidis, S. haemolyticus, S. hominis, S. warneri, S. capitis, S. lugdunensis, S. simlulans and S. xylosus*. All of these are opportunistic pathogens, especially in patients with intravascular catheters, or implanted prosthetic devices, or who are immunosuppressed (Colley *et al*., 2006). Staphylococci are also transmitted from person to person. Upon transmission, the organisms may become established as part of the recipient's normal flora and later be introduced to sterile sites by trauma or invasive procedures (Forbes *et al*., 2007).

3.2 Classification of Staphylococci

Staphylococci can be classified in various ways depending on their characteristic pigment production, coagulase production and several other biochemical properties.

3.2.1 Classification on the basis of pigment production

i. *S. aureus* producing golden yellow colonies and are pathogenic.
ii. *S. albus* producing white colonies and are non-pathogenic.
iii. *S. citreus* producing yellow colonies and are non-pathogenic.

(Ananthanarayan and Panikar, 1986)

However, since pigment production is not constant and an uncertain character, this classification is now obsolete (Chakraborty, 2005).

3.2.2 Classification on the basis of coagulase production

i. Coagulase positive: *S. aureus*
ii. Coagulase negative: *S. epidermidis, S. saprophyticus* etc.

3.2.3 Baired- Parker classification

Baired-Parker's classification is based on several biochemical tests such as coagulase and phosphatase tests, acid production from Arabinose, Lactose, Maltose and Mannitol as well as acetoin and pigment production.

Table 1: Baired- Parker's classification of Staphylococci

Tests	Subgroups of *Staphylococcus*					
	I	**II**	**III**	**IV**	**V**	**VI**
Coagulase	+	-	-	-	-	-
Phosphatase	+	+	+	-	-	-
Voges-Prouskauer (VP)	+	+	-	+	+	+
Lactose	A	A	V	-	A	V
Maltose	A	A	-	V	A	V
Mannitol	A	-	-	-	-	A

Note: A= acid production, F= fermentative, V= variable

(Gupta, 1993)

3.3 *Staphylococcus aureus*

Staphylococcus aureus is a gram positive bacterium which is spherical in shape and gets its name from the golden colour of its pigmentation produced on agar media. These bacteria are catalase positive and relatively resistant to reduced water potential and tolerate drying and high salt fairly well (Brock, 2003).

3.3.1 Morphological and cultural characteristics

S. aureus is approximately 1 µm in diameter, and divides to form the clusters characteristic of the genus. In liquid media, singles, pairs, and short chains are also seen.

On blood or nutrient agar, incubated in air for 18-24 hours at the optimal growth temperature of 37^0C, it forms colonies 1-3 mm in diameter, although dwarf colonial forms are not uncommon. Colonies are smooth, low convex, glistening, densely opaque

and of butyrous consistency, sometimes surrounded by a narrow zone of haemolysis on blood agar, depending on the strain. Older colonies become translucent and sticky. Occasional strains are capsulated; their colonies are large, convex and glistening, becoming so slime that they run over the surface of a tilted agar plate (Collee *et al*., 2006).

Pigmentation is characteristic of this species when grown aerobically, and ranges from cream through buff to gold. Pigmentation is enhanced on fatty media such as Tween agar, by prolonged incubation, and by leaving plates at room temperature. The colonies will be smaller and greyish in colour when grown anaerobically (Collee *et al*., 2006).

S. aureus ferments mannitol as well as it can tolerate high salt concentration and hence on the selective media like mannitol salt agar (MSA) which contains mannitol 1% and sodium chloride 7.5% with phenol red as indicator of acid production, it forms 1mm diameter yellow colonies surrounded by yellow medium due to acid formation (Collee *et al*., 2006).

On MacConkey or CLED agar, it acquires the appropriate colour of the indicator, depending on whether or not the particular strain ferments lactose. However, the colonies formed on these media are very small (pinhead size).

3.3.2 Biochemical characteristics

Coagulase production is the most identifying characteristic of *S. aureus* which differentiate it from the other species of staphylococci. It gives both tube (free coagulase) and clumping factor (bound coagulase) tests positive and ferments a range of sugars, including mannitol. Besides, it also gives acetoin production (Voges-Proskauer), gelatinase, and alkaline phosphatase tests positive. It produces a deoxyribonuclease (DNase) and a heat-stable nuclease (thermonuclease, TNase). It is also catalase positive.

3.3.3 Virulence factors and pathogenesis

Virulence factors are the weapons for the bacteria which help to establish them at certain habitat (location) as well as help them to combat with the host defense mechanism.

It appears that *S. aureus* has gained about 300,000 DNA base pairs, many of which are factors that lead to its pathogenicity and ability to avoid the body's defense system (Seifert and DiRita, 2006).

Virulence seems to commonly arise via mechanisms of conjugation, pathogenicity island transfer, bacteriophage insertion, and loss of metabolic coding information. In addition, pathogenicity islands via conjugation have been transferred from other bacteria adding new virulence factors. The most significant lateral transfer appears to be the addition of chromosomal DNA cassettes (pathogenicity islands) from neighboring bacteria (Seifert and DiRita, 2006).

S. aureus expresses many potential virulence factors and for the majority of diseases caused by *S. aureus,* pathogenesis is multi-factorial. So, it is difficult to determine precisely the role of any given factor. However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants which suggests their role in a particular disease (Todar, 2008).

Some virulence factors associated with *S. aureus* infections can be categorized under following headings (Todar, 2008):

i. Surface proteins that promote colonization of host tissues

S. aureus cells express surface proteins that promote attachment to host proteins such as laminin and fibronectin that form the extracellular matrix of epithelial and endothelial surfaces. In addition, most strains express a fibrin/fibrinogen binding protein (clumping factor) which promotes attachment to blood clots and traumatized tissue. Moreover, an adhesin that promotes attachment to collagen has been found in strains that cause

osteomyelitis and septic arthritis. Interaction with collagen may also be important in promoting bacterial attachment to damaged tissue where the underlying layers have been exposed.

ii. Invasins that promote bacterial spread in tissues

These include staphylokinase, hyaluronidase, lipase, deoxyribonuclease, phosphatase etc.

a.Staphylokinase

Many strains of *S. aureus* express a plasminogen activator called staphylokinase. This factor lyses fibrin, hence, also called fibrinolysin. As it forms a complex and causes dissolution of fibrin clots by its proteolytic activity, it serves as a spreading factor.

b. Hyaluronidase

This is another enzyme that helps in spreading of the organisms from he localized part to surrounding tissues. It acts mainly on the hyaluronic acid and breaks down the connective tissue of host.

S. aureus can also express proteases, a lipase and a deoxyribonuclease (DNase). However, these enzymes provide basically the nutrients for the bacteria, and play a very minor role in pathogenesis.

iii. Surface factors that inhibit phagocytic engulfment

a. Capsular polysaccharide

The majority of clinical isolates of *S. aureus* express a surface polysaccharide. However, this capsule is distinct from the surface carbohydrate involved in adherence and biofilm formation. This has been called a microcapsule because it can be visualized only by electron microscopy unlike the true capsules of some bacteria which are readily visualized by light microscopy. The function of the capsule in virulence is not entirely clear. However, it does play some role in antiphagocytic activity.

b. Protein A

Protein A is a surface protein covalently bound to the peptidoglycan layer and found in more than 90 % of *S. aureus* strains. It binds to the Fc portion of the IgG molecules except IgG3, preventing specific antibodies from binding to the bacteria and hindering Fc-mediated opsonization.

iv. Biochemical properties that enhance their survival in phagocytes

a. Catalase production

Catalase is an enzyme that splits H_2O_2 to H_2O and O_2. H_2O_2 is a toxic substance produced inside the phagocytic cells during phagolysosome formation. Catalase producing organisms, hence, skip from the toxic substance produced by phagocytic cells and can survive.

v. Membrane-damaging toxins that lyse eukaryotic cell membranes

a. Alpha toxin (alpha-hemolysin)

It is the best characterized and most potent membrane-damaging toxin of *S. aureus*. It is expressed as a monomer that binds to the membrane of susceptible cells. Subunits then oligomerize to form heptameric rings with a central pore through which cellular contents leak. Also, by inducing cellular damage that triggers cytokine production alpha toxin might contribute to shock. Alpha toxin is also called alpha-hemolysin because it can lyse red blood cells.

Some strains of *S. aureus* also produce other toxins- beta toxin, gamma toxin, delta toxin. These toxins can damage membranes of cells other than red cells and may well have a role similar to alpha toxin.

b. Leukocidin

Leukocidin is a multicomponent protein toxin produced as separate components which at together to damage membranes. Leukocidin forms a hetero-oligomeric transmembrane pore composed of four LukF and four LukS subunits, thereby forming an octameric pore in the affected membrane.

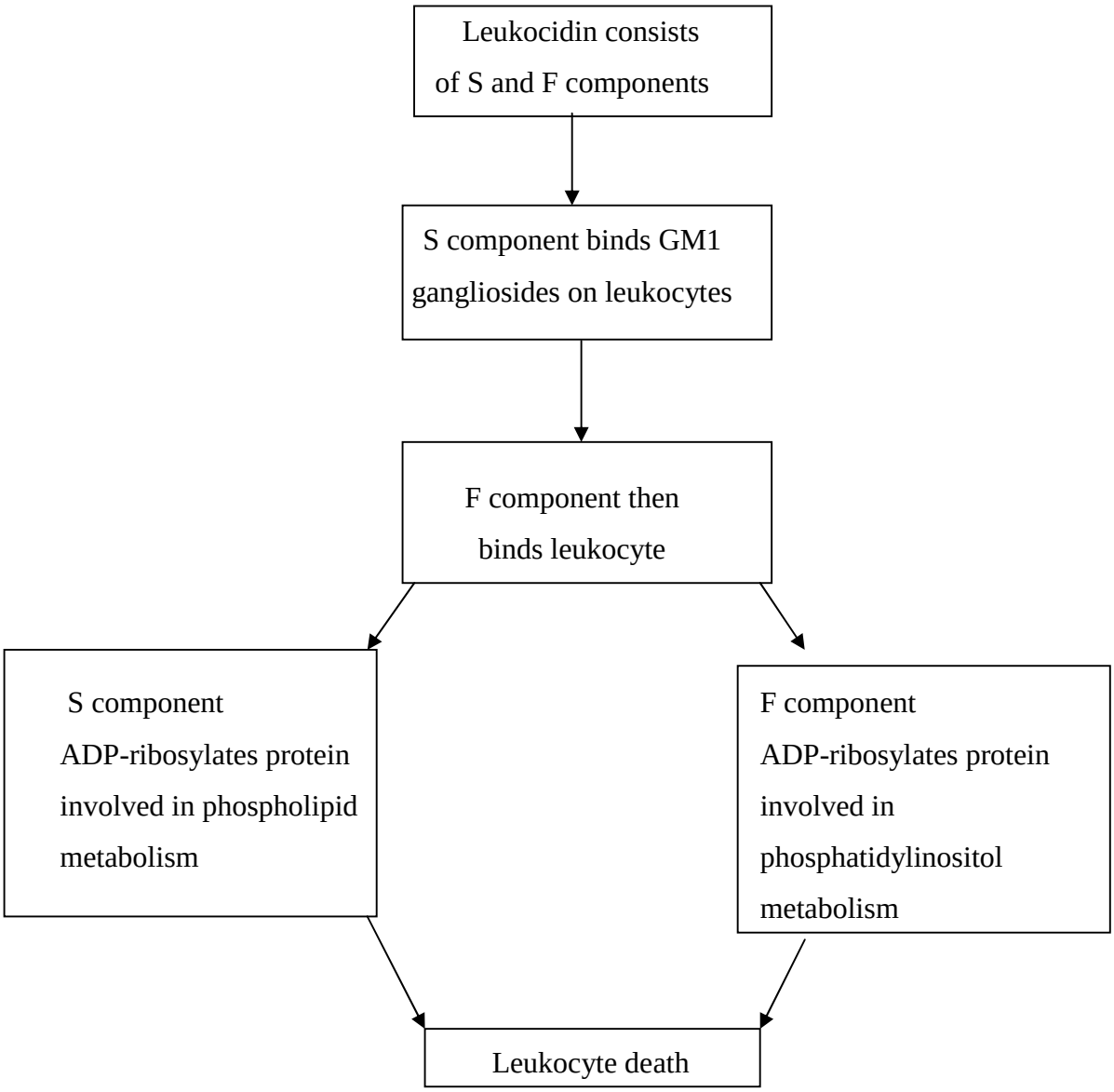

Fig 1: Structure and action of leukocidin

(Salyers and Whitt, 2002)

vi. Exotoxins that damage host tissues or otherwise provoke symptoms of disease

a. Enterotoxins

There are six antigenic types of enterotoxins (named SE-A, B, C, D, E and G). Enterotoxins cause diarrhoea and vomiting when ingested and is responsible for staphylococcal food poisoning.

b. Toxic shock syndrome toxin (TSST-1)

TSST-1 is expressed systemically and is the cause of toxic shock syndrome. Both enterotoxins and TSST-1 are the superantigens. Superantigens stimulate T cells non-specifically without normal antigenic recognition. Up to one in five T cells may be activated, whereas 1 in 10,000 is stimulated during a usual antigen presentation. Cytokines are released in large amounts, causing the symptoms of TSS. Superantigens bind directly to class II major histocompatibility complexes of antigen-presenting cells outside the conventional antigen-binding groove.

c. Exfoliatin toxin

The exfoliatin toxin, associated with scalded skin syndrome, causes separation within the epidermis, between the living layers and the superficial dead layers.

vii. Inherent and acquired resistance to antimicrobial agents

Beginning with the use of the penicillin in the 1940's, drug resistance has developed in the staphylococci within a very short time after introduction of an antibiotic into clinical use. Resistance in bacteria may arise in two ways: (1) mutation in chromosomal genes followed by selection of resistant strains and (2) acquisition of resistance genes as extrachromosomal plasmids, transducing particles, transposons, or other types of DNA inserts. *S. aureus* expresses its resistance to drugs and antibiotics through a variety of mechanisms. For example, almost 90% *S. aureus* possesses plasmid that contains genes encoding the enzyme beta-lactamase responsible for beta-lactam resistance.

3.4 Disease due to *Staphylococcus aureus* infection

Staphylococcus aureus is a bacterium that can frequently live harmlessly in the nose and occasionally on the skin in a state known as colonization (Talaro, 2008). Most likely, *S. aureus* was originally designed to live in harmony with man as a harmless normal flora. Perhaps it played a positive role in the recycling of cell components in nose or skin as its "cousin" *S. epidermidis* did in its original good design. However, a patient becomes clinically infected if the organism invades the skin or deeper tissues and multiplies. In general, *S. aureus* causes two forms of diseases:

1. **Acute inflammation** that usually begins at or near the site of entry of the organisms to the tissue. In most instances it is relatively mild and localized but at times spreads widely by direct extension and occasionally leads to generalized infections.

2. **Acute toxaemia** that results from the absorption of extracellular products formed by staphylococci multiplying at a lesion site, a carrier site or outside the body.

Broadly, the diseases caused by *S. aureus* can be studied under following headings.

A. Localized infections

a. Pyoderma (impetigo): Staphylococcal impetigo is chiefly a disease of children but may occur in adults living under conditions of poor hygiene. Impetigo presents in 2 basic forms; simple or crusted lesions are formed when vesicles develop, burst and discharge copious amount of serous fluid. This forms the characteristic 'honey coloured stuck-on crusts'.

b. Folliculitis: Folliculitis is a mild form of infection but may cause problems at particular sites, for example, the eye.

c. Boil (furuncle): The characteristic staphylococcal lesion is the boil (furuncle), a sub epidermal collection of pus, often around the root of a hair follicle.

B. Deep infection:

a. Osteomyelitis and septic arthritis: *S. aureus* is most common bacterial cause of acute osteomyelitis, particularly in children. The infection is usually due to haematogenous spread of organism.

S. aureus is the primary cause of septic arthritis, a disease due to invasion of synovial membrane, in both children as well as in adults.

b. Pneumonia: *S. aureus* invade the lungs from the bloodstream, giving rise to abscesses; more often it causes a primary pneumonia. Staphylococcal pneumonia occurs in 3 classes of patients:

i. Young infants,
ii. Healthy young adults secondary to influenza, and
iii. Adults suffering from serious diseases.

The onset of the staphylococcal disease is rapid, sputum is watery, profuse and evenly blood-tinged. Staphylococcal pneumonia is rare in young adults in the absence of influenza, except in those who are HIV-positive.

c. Acute septicaemia and endocarditis: Acute septicaemia may occur in association with local suppuration, such as wound sepsis, pneumonia or osteomyelitis, or it may occur spontaneously. Septicaemia often develops in patients who are predisposed to it by a serious underlying disease such as, neoplasia, liver disease, diabetes, rheumatoid arthritis, or certain extensive skin diseases or by septic complications of procedures for their relief, notably surgical operations and intravenous cannulation (Lautenschlager, Herzog and Zimmerli, 1993; Espersen *et al*., 1994).

Endocarditis develops in many cases of septicaemia. Septicaemia without an obvious primary source is more commonly associated with endocarditis and with the subsequent

development of pyaemic abscesses elsewhere in the body. *S. aureus* is one of the important causes of endocarditis associated with intravenous drug abuse.

d. Meningitis: Meningitis due to *S. aureus* may result from haematogenous spread, especially in older patients with underlying disease, but is more frequently associated with trauma and foreign body infection (Jensen *et al.*, 1993).

C. Toxin-mediated disease

a. Staphylococcal scalded skin syndrome (SSSS): It is the cutaneous manifestation of infection with an exfoliatin-producing strain of *S. aureus*. The disease is characterized by separation of the superficial layers of the skin by sideways pressure or the formation of bullae (blisters) as a result of the action of epidermolytic toxins. Scalded skin syndrome is commonly seen in neonates and young children.

b. Toxic shock syndrome (TSS): The toxin responsible for the disease is referred as toxic shock syndrome toxin (TSST-1). The disease is characterized by syndrome of high fever, headache, confusion, conjunctival reddening, subcutaneous oedema, vomiting and diarrhoea, and profound hypotensive shock in children and adults. In more severe cases, acute renal failure, disseminated intravascular coagulation, peripheral gangrene, and even death occurs. A strong association is found with the use of highly absorbent intravaginal tampons during menstruation, especially when these are used continuously and changed infrequently (Davis *et al*., 1980; Shands *et al*., 1980).

c. Staphylococcal food poisoning: It is one of the most common food-borne illnesses. It is an intoxication rather than infection, caused by heat- and protease-stable enterotoxin. It is characterized by acute onset of nausea and vomiting, sometimes followed by diarrhoea.

3.5 Laboratory diagnosis

Laboratory diagnosis of *S. aureus* infection depends upon the site of infection. Appropriate specimen collection is the key to proper diagnosis and treatment.

3.5.1 Specimen collection

In case of wound infection, pus/swab from wound is preferred. In pneumonia, sputum specimen (whether expectorated purulent sputum, respiratory lavage or bronchoscopy guided biopsy for culture) is required. In urine infection, urine specimen using aseptic condition should be followed. Similarly, in case of bacteraemia (or septicaemia) and meningitis, blood and CSF samples respectively are collected. Faces, vomits from patients or the remaining of suspected foods may be the choice of sample in case of food poisoning. In the same way, ascitic fluids, pleural fluids etc may be the choice of sample in some deep-seated infections.

3.5.2 Specimen transportation and storage

All the clinical samples should be processed as soon as possible. In case delay, specimens may be stored at refrigerator temperature. However, CSF sample should be kept at room temperature. No special considerations are required for specimen transportation.

3.5.3 Microscopic examination

Direct microscopic examination in some cases may help in rapid assessment of the infections. Cerebrospinal fluid (CFS), ascitic fluid, pleural fluids, blood etc are sterile body fluids and detection of any organism in the Gram smear of these fluids is sign of infection. Staphylococcal cells may appear in pairs, chains or in clusters with the purple (Violet) colour of cocci showing gram positive reaction.

Microscopic examination may be done by taking colonies from the agar plates. Microscopy of Gram stained smear slide shows the shape and arrangements of the bacteria. The shape and configuration of the Gram positive cocci helps to distinguish staphylococci from streptococci. Staphylococci are perfectly spherical cells about 1 µm in diameter. The staphylococci grow in clusters because the cells divide successively in three perpendicular planes with the sister cells remaining attached to one another following each successive division. Since the exact point of attachment of sister cells

may not be within the divisional plane and the cells may change position slightly while remaining attached, the result is formation of an irregular clusters of cells.

3.5.4 Culture and isolation

Microscopic examination may not provide complete identification of any organism and is not a confirmatory test. Study of cultural characteristics and different biochemical tests is necessary for the compete identification of an organism. And for this, isolation of pure isolated colonies of that organism is must.

Culture and isolation of any organism needs specific culture media as well as specific environmental condition and required incubation time period. The media used may be selective or non-selective. Generally, non-selective media are used first, however, in case mixed growth is obtained, proper selective media can be used to get the pure and isolated colonies of required organism. In case of staphylococcal infection, non-selective basal media like nutrient agar or blood agar may be used whereas in case of mixed growth obtained, selective media like mannitol salt agar or Colombia colistin nalidixic acid agar may be used. For, ascitic fluids, pleural fluids or CSF which are normally the sterile body fluids, enrichment broth should be used in order to increase the bacterial count and only then it should be subcultured on other basal agar media.

3.5.5 Identification

For the complete identification of *S. aureus*, study of colony morphology on different agar plates as well as study of its biochemical tests is necessary.

3.5.5.1 Colony morphology

Colonies on non selective media like blood agar or nutrient agar are seen 1-3 mm in diameter. Colonies are smooth, convex, opaque, and of butyrous consistency, sometimes surrounded by a narrow zone of haemolysis on blood agar. Older colonies become translucent and sticky. Occasional strains are encapsulated and their colonies appear slightly larger, convex and glistering. Pigmentation, which is characteristic of *S.*

aureus can be seen when grown aerobically, and ranges from cream through buff to gold.

On MacConkey or CLED agars, it acquires the appropriate colour of the indicator, depending on whether or not the particular strain ferments lactose. The colonies, however, on MacConkey are small as compare to those on NA or BA.

On selective media like mannitol Salt Agar (MSA), colonies appear yellow surrounded by yellow halo zone due to mannitol fermentation. On tellurite glycine agar, *S. aureus* forms black colonies due to tellurite reduction.

3.5.5.2 Biochemical Tests

a. Catalase test

This is used to test whether the organism can produce the enzyme catalase or not. Catalase producing organism splits hydrogen peroxide into water and oxygen and the gas evolves as bubbles.

However, the release of bubbles should be observed within 10 seconds. False positive result may be seen if the culture is taken from blood agar plate or iron wire is used. Usually, 3 % H_2O_2 is used for this purpose.

b. Coagulase test

Coagulase test is one of the identifying tests of *S. aureus*. It is the enzyme produced by *S. aureus* which causes plasma to clot by converting fibrinogen to fibrin. Two types of enzymes are produced by most stains of *S. aureus*: bound coagulase (clumping factor) and free coagulase.

Slide coagulase test

These rapid tests detect 'bound coagulase' (clumping factor). However, 15% of ordinary strains of *S. aureus* and many more of MRSA give negative reactions in them, and a few species of coagulase-negative staphylococci may give positive reactions. Hence, tube coagulase test is done as the definitive test.

The slide test is performed by making a suspension of culture in physiological saline. A drop of EDTA plasma is added. The mixture is stirred well and observed for clumping within 10 seconds (a positive reaction).

Tube coagulase test

This test is for free or extra cellular coagulase. It is one of the confirmatory tests of *S. aureus*. All the staphylococci isolates that give negative slide coagulase test must be tested for tube coagulase.

In this test, plasma is diluted using physiological saline in a test tube and to this pure culture or broth culture of organism is inoculated. The tube is then incubated at 37^0C for up to 4 hours and gel formation is observed at intervals of 30 minutes. The incubation temperature may be increased up to 18-24 hours.

c. Oxidation fermentation (O/F) test

The test is used to differentiate whether the organism is oxidative and can oxidize carbohydrates aerobically or the organism is fermentative and can ferment carbohydrate under anaerobic condition.

In this test, the test organism is inoculated into two tubes each containing O/F medium, one of which is then overlaid with paraffin oil so as to create anaerobic condition. Fermenting organisms like *S. aureus* produces acid throughout the medium in both the tubes changing the colour of media to yellow because of indicator in the media. However, oxidative organism turns the colour of medium yellow only in the aerobic tube.

d. Voges-Proskauer (VP) test

This test is based on the fact that some organisms produce a neutral product, acetoin. The test is performed using a tube containing MR/VP broth. The test organism is inoculated into it and incubated for 24 hours at 37^0C. After incubation, a few drop of Barrit's reagent (3 volume of 5% alpha-naphthol and 1 volume of 40% KOH solution)

is added into it. Reddening of the supernate within 5-10 minutes is indicative of positive test.

e. Deoxyribonuclease (DNase) test

The test is based on the production of the enzyme deoxyribonuclease (DNase). Most strains of *S. aureus* hydrolyze DNA and give positive reactions in this test but some MRSA strains do not and some coagulase-negative staphylococci give weak reaction. The test may be helpful in identifying strains that give doubtful reaction in the tube coagulase test.

In the test, the test organism is cultured on the medium that contains DNA and it is incubated at 37^0C for 24 hours. The organisms which produce the enzyme deoxyribonuclease (DNase) hydrolyze DNA present in the medium which can be visualized flooding 1 N HCl solution on the medium. The clear zone around the colony is indicative of the positive test.

3.6 Antimicrobial susceptibility testing

After the proper diagnosis of a bacterial infection one should go through the antimicrobial susceptibility testing. This testing provides a good knowledge of the appropriate antibiotics effective against the infection in that individual from which the clinical specimen is collected.

Usually, it is performed in the laboratory by Kirby-Bauer disc diffusion method as recommended by clinical and laboratory standard institute (CLSI), formerly known as NCCLS. The media preferred for this purpose is Muller-Hinton agar (MHA). Agar surface of the MHA plate is swabbed with pure culture of the isolated organism using sterile cotton swab and after placing antibiotic discs on it, the plate is incubated overnight at suitable temperature. After that the inhibition zone size around the antibiotic disc is measured and interpreted.

3.7 Methhicillin resistance *S. aureus*

Methicillin is a narrow spectrum beta-lactam antibiotic of the penicillin class. In other word, it is a penicillin derivative synthesized in 1959 in order to combat the problem of penicillin resistance by beta-lactamase producing organisms. It has the phenol group of benzylpenicillin distributed with methoxy groups. The methoxy groups produce steric hindrance around the amide bond reducing its affinity for staphylococcal beta-lactamases. Methicillin has been recently renamed meticillin to comply with European law, which requires the use of the recommended international non-proprietary name (rINN). International convention has now renamed this agent as meticillin (Rao S, 2009).

Methicillin resistant *Staphylococcus aureus* (MRSA) are strains of the *Staphylococcus aureus* that are resistant to the action of methicillin and related beta-lactam antibiotics (e.g. penicillin, oxacillin, amoxacillin etc). Although the media discusses MRSA as a single new strain, in reality MRSA represents more than 1100 distinct strains of *S. aureus* (Wim and Neeling, 2005).

In recent years, strains of *S. aureus* have emerged that are resistant to virtually all antibiotics except Vancomycin. These have been called methicillin resistant *Staphylococcus aureus* (MRSA) strains, but the description "multiple resistant *S. aureus*" strains would be more appropriate because many MRSA strains are also resistant to tetracycline, macrolids, lineosamides, fluroquinolones, and aminoglycosides. Resistance to trimethoprim/sulfomethoxazole is also is also becoming common in MRSA strains. In some hospitals, nearly 90% of *S. aureus* isolates are MRSA.

MRSA is prevalent in health care environments because individuals tend to be older, sicker and weaker than the general population, which heightens their vulnerability to infection through weakened immunity. In addition, these environments involve a great

many people living and working closely –perfect for transferring MRSA (Coella *et al*., 2005).

3.7.1 Invasive MRSA infections

A case of invasive MRSA infection was defined by the isolation of MRSA from a normally sterile body site in a resident of the surveillance area, including residents institutionalized in long-term care facilities, prisons, etc. normally sterile sites include blood, cerebrospinal fluid, pleural fluid, joint/synovial fluid, pleural fluid, pericardial fluid, peritoneal fluid, internal body site (lymph node, brain, heart, liver, spleen, vitreous fluid, kidney, pancrease, or ovary), or other normally sterile

3.7.2 Prevalence of MRSA

The incidence of MRSA infections has increased significantly since the organism first emerged. In 1974 fewer than 2 percent of staphylococcal infections in the United States were caused by MRSA, but by 2004 more than 60 percent were the result of MRSA. Likewise, in 1993 in the United Kingdom, roughly 50 people died from MRSA infection, compared with more than 1,600 people in 2006.

In 2005 in the United States, methicillin-resistant *Staphylococcus aureus* was responsible for an estimated 94,000 life-threatening infections and 18,650 deaths, which surpassed deaths from HIV/AIDS (approximately 17,000) (CDC, 2007) and it arises the need for improved surveillance to prevent and control the spread of this potentially lethal organism.

In a study carried out at the Department of Microbiology and Department of Pathology, Iran, 53 out of 175 strains of *S. aureus* were found to be resistant to methicillin using E-test whereas disk diffusion method using oxacillin or cefoxitin showed 52 strains to be methicillin resistant (Rahbar *et al.*, 2006).

Forty strains out of 79 strains of *S. aureus* were identified as MRSA on the basis of resistance to oxacillin disc in a study carried at Mymensingh Medical College, Bangladesh (Khan *et al.*, 2007).

In another study done at the tertiary care hospital of northern India, out of 783 *S. aureus* samples isolated, 301 strains were found to be MRSA (Tiwari *et al*., 2008)

In contest of Nepal, various degrees of methicillin resistance strains of *S. aureus* have been obtained. In a study enrolling 250 clinical samples, out of which 52 (20.8%) were *S. aureus*, 13 strains were found to be methicillin resistant (Lamichhane *et al*., 1999).

Among 908 pus samples handled both from outpatients as well as from admitted patients visiting Bir hospital, 284 isolates were found to be *S. aureus* and of which 156 strains were methicillin resistant (Rajbhandari *et al*., 2002).

In another study, out of 210 clinical samples collected and analyzed, 65 were found to be *S. aureus* and among which again, 19 strains were found to be MRSA (Thapa *et al*., 2004).

In 2006, the study carried out again at Bir hospital showed 81 strains to be MRSA from 264 *S. aureus* isolates collected handling altogether 1690 different clinical samples (Sapkota *et al*., 2006).

In a study in a tertiary-care hospital of Eastern Nepal, out of total 750 *Staphylococcus aureus* isolated from various clinical samples, 196 (26.14%) were found to be methicillin resistant. Among the total isolates of MRSA, seventy percent isolates were from inpatient departments (Kumari *et al*., 2008).

3.7.3 Classification of MRSA

MRSA infections can be classified into two:

i. Community acquired MRSA (CA-MRSA), and

ii. Hospital acquired MRSA (HA-MRSA).

i. Community acquired MRSA (CA-MRSA)

CA-MRSA is acquired by persons who have not been recently (within the past year) hospitalized nor had a medical procedure (such as dialysis, surgery, catheters). These infections manifest usually as skin infections, such as pimples and boils and occur in otherwise healthy people (Buckingham *et al*., 2004). About 75 percent of CA-MRSA infections are localized to skin and soft tissue and usually can be treated effectively. However, CA-MRSA strains display enhanced virulence, spread more rapidly and cause more severe illness than traditional HA-MRSA infections, and can affect vital organs leading to widespread infection (sepsis), toxic shock syndrome and pneumonia. It is not known why some healthy people develop CA-MRSA skin infections that are treatable whereas others infected with the same strain develop severe, fatal infections (Todar, 2008).

ii. Hospital acquired MRSA (HA-MRSA)

Hospital- acquired MRSA (HA-MRSA) infection is acquired by persons admitted to hospitals for more than 48 hours or those have medical history of MRSA infections or colonization during previous admission. Common sites of HA-MRSA are surgical wound infections, urinary tract infections, and pneumonia. A ***surgical wound infection (SWI)*** is a term that was changed according to CDC (Center of Disease Control) to ***surgical site infections (SSI).***

In case of HA-MRSA, patients who already have an MRSA infection or who carry the bacteria on their bodies but do not have symptoms (are colonized) are the most common sources of transmission. The main mode of transmission to other patients is through human hands, especially healthcare workers' hands. Hands may become contaminated with MRSA bacteria by contact with infected or colonized patients.

3.7.4 Evolution of methicillin resistant *S. aureus*

Methicillin resistance in *S. aureus* is primarily mediated by the mecA gene, which codes for the modified penicillin-binding protein 2a (PBP 2a or PBP 2').

Scientists believe that *mecA* may have come from a *Staphylococcus* sp, *S. sciuri*, which is found primarily in squirrels. A possible way of how a squirrel staphylococcal gene migrated into the human is that although *S. aureus* and *S. epidermidis* are often portrayed as human specific pathogen, they can also be isolated from dogs and cattle, and possibly it is one of these animal reservoirs that a squirrel staphylococcal strain and S. aureus met and became friendly enough to exchange DNA (Salyers *et al*., 2002).

Examination of a large number of MRSA isolates has led to the conclusion that the original acquisition of the *mecA* gene has occurred

3.7.5 Cell wall structure and molecular basis of methicillin resistance

The staphylococcal cell is surrounded by a mesh-like structure 20-40 nm thick, called peptidoglycan, that is composed of a series of short glycan chains of approximately 20 alternating N-acetylmuramic acid and beta-1,4-N-acetylglucosamine residues. Attached to each N- acetylmuramic acid residue is a pentapeptide chain referred to as the stem peptide. The glycan chains in peptidoglycan are linked together via the 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 (position 4) an another. Pentaglycine cross-bridges are performed in the cytoplasm by the FemX, FemA and FemB proteins, which attach the glycine residues to the L-lysine residue of the stem peptides. The cross-linking or transpeptidation reactions take place on the external surface of the cytoplasmic membrane in a reaction catalyzed by penicillin-binding proteins (PBPs). There are four PBPs in *S. aureus,* PBP1, PBP2, PBP3, and PBP4. High molecular weight PBPs have two protein domains, one involved in transpeptidation (cross-linking) the other involved in transglycosylation (extending the glycan chain). The beta-lactam antibiotics, which resemble the terminal D-alanyl-D-alanine bond of the stem peptide, inhibit the transpeptidation domain of PBPs (and carboxypeptidase activity of low molecular weight PBPs) thus interfering with the cross-linking reaction. Without cross-linking of the peptidoglycan, the cell wall becomes mechanically weak, some of the cytoplasmic contents are released and the cell dies.

Staphylococcal resistance to oxacilllin/methicillin occurs when an isolate from infected patient carries an altered 76 KDa penicillin-binding protein, PBP2a (also referred to as PBP2'), which is encoded by a large stretch of foreign DNA (40-60Kb), referred to as the mec element (the mecA gene). PBP 2a is located in the bacterial cell wall and has low binding affinity for beta-lactams, however, it can takeover the transpeptidation (cross-linking) reactions of the PBPs. mecA expression can be constitutive or inducible. *mecA* is carried on a mobile genetic element, the staphylococcal cassette chromose mec (SCCmec) and at least five types of SCCmec elements have been reported (Rao S, 2009).

Adjacent to *mecA* on the staphylococcal chromosome are two genes, *mecR1* and *mecI*, that are co-transcribed divergently from *mecA*. The *mecR1* gene encodes a membrane-bound signal transduction protein (MecR1) while *mecI* encodes a transcriptional regulator (MecI). Between *mecA* and *mecR1* are the promoters for genes and an operator that encompasses the -10 sequence of *mecA* and the -35 sequence of *mecR1* (Sharma *et al*., 1998).

Unlike beta-lactamase synthesis, expression of PBP2a is not strongly inducible in isolates carrying the normal regulatory genes (*mecA* and *mecR1-mecI*) and induction is much slower (15 minutes for beta-lactamase expression compared to up to 48 hours for PBP2a synthesis). This is because MecI is a tight regulator of mecA transcription and most beta-lactam antibiotics do not efficiently activate MecR1 (Kuwahara-Arai *et al*., 1996). However, selective pressure though antibiotic usage has promoted S. aureus isolates that have mutations or deletions in *mecI* or the *mecA* promoter/operator region giving rise to an inactive repressor and constitutive PBP2a expression (Kobayashi *et al.*, 1998).

3.7.6 Internal factors affecting methicillin resistance: Since PBP2a is essential in conferring methicillin resistance, any factor that interferes with the expression of the

mecA gene or with the activity of PBP2a will affect methicillin resistance. Studies have shown that PBP2a requires:

i. Glycan chains to be of certain lengths: PBP2a is dependent upon the transglycosylase activity of PBP2. Beta-lactams inhibit the transpeptidase domain of high molecular weight PBPs but do not affect the transglycosylase domain. Inactivation of the transglycosylase domain of PBP2, results in an increase in glycan chains of shorter lengths and a marked decrease in methicillin resistance. Therefore, compounds that target the transglycosylase domains of PBPs could serve useful therapeutic agents. (Pinho MG *et al.*,2001)

ii. The stem peptide to have the normal peptide configuration

The addition of glycine to the growth medium leads to stem peptides of peptidoglycan ending in two glycine residues instead of two alanine residues. This leads to a decrease in methicillin resistance and conversion of a highly resistant homogeneous strain to a heterogeneous phenotype. These results illustrate that PBP2a requires the stem peptides to have the correct length and contain the normal series of peptides. (Ludovice AM *et al*., 1998)

iii. Requires the pentaglycine cross-bridge to be intact

FemA, FemB, and FemX (FmhB) are involved in building the pentaglycine cross-bridges that link the glycan chain together. FemX adds the first glycine, FemA adds glycines 2 and 3, and FemB adds glycines 4 and 5. Fem A and FemB are not interchangeable, consequently, inactivation of the genes coding for either of these proteins results in cell walls that contain mono-or triglycine cross-bridges, respectively. Inactivation of either of the femA or femB genes is thought to be lethal but compensatory mutations can restore cell viability, although growth is severely affected. Significantly, inactivation of either femA or femB also results in a large reduction in methicillin resistance. (Labischinski H *et al*., 1999)

3.7.7 External factors that affect methicillin resistance

External factors that affect methicillin resistance include among others, salt concentration, pH, medium composition, osmolarity and temperature (Matthews *et al.*, 1984). Some of these external influences are exploited in the clinical laboratory to enhance the detection of strains exhibiting heterogeneous methicillin resistance; isolates are grown in the presence of high NaCl concentrations (2%) and at lower temperature (30-35^{0}C) maintaining neutral pH and incubation time extending to 24 hours. (Rao S, 2009)

3.7.8 Transmission of MRSA

Staphylococci are common in skin folds, such as the perineum and axillae, and in the anterior nares. They may also colonize chronic wounds, for example in eczema, varicose and decubitus ulcers. MRSA may spread in the same ways as sensitive strains of *Staphylococcus.*

Transmission of MRSA occurs mainly by contact transmission and droplet infection. Contact transmission occurs via hands which may become contaminated by contact with colonized or infected individuals, colonized or infected body sites of other persons, devices and environmental surfaces already contaminated with body fluids containing MRSA. (Bassim *et al*., 2005)

Surgical wound infection and contamination occurs mainly through contact with hands of health care workers or environmental surfaces contaminated with body fluids containing MRSA. Droplet infection is another type of transmission which causes pneumonia and in such a case; the patient is infectious through droplet infections to the surrounding patients and health care workers. (Bassim *et al.,* 2005)

Other factors contributing to transmission include skin-to-skin contact, crowded environment conditions, and poor hygiene. So, basically, the transmission of MRSA may be classified into two:

i. Endogenous spread (or transmission)

This occurs when a person with staphylococci spreads the bacteria from one part of their body to another

ii. Exogenous spread (or transmission): This occurs when organisms are transferred from person to person by direct contact with the skin or via contaminated environments or equipment. Skin scales may contaminate all surfaces if they become airborne, for example during activities such as bed making, or if the affected person is heavily colonized, or has a condition such as eczema which causes skin shedding which will result in widespread distribution of many skin organisms. Staphylococci that are shed into the environment fall on horizontal surfaces and may survive for long periods in dusts (Coella *et al.*, 1997).

3.7.9 Detection of resistance

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by *S. aureus.* Different methods used for the detection of MRSA are as follow:

A. **Phenotypic detection system**

a. Agar dilution test

A minimum of 4 to 5 colonies isolated from an overnight growth are transferred to sterile saline. The suspensions is adjusted to a 0.5 McFarland standard and spot inoculation on Mueller-Hinton agar plates supplemented with 2% NaCl and containing 0.25 microgram oxacillin/ml in serial doubling dilutions. The oxacillin Mueller-Hinton plates are incubated at 35^0C for 24 hours. MIC of ≥4 microgram/ml is considered resistant and MIC of ≤2 is considered susceptible. (Rao S, 2009)

b. Broth microdilution

This involves the use of Mueller-Hinton broth with 2% NaCl, an inoculum density of $5*10^5$ cfu/ml and incubation at 33-35^0C for 24 hours. (Rao S, 2009)

c. Breakpoint methods

Breakpoint method include both agar and broth methods and are essentially similar to dilution MIC methods but test only the breakpoint concentration (2 mg/L oxacillin, 4 mg/L methicillin). (Rao S, 2009)

d. E-test oxacillin MIC test

The inoculum is standardized to 0.5 McFarland turbidity standards and plated on Mueller-Hinton agar supplemented with 2% NaCl. E-test strips are placed and incubation at 35^0C for a full 24 hours. The E-test has an advantage over other MIC methods in that it is as easy to set up as a disc diffusion test. (Rao S, 2009)

e. Oxacillin screen agar

Mueller-Hinton agar (MHA) plates containing 4% NaCl and 6 microgram/ml of oxacillin are inoculated with 10 microlitre of 0.5 McFarland suspension of the isolate by streaking in one quadrant and incubated at 35^0C for 24 hours. Plates are observed carefully in transmitted light for any growth. Any growth after 24 hours is considered oxacillin resistant. Induction with oxacillin requires an extended period for full expression. Hence, oxacillin–containing media achieve sufficiently high sensitivities only after 48 hours of incubation.

f. Disc diffusion tests

A direct colony suspension of each *S. aureus* isolate is prepared to a 0.5 McFarland standard and plated on Mueller-Hinton agar containing 2-4% NaCl. An oxacillin (1 microgram) disk is placed on the surface and incubated at 35^0C for 24 hours. Oxacillin disk is more resistant to degradation in storage and more likely to detect heteroresistant strains. The zone of inhibition must be read with transmitted light and not reflected light. Zone diameter of ≤10mm is considered as resistant, ≥13 mm as susceptible whereas 11-12 mm is considered as intermediate. If intermediate results are obtained for *S. aureus*, testing for *mecA*, PBP 2a, cefoxitin disk test, oxacillin MIC test or oxacillin-salt agar screen test may be performed. Any discernable growth within the zone of

inhibition when seen using transmitted light is indicative of oxacillin resistance. It may be possible that some of the oxacillin disk test positive isolates are hyper beta-lactamase producers, thereby accounting for non-*mecA*-mediated methicillin resistance. In disk diffusion tests, hyper-producers of penicillinase may show small methicillin or oxacillin zones of inhibition, whereas most true methicillin-/oxacillin-resistant isolates give no zone. A 5 microgram methicillin disk can also be used but is not a popular choice. Zone diameter of ≤9 mm is considered resistant, ≥14 mm is considered susceptible whereas a diameter of 10-13 mm is considered intermediate. (Rao S, 2009)

The addition of up to 5% NaCl to the test medium for MRSA has been widely used. Addition of NaCl to Mueller-Hinton, Columbia and DST media improves the detection of methicillin resistance (Brown *et al*., 1986; Milne *et al*., 1993). However, the growth of few strains is adversely affected by 5% NaCl in all media, resulting in false susceptible reports, though this type of problem is particularly evident with coagulase negative staphylococci (CoNS). Moreover, concentration of 2% of NaCl is as effective as 5% NaCl in enhancing the expression of resistance but is markedly less inhibitory to those strains that will not tolerate 5% NaCl (Huang *et al*., 1993). Lee *et al*., in 1998, have also reported that MHA with 2% NaCl and manitol salt agar detect heterogeneous *S. aureus* more accurately than MHA.

With strains of MRSA that are particularly heterogeneous, increasing the inoculum size increases the chances of detecting the minority of cells comprising the resistance sub-population, provided the other conditions are favourable for expression of resistance.
Louie *et al*., in 2000, reported the oxacillin disk diffusion method to be the least reliable method for the detection of methicillin resistance. However, the study carried out by Rahbar M and Safadel N in 2006 on evaluation of cefoxitin disk diffusion test for routine detection of MRSA, revealed that sensitivity and specificity for both cefoxitin and oxacillin disk diffusion methods were 98% and 100% respectively; though they mentioned cefoxitin disk diffusion method more reliable for identification of intermediate resistant strains of *S. aureus*. One more difficulty that arises during the use

of oxacillin disk is the consideration of several factors including concentration of NaCl, temperature, inoculums, and test agent.

Cefoxitin disc diffusion test

Cefoxitin, which is a potent inducer of the *mecA* regularly system is being widely used as a surrogate marker for detection of *mecA* gene-mediated methicillin resistance. MRSA strains exhibiting inducible resistance to methicillin grow much more readily in the presence of cefoxitin than oxacillin, due to an enhanced induction of PBP 2a by cefoxitin. CLSI has recommended cefoxitin disc diffusion method for the detection of MRSA. A 0.5 Mc Farland standard suspension of the isolate is made and lawn culture done on MHA plate. A 30 microgram cefoxitin disc is placed and plates are incubated at 37^0C for 18 hours and zone diameters are measured. The zone diameter must be measured in reflected light. An inhibition zone diameter of ≤ 21 mm is reported as methicillin resistant and ≥ 22 mm is considered as methicillin susceptible. (Rao S, 2009)

Recent studies indicate that disk diffusion testing using cefoxitin disc is far superior to most of the phenotypic methods like oxacillin disc diffusion and oxacillin screen agar testing and is recommended by CLSI.

g. PBP 2a latex agglutination kit

The method involves extraction of PBP2a from suspensions of colonies and detection by latex agglutination. The kit contains latex particles sensitized with a monoclonal antibody against PBP2a. Visible agglutination indicates a positive result and the presence of PBP2a, the *mecA* gene product. The test is rapid (10 minutes for a single test) and very sensitive and specific with *S. aureus*, but may not be reliable for colonies grown on media containing NaCl. Isolates producing small amounts of PBP 2a may give weak agglutination reactions or agglutinate slowly. Reactions tend to be stronger if PBP 2a production is induced by growth in the presence of penicillin. Rare isolates may give negative reactions. (Rao S, 2009)

B. Molecular method

Detection of *mecA* gene by PCR is considered as the gold standard. DNA extraction is performed on the isolate and *mecA* gene is amplified using specific primers. The master mixture containing PCR buffer, dNTP mixture, primer, Taq DNA polymerase, and $MgCl_2$ and template DNA is subjected to hot start PCR. This is followed by 30 cycles of denaturation at 94^0C for 45 seconds, annealing at 50^0C for 45 seconds, and extension at 72^0C for 1 mintute and final extension step at 72^0C for 3 minutes. PCR products are then visualized on 2% agarose gel with ethidium bromide dye under UV transilluinator. (Rao S, 2009)

3.7.10 Preventive strategies

The main objectives of prevention are:

a) To prevent the access of *S. aureus* to susceptible sites from which it can invade the tissue
b) To lessen the chance that organism that do reach the tissues can cause sepsis, and
c) To reduce as far as possible the numbers of *S. aureus* in the immediate neighborhood of the patients.

Two main preventive strategies can be applied for the control of MRSA infections:

1) Prevent or minimize transmission.
2) To use appropriate antibiotic protocol aiming for reduction of emergence of this resistant strain.

3.7.11 Methods to control transmission

i. Standard precaution could control the spread of MRSA in most instances.
ii. Also contact precautions are required to control the spread of MRSA infections in surgical wounds.

i. Standard precautions include:

1. Hand washing: Wash hands after touching blood, body fluids, secretions, excretions,, and contaminated items, whether or not gloves are worn. Wash

hands immediately after gloves are removed, between patients' contacts, and when otherwise indicated to avoid transfer of microorganisms to other patients or environments. It may be necessary to wash hands between tasks and procedures on the same patient to prevent cross-contamination of different body sites. (Buckingham S *et al*., 2004)

2. Gloving: Wear gloves (clean non sterile gloves are adequate).
3. Masking and eye protection: Wear a mask and eye protection or a face shield to protect mucous membranes of the eyes, nose, and mouth during procedures and patient-care activities that are likely to generate splashes or sprays of blood, body fluids, secretions, and excretions. (Collignon P *et al*., 1998)
4. Gowning: Wear a gown (a clean non sterile gown is adequate).
5. Appropriate device handling
6. Appropriate handling of laundry

If MRSA is judged by the hospital's infection control program to be special clinical or epidemiological significance, then contact precautions should be considered.

ii. Contact Isolation Precautions consists of:

1) Placing a patient with MRSA in a private, isolated room. When such a room is not available, the patients may be placed in a room with a patient (s) who has active infection with MRSA, but with no other infection.
2) Wearing gloves (clean non-sterile gloves are adequate) when entering the room. During the course of providing care for a patient, change your gloves after having contact with infective material that may contain high concentrations of microorganisms (e.g., fecal material and wound drainage). Remove gloves before leaving the patient's room and wash hands immediately with an antimicrobial agent.
3) Wearing a gown when entering the room if you anticipate that your clothing will have substantial contact with the patient, environmental surfaces, or items in the patient's room, or if the patient is incontinent, or has diarrhoea, an ileostomy, a colostomy, or wound drainage not contained by a dressing.
4) Limiting the movement and transport of the patient from the room to essential purposes only.

5) Ensuring that patient-care items, bedside equipment, and frequently touched surfaces receive daily cleaning.

6) When possible, dedicating the use of non-critical patient-care equipment and items such as stethoscope, sphygmomanometer, bedside commode, or electronic rectal thermometer to a single patient (or cohort of patients infected or colonized with MRSA) to avoid sharing between patients. (Goetz A *et al*., 1999)

3.7.12 Preventive measures for MRSA carrier

1. The staff member (health care workers) may have to temporary cease work with surgical patients until cleared of the organisms.
2. Use an antiseptic as chlorhexidine on the affected skin.
3. Mupirocin topical application for nasal carriage.
4. Ciprofloxacin and rifampin for chronic carriers.

3.7.13 Treatment

Whether or not to give specific treatment for a particular staphylococcal infection and the choice of anti-microbial therapy and other measures such as surgical treatment, are matters for clinical judgment. Antibiotics alone will not cure closed septic lesions, such as abscesses, without surgical drainage; indeed, for small superficial lesions adequate surgical drainage alone may be sufficient (Collier *et al*., 1998). If specimen results indicate MRSA it does not automatically mean antibiotics are required.

Treating MRSA infection depends on:

i. Clinical signs;
ii. Local policy;
iii. The individual's risk to others-for example, where they are being nursed;
iv. The individual's own risk to others-for example, the presence of invasive devices, the need for surgery and whether they are immunocompromised; and
v. Whether the individual is colonized or infected.

Individuals with a clinical infection will usually require a course of systemic antibiotics. The choice of antibiotics depends upon the site of infection and on the particular strain of MRSA. Some antibiotics may only be given intravenously, and may be toxic and expensive. (Coella *et al*., 1997)

There should be clear local guidance for the application of topical treatment for colonized patients. Applying an aseptic lotion, containing triclosan or chlorhexidine, may eradicate skin colonization. To further reduce colonization, some policies recommend applying hexachlorophene talcum powder to the axillae and groin, but this should not be used on broken areas of skin. (Coella *et al*., 1997)

Applying mupirocin ointment to the anterior nares three times daily for five to seven days may eradicate nasal colonization. Prolonged use of mupirocin can cause resistance to develop, which may limit its subsequent use to control outbreaks. Use of mupirocin should be restricted to no more than two five-to-seven day courses. (Coella *et al*., 1997)

Applying topical antiseptics-such as povidone iodine, silver sulphadiazine or mupirocin –may help to eliminate wound colonization. It is important to check that the agent used is appropriate for the wound. One should also avoid prolonged application of these topical agents. However, the value of applying topical antiseptics to chronic wounds-for example, pressure sores and leg ulcers- continues to be the subject of heated debate. Use of antibiotic creams for colonized wounds should not be due to resistance. Local wound care policies should be followed. (Coella *et al*., 1997)

The presence of an invasive device –such as a PEG tube, trachaeostomy or urinary catheter-often extends the period of colonization. Topical agents may not be appropriate here as there is a risk of degeneration of jejunostomy tubes and continuous ambulatory peritoneal dialysis (CAPD). Use an aseptic technique when handling the device and remove it as soon as clinically possible. (Coella *et al*., 1997)

3.7.14 Ethical Considerations

Patients and staffs colonized or infected with MRSA must be treated sensitively and fairly. Hospitals, nursing and residential homes and other care settings should have procedures in place for managing infections in general, not just MRSA. Patients should not be refused treatment, investigations, therapy or residential care because of MRSA. Nurses should not refuse to care for a person with MRSA, or indeed any other kind of infectious disease. They should have the knowledge, policy, procedures and resources to care for them safely. Likewise insurance policies that cover care homes for infectious disease should not specifically exclude MRSA. In trying to control the spread of MRSA, there may be potential breaches of confidentiality. Notices and information stickers should be discreet and the patient should be involved in any decision to pass on information about diagnosis (Coella *et al*., 1997).

3.8 Vancomycin Resistant *Staphylococcus aureus*

Most *Staphylococcus aureus* strains are sensitive to antibiotic vancomycin, a glycopeptide. This is often the drugs of choice for serious infections caused by methicilin Resistant *Staphylococcus aureus* (MRSA). Concern arose for the anticipated emergence of *S. aureus* with diminished vancomycin susceptibility when vancomycin-resistant enterococci (VRE) were initially described in the late 1980s. Methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) strains that have acquired the vanA operon from glycopeptide-resistant enterococci are designated vancomycin-resistant S. aureus (VRSA). The first two VRSA isolates were recovered in the United States in 2002. Vancomycin acts by binding to the C-terminal acyl-D-alanyl-D-alanine (acyl-D-Ala-D-Ala) of pentapeptide peptidoglycan precursors and inhibits transglycosylation and transpeptidation reactions, thus preventing cell wall formation and with the acyl-D-Ala-D-Ala residues being incorporated into peptidoglycan precursors as dipeptides synthesized by the host D-Ala:D-Ala ligase. VanA-type resistance is characterized by high-level inducible resistance to vancomycin and teicoplanin due to synthesis of peptidoglycan pentadepsipeptide precursors ending in D-Ala-D-lactate (D-Ala-D-Lac). This alteration is responsible for the diminished binding

affinity of glycopeptides for their target. However, the first reported case of diminished vancomycin susceptibility in a clinical isolate of *S. aureus* in 1997 was mediated not via acquisition of vanA by a strain of methicillin-resistant *S. aureus* (MRSA) strain, but by an unusually thickened cell wall containing dipeptides capable of binding vancomycin, thereby reducing availability of the drug for intracellular target molecules. This was the first observation of vancomycin-intermediate *S. aureus* (VISA). Since the first two VISA isolates in the United States were also resistant to teicoplanin, the term glycopeptide-intermediate S. aureus (GISA) was used to indicate this broader resistance profile.

The concentration of vancomycin required to inhibit these strains (minimal inhibitory concentration - MIC) is 0.5-2 micrograms/mL. *S. aureus* strains for which vancomycin MICs are 4-8 micrograms/mL are classified as Vancomycin Intermediate *Staphylococcus aureus* (VISA), and strains for which vancomycin MICs is equal or exceeds 16 micrograms/mL are classified as Vancomycin Resistant *Staphylococcus aureus* (VRSA) (CLSI, 2006).

A pathogenic vancomycin-resistant *Staphylococcus aureus* (VRSA) isolate (MIC $\geq$64 $\mu g\,ml^{-1}$) was obtained from a Kolkata hospital in June 2005. Species identification was confirmed by Gram staining, standard biochemical tests and PCR amplification of the *nuc* gene, which encodes the thermostable nuclease that is highly specific for *S. aureus* (Saha *et al*., 2008).

The emergence of vancomycin-intermediate Staphylococcus aureus (VISA) and heterogeneous vancomycin-intermediate Staphylococcus aureus (hVISA) over the past decade has provided a challenge to diagnostic microbiologists to detect these strains. These strains have been detected in many cases and are associated with glycopeptide treatment failure. It is now becoming clear that sequential point mutations in key global regulatory genes contribute to the hVISA and VISA phenotypes, which are associated predominately with cell wall thickening and restricted vancomycin access to its site of

activity in the division septum; however, the phenotypic features of these strains can vary because the mutations leading to resistance can vary (Howden *et al*., 2010).

Although the cases of vancomycin resistant *S. aureus* (VRSA) and vancomycin intermediate *S. aureus* (VISA) are emerging, the frequency of such emerging strains is very low. In Nepal, no such strains of S. aureus have been detected till date. One of the key reasons for this is the less frequent use of this drug.

CHAPTER IV

4. MATERIALS AND METHODS

4.1 Materials

A complete list of materials, equipments, media, chemicals, reagents and antibiotics used in this study are listed in Appendices I, II, III and IV.

4.2 Methods

Altogether 744 samples were collected and analyzed by conventional culture method and identified by various biochemical tests. The antibiotic sensitivity test was done by using Kirby-Bauer disk diffusion method and the prevalence of MRSA was found bu using oxacillin disk.

4.2.1 Sample collection

Most of the clinical samples such as pus/swab from wound, throat, eye, ear as well as shunt tips were collected aseptically by experienced medical officers, nurses or laboratory technicians. CSF and other body fluids like ascitic fluids were collected by skilled hospital personnel and medical officers. Semen was collected by the patients themselves as per instruction from the laboratory technicians to avoid the contamination.

All the samples were labeled appropriately with patient's identification number. The samples were processed immediately as soon as possible. In case of delay, they were stored at the refrigerated temperature.

4.2.2 Sample processing

All the samples were processed using standard protocol. All the samples except body fluids were inoculated directly onto Nutrient Agar, Blood Agar, and Mac Conkey Agar. Suspected *S. aureus* colonies are then inoculated onto Mannitol Salt Agar and incubated. For body fluids like ascitic fluid, it was inoculated first into the Brain Heart Infusion broth for enrichment.

4.2.3 Isolation of *S. aureus*

The plates were incubated at 37^0C for 24 hours. *S. aureus* colonies were identified on the basis of colony characteristics on, Nutrient Agar, Blood Agar, Mac Conkey Agar, and Mannitol Salt Agar; gram's reaction and biochemical tests.

4.2.3.1 Subculture on NA

Mannitol fermenting colonies from MSA were sub-cultured on NA and incubated for 24 hours at 37^0C. Golden yellow colony having round, convex, opaque, and smooth-glistering surface with colony diameter about 2-3 mm were indicative of Staphylococci.

4.2.3.2 Gram's staining

Procedure

i. A sterile inoculating loop was taken and with the help of that loop, pure isolated colony from NA plate was touched slightly and it was then transferred to a clean and greese free glass slide containing a drop of distilled water.
ii. A uniform smear was made on the glass slide, air dried and then heat fixed.
iii. The smear was flooded with crystal violet solution for 1 minute and rinsed with distilled water.
iv. It was then loaded with Gram's Iodine solution for 1 minute and again rinsed with distilled water.
v. The smear was treated with acetone alcohol solution for 10-15 seconds for decolourization action and rinsed with distilled water.
vi. Finally, the smear was treated with counter stain safranine for 1 minute and ringed with distilled water.
vii. The back side of the slide was blotted with blotting paper, air dried and then examined firs under 40 X objective and then under oil immersion objective of the microscope.

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

4.2.4 Further identification test

CATALASE TEST

Procedure

i. A clean and greese free glass slide was taken. On the slide, a drop of freshly prepared 3% H_2O_2 solution was put.
ii. The pure colony from NA plate was taken with the help of a sterile glass rod and it was then transferred on the H_2O_2 solution.

The rapid evolution of gas bubbles on the slide was an indicative of a catalase positive test.

COAGULASE TEST

i. Slide coagulase (clumping factor/ bound coagulase) test

Procedure

i. A clean and greese free slide was taken and a drop of physiological saline was dropped on it.
ii. A colony of the test organism was transferred on it with help of a glass rod and a thick suspension was made.
iii. To the suspension, a drop of plasma was added and mixed gently.

Clumping observed within 5-10 seconds was an indicative of slide coagulase (clumping factor) positive test. However, both the slide coagulase positive as well as negative organisms were subjected to tube coagulase test for further confirmation.

ii. Tube coagulase (free coagulase) test

Procedure

i. Clean tests tubes were taken and labelled as T (Test), P (Positive) and N (Negative).
ii. 0.2 ml of fresh plasma was pipetted in each test tube.
iii. 0.8 ml of test broth culture was added to the tube labelled as ‘T’, 0.8 ml of standard culture of S. aureus was added to the one tube labelled as ‘P’ and 0.8 ml of sterile broth was added to the another tube labelled as ‘N’.

iv. After mixing gently, all the tubes were incubated at 37^0C and the clot formation was examined at an interval of one hour for up to 4 hours, by tilting the tubes.

v. The negative tubes were kept at room temperature overnight and were reexamined.

Stiff gel formation or clot floating in the tube was indicative of positive tube test.

VOGES-PROSKAUER (VP) TEST

Procedure

i. A tube containing 2 ml of sterile MRVP broth was taken.

ii. In the test tube, a loopful of test organism was inoculated and incubated at 37^0C for 24 hours.

iii. After incubation, few drops of Barrit's reagent (consisting 3 volumes of 5% α-Naphthol and 1 volume of 40% KOH solution) was added to the tube.

iv. The tube was shaken vigorously for aeration and observed for the pink-red colour changed during the 30-minute period.

Pink-red colour observed in the tube within 30 minutes was indicative of positive test.

OXIDATION-FERMENTATION (O/F) TEST

Procedure

i. Two tubes each containing sterile O/F medium were taken and the test organisms were inoculated in each of the tube with the help of a sterile inoculating wire.

ii. In one of the two tubes, liquid paraffin was added to form a layer of about 1 cm in depth.

iii. Both the tubes were then incubated at 37^0C for 24 hours.

iv. Both the tubes were examined for the colour change.

Yellow colour observed in both the tubes were indicative of fermentative reaction.

4.2.5 Antibiotic susceptibility test

All identified *S. aureus* isolates from different clinical isolates were subjected to *in-vitro* antimicrobial susceptibility test by Kirby-Bauer disc diffusion method as

recommended by CLSI. The antibiotic used in this study were amoxycillin (30 mcg), ciprofloxacin (5 mcg), chloramphenicol (30 mcg), cotrimoxazole (25 mcg), cloxacillin (5 mcg), erythromycin (15 mcg), oxacillin (1 mcg), tetracylin (30 mcg), vancomycin (30 mcg).

Procedure

i. Sterile petri plates containing Mueller-Hinton Agar (MHA) were taken.

ii. 4-5 pure isolated colonies of the test organisms were taken with the help of sterile inoculating loop and transferred into respective tubes containing sterile nutrient broth.

iii. The tubes were then incubated at 37^0C for up to 4 hours to obtain the turbidity equivalent to the density of Mc Farlands' Nephelometer Standard No. 0.5 (1.5 x 10^8 CFU/ml of cell density).

iv. In case the turbidity didn't match the standard, it was adjusted by adding more pure colonies of bacterial isolates or by adding more sterile nutrient broth solution.

v. 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.

vi. Swabbing was done evenly over the surface of the MHA plate by rotating the plates.

vii. 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.

viii. Appropriate antibiotic discs were taken out of the respective vials with the help of sterile forceps and placed carefully on the agar surface at an equal distant.

ix. The discs were pressed gently with the forceps so as to make complete contact with the agar surface.

x. The plates were then incubated at 35^0C for 24 hours.

xi. After incubation, the diameter of the zone of inhibition of each antibiotic disc was measured.

The standard zone size at which the organism is considered resistant, intermediate or susceptible is given in the zone-size interpretative chart (Appendix-IV).

4.2.6 Purity plate

To check the purity of inoculum used biochemical tests, the same inoculum was simultaneously inoculated on one half of a NA plate before and after the tests. Pure culture of same organism obtained on the media in both halves of the plate confirmed the aseptic measures taken during each test.

4.2.7 Quality control

Quality control for all the tests is the utmost important factor for the data to be reliable. Hence, the quality control was maintained throughout this study.

To maintain quality control of chemical reagents, antibiotics and media, they were prepared and stored as per instructions provided by the respective companies. Similarly, antibiotics were also stored as per the instruction provided by the respective companies. Furthermore, to assure the quality control of the already prepared and stored chemical reagents, each batch of reagent prepared in the laboratory was tested with positive and negative control organisms. With each batch of biochemical test performed, a positive and a negative control were used.

For antibiotic susceptibility test by Kirby-Bauer disc diffusion, standard culture of *S. aureus* ATCC 25923 was used as a reference strain.

4.2.8 Data analysis

All the raw data collected in the microbiology laboratory were statistically analyzed using computer based software program SPSS version 16.

CHAPTER V

5. RESULTS

Out of 744 clinical samples obtained, 453 (60.8%) were from male patients and 291 (39.2%) were from female patients. Likewise, 557 (74.8%) were from outpatients and 187 (25.2%) were from admitted patients. (Fig 3 and 4)

5.1 Isolation of *S. aureus* from the samples

In this study, only the growth of *S. aureus* was taken into consideration. Out of 744 different clinical specimens, *S. aureus* was isolated from 125 (16.8%) samples. (Fig 2)

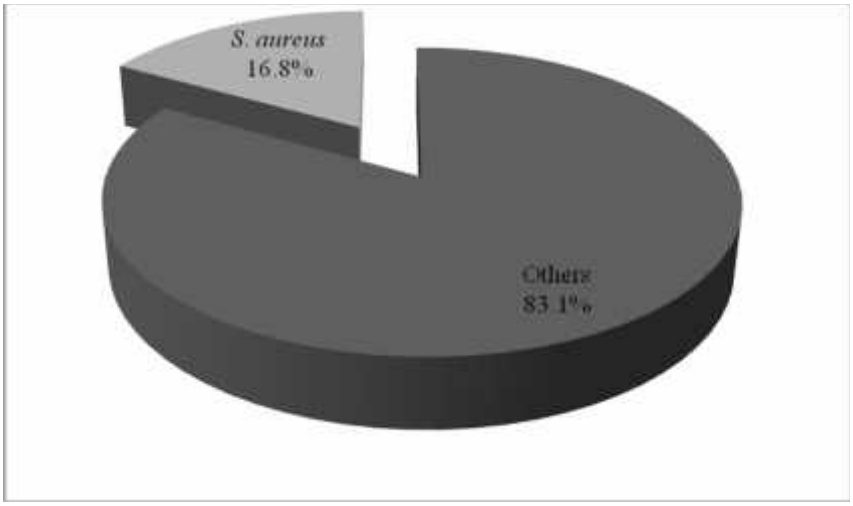

Fig 2: Total isolates of *S. aureus* from pus sample

In the above figure, others (83.1%) include bacterial isolates other than *S. aureus* as well as culture negative specimens.

5.2 Comparison of *S. aureus* isolates in outpatients and admitted patients

All together 557 samples of outpatients and 187 samples of admitted patients were analyzed by standard method. In total, 125 strains of *S. aureus* were isolated, out of

which 64.8% (n=81) were from outpatients whereas 35.2% (n=44) were from admitted patients. (Fig 3)

5.3 Number of *S. aureus* isolated from different clinical specimens

Majority of the specimens comprised of pus/wound swabs (n=549, 73.7%) showing *S. aureus* isolates in 108 (19.6%) specimens, followed by body fluids (n=57, 7.6%) showing *S. aureus* isolates in 3 (5.2%), whereas the least number (n=8, 1.07%) of samples were recorded of showing single strain of *S. aureus*. (Table 2)

Table 2: Number and percentage of *S. aureus* isolates in different clinical samples

Clinical sample	Total no. of sample	No. of *S. aureus*
Pus/wound swab	549	108 (19.6%)
Nasal swab	19	4 (21.0%)
Ear swab	8	1 (12.5%)
Tracheal swab	14	1 (7.1%)
Throat swab	20	1 (5.0%)
Body fluids	57	3 (5.2%)
Devices	49	4 (8.1%)
Others	28	3 (10.7%)
Total	744	125 (16.8%)

5.4 *S. aureus* isolates from different age groups of male and female patients

Out of 125 *S. aureus* strains isolated, 53.6% (n=67) were from male patients while 46.4% (n=58) were from female patients (Fig 4).

In male, maximum number of sample was observed in age group 21-30 years, and also maximum number of *S. aureus* strains (n=24) was isolated from the same age group. Likewise, in female, highest number of sample (n=81) was analyzed in age group 31-40

years, showing 10 strains of *S. aureus*, whereas the highest number of *S. aureus* strain (n=15) was observed in age group 21-30 (Table 3).

Table 3: Age-wise and sex-wise distribution of total clinical samples and *S. aureus* isolates

Age (years)	Male		Female	
	Total no. of sample	**No. of *S. aureus***	**Total no. of samples**	**No. of *S. aureus***
<10	7	3 (42.8%)	5	2 (40%)
11-20	68	11 (16.1%)	37	14 (37.8%)
21-30	138	24 (17.3%)	68	15 (22.0%)
31-40	62	7 (11.2%)	81	10 (12.3%)
41-50	85	5 (5.8%)	42	5 (11.9%)
51-60	46	4 (8.6%)	45	5 (11.1%)
>60	47	13 (27.6%)	13	7 (53.8%)
Total	453	67 (14.7%)	291	58 (19.9%)

5.5 Antibiogram of *Staphylococcus aureus*

All the total 125 strains of *S. aureus* isolated were tested with specific antibiotics by using Kirby Bauer disc diffusion method. Antibiotic resistance pattern of those 125 *S. aureus* strains showed that the highest number of isolate was resistant to Co-trimoxazole (70, 56%), followed by amoxycillin (63, 50.4%) and erythromycin (57, 45.6%). Likewise, highest number of strain was susceptible to chloramphenicol (97, 77.6%), followed by oxacillin (89, 71.2%) and ciprofloxacin (84, 67.2%). (Table 4)

Table 4: Antibiotic Sensitivity Pattern of *S. aureus* isolates

Antibiotics used	**Total no. of *S. aureus***	**Sensitive**	**Resistance**
Amoxycillin	125	62 (49.6%)	63 (50.4%)
Chloramphenicol (30 mcg)	125	97 (77.6%)	28 (22.4%)
Ciprofloxacin (5 mcg)	125	84 (67.2%)	41(32.8%)
Cloxacillin (5 mcg)	125	80 (64%)	45(36%)
Co-trimoxazole (25 mcg)	125	55 (44%)	70 (56%)
Erythromycin (15 mcg)	125	68 (54.4%)	57(45.6%)
Oxacillin (1 mcg)*	125	89 (71.2%)	36 (28.8%)
Tetracyclin (30 mcg)	125	78 (62.4%)	47(37.6%)
Vancomycin (30 mcg)	125	125 (100%)	0 (0%)

In the same way, antibiogram of total *S. aureus* isolates showed 36 (28.8%) strains to be oxacillin resistant. Vancomycin was found to be 100% effective.

5.6 Distribution of MRSA in different age groups

Highest percentage (40%, n=8)) of MRSA was isolated from the age group of above 61 years whereas the highest number (n=12, 30.7%) of MRSA isolates belonged to age group of 21-30 years (Table 5).

Table 5: Age-wise distribution of MRSA

Age group (years)	Total no. of *S. aureus* isolates	Total no. of MRSA
<10	5	1 (20.0%)
11-20	25	8 (32.0%)
21-30	39	12 (30.7%)
31-40	17	3 (17.6%)
41-50	10	2 (20.0%)
51-60	9	2 (22.2%)
>60	20	8 (40.0%)
Total	125	36 (28.8%)

5.7 Distribution of MRSA in outpatients and admitted patients of different age groups

Out of total 36 MRSA strains isolated, 25 strains were from outpatients whereas only 11 strains of MRSA were isolated from admitted patients.

Among outpatients, 9 strains (32.1%) were isolated from age group 21-30 years, which was the highest in number but the highest percentage (40%, n=6) of MRSA was isolated from the age group of 11-20. Likewise, maximum number of MRSA strain isolated from admitted patients was isolated to be 3 in each age group 21-30 and above 60 years; however, the highest percentage of MRSA was isolated from the age group of above 60 years. (Table 6)

Table 6: Distribution of *S. aureus* and MRSA in outpatients and admitted patients of different age-groups

Age (years)	Outpatients		Admitted patients	
	Total no. of *S. aureus*	**No. of MRSA**	**Total no. of *S. aureus***	**No. of MRSA**
<10	0	0	5	1 (20.0%)
11-20	15	6 (40.0%)	10	2 (20.0%)
21-30	28	9 (32.1%)	11	3 (27.2%)
31-40	13	2 (15.3%)	4	1 (25.0%)
41-50	9	2 (22.2%)	1	0 (0%)
51-60	4	1 (25.0%)	5	1 (20.0%)
>60	12	5 (41.6%)	8	3 (37.5%)
Total	81	25 (30.8%)	44	11 (25.0%)

CHAPETR VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Staphylococcus aureus is the most virulent species of Staphylococci and is an opportunistic pathogen. It remains as the normal flora on different parts of the body of human beings throughout the life but may cause a variety of diseases ranging from benign local skin infections such as folliculitis, pustules, boils, carbuncules, impetigos

and infections of wound to the life threatening diseases like pneumonia, meningitis, osteomyelitis, endocarditis and bacteraemia in immunocompromised patients. It may cause several toxin mediated infections and diseases such as food poisoning, skin scalded syndrome and toxic shock syndrome. *S. aureus* is one of the major pathogens in human in both communities and hospitals.

In most of the study, the frequency of infection caused by *S. aureus* reaches the peak. Kumari *et al* (2008), in their report, have mentioned that out of total 98 gram positive isolates, *S. aureus* occupied 83.67%. Similar results were obtained in a study by Karkee *et al* (2008) and Khan *et al* (2007). The high frequency associated with the infection caused by *S. aureus* might be due to its ubiquitous nature and large number of virulence factors associated with it.

Methicillin became the drug of choice as many of the strains of *S. aureus* became resistant to the drug penicillin in early days because of the penicillinase enzyme they produce. However, within very narrow range of time, methicillin-resistant strains developed. Treatment of infection caused by these strains became somewhat problematic as these strains were resistant to chemically other unrelated groups of antibiotics as well. Since the emergence of MRSA strains in 1961, the trends seem to be fluctuating. In Nepal, it ranges from 11.7 to 54.9% (Pokharel *et al*., 1993; Lamichhane *et al*., 1999; Rajbhandari *et al*., 2002; Thapa *et al*., 2004; Sapkota *et al*., 2006; Kumari *et al*., 2008). Indiscriminate use of multiple antibiotics especially in developing countries, prolonged hospitalization, intravenous drug abuse, and transfer of MRSA through nose are supposed to be few implicated risk factors associated with high frequency of MRSA infections. Although, previously it was found only as hospital and health-care associated infection, soon it was discovered to be community acquired as well.

The aim of this study was to discover the present antibiogram pattern of *S. aureus* as well as prevalence of MRSA infection and different factors associated with it. In this study, altogether 744 different clinical specimens were studied which comprised 74.8% from outpatients and 25.2% from admitted patients. Similarly, the samples obtained

were 60.8% from male and 39.2% were from female patients. *S. aureus* was isolated from 16.8% of the total samples.

Out of 744 clinical samples collected, the highest number of sample was from pus/wound swabs (n=549) and also the highest number of *S. aureus* isolates was from the same category (n=108, 86.4%).

Similar results were obtained by Khan *et al* (2007). According to them, out of 550 clinical specimens from hospitalized patients, more than 83% of samples were from wound swabs and 53.3% of *S. aureus* isolates were from wound swabs. The result was also in consistent with Mehta *et al* (1996) where isolation rate of *S. aureus* was found to be 73%.

In a study by Kumari *et al* (2008) in a tertiary-care hospital in Eastern Nepal, the findings were somewhat similar. They also found the highest number of *S. aureus* isolates (n=480, 64%) from pus and wound swab samples. Likewise, our findings also correlate with the findings of Sapkota *et al* (2006) who reported 53.4% of *S. aureus* isolates were from pus swab from wound.

Higher number of wound specimens might be due to the higher number of surgical wound infections and its substandard hygienic condition. Furthermore, wound infection is usually caused by multi-drug resistant bacteria. So, to limit morbidity of the surgical patients, clinicians send wound specimens for culture and sensitivity to select proper antibiotic with priority. Similarly, higher incidence of *S. aureus* infection could be explained by the fact that *S. aureus* remains as a skin flora, and the bacteria can easily be transmitted to the site of infection, particularly when the wound is present in the exposed surface of the patient.

In context to *S. aureus* isolates, in overall, the highest positivity of 41.6% (5/12) was observed in the age group of below 10 years. The results, however, were inconsistent with the findings of Rajbhandari *et al* (2002) and Sapkota *et al* (2006). In both the studies, highest number of isolation was from the age group 20-29 years.

Highest incidence of *S. aureus* infection in such lower age group patients can be correlated with the less developed immunity of that group of patients.

Likewise, out of total 125 *S. aureus* isolates, 64.8% were from the outpatients patients. The result, hence, showed a higher incidence of *S. aureus* infection in the outpatients patients as compared to that observed in admitted patients.

Similar findings were observed in their report by Rajbhandari *et al* (2002) and Thapa *et al* (2004). Sapkota *et al* (2006) had also reported the same.

Again, in this study, 2% NaCl MHA was used for isolation of MRSA strains. In the same way, oxacillin disks of Oxoid Company were used for the detection of MRSA. Initially, oxacillin disks of HiMedia Company were used for that purpose but all the strains of *S. aureus* isolated were found to be resistant. Those strains were also tested using methicillin disks of the same company with little higher strength (30 mcg) and the results were again found the same. So, ultimately, the study was carried using oxacillin disks of Oxoid Company.

Antibiogram of all *S. aureus* strains isolated showed chloramphenicol (77.6%) to be the most effective drug, followed by oxacillin (71.2%) and ciprofloxacin (67.2%). Likewise, the most resistance drug was co-trimoxazole (56%), followed by amoxycillin (50.4%) and erythromycin (45.6%). The results obtained in our study are also somewhat in accordance to the findings of Karkee *et al* (2008), who reported the percentage resistivity of the drugs co-trimoxazole and amoxycilin to be 51.04 and 46.85 respectively.

Our findings synchronize with that of Abussaud *et al* (1996), who found that the most effective antibiotic against *S. aureus* was chloramphenicol (78%).

In a study, Thapa *et al* (2004) found, the most effective drug against *S. aureus* being ciprofloxacin (78.46%), followed by tetracycline (73.84%) and methicillin (70.77%).

The results obtained, however, are inconsistent with the findings of Kumari *et al* (2008), who reported, for *S. aureus*, the most sensitive antibiotic was ofloxacin (69.51%) followed by ciprofloxacin (63.41%), cephalexin (60.97%), cloxacillin (60.97%), chloramphenicol (54.87%), erythromycin (52.43%) and cotrimoxazole (43.90%).

In another study by Onche and Adedeji (1994), cephalosporins were found to be most potent against *S. aureus* as 10 of 17 cultures (58.8%) responded favourably to cephalexin. The traditionally potent cloxacillin was found completely resisted by it.

Antibiogram of total 125 *S. aureus* strains in our study showed 28.8% (n=36) were methicillin resistant by the use of oxacillin disk test. Out of 36 strains of MRSA, majority of them (33, 91.6%) were isolated from pus/wound swabs.

The result obtained is in agreement with the findings of Kumari *et al* (2008), who reported 26.14% MRSA isolates out of 750 *Staphylococcus aureus* strains isolated from various clinical samples during a period of one year in a tertiary-care hospital in Eastern Nepal, the maximum isolation of MRSA being isolated from pus and wound swabs. Likewise, our finding is also in agreement with the reports by Uday *et al* (1997) and Mehta *et al* (1998) from India, who found 20% and 32.8% MRSA respectively.

Similarly, our findings also synchronize with the findings of Sapkota *et al* (2006), who reported 31.1% of MRSA out of 264 *S. aureus* isolates; 25% of MRSA out of 52 *S. aureus* isolated reported by Lamichhane *et al* (1999) and 29.23% of MRSA out of 65 *S. aureus* isolates reported by Thapa *et al* (2004). On the contrary, report shown by Rajbhandari *et al* (2002) has alarmingly high incidence of MRSA infection (54.9%).

Among the total MRSA isolated, the highest percentage (8/20, 40%) was from the age group of above 60 years. The higher vulnerability of the higher age group patients towards the MRSA infection can be correlated with the decreased immune system in that group of patients.

Vancomycin was found to be 100% sensitive. The finding is in accordance with those of the findings by Lamichhane *et al* (1999), Rajbhandari *et al* (2002), Thapa *et al* (2004) and Sapkota *et al* (2006). Similarly, the result also synchronizes with the findings of Khan *et al* (2007) and Kumari *et al* (2008).

Since vancomycin is used as the drug of choice or reserved drug for treating multidrug-resistant MRSA infections, it carries really good information to the healthcare personnel. The development of least resistivity towards this drug can be correlated with the uncommon prescription of the drug, which is almost due to the higher price of the antibiotic and its unavailability in the market.

6.2 Conclusion

In this study, the prevalence of staphylococcal infection as well as infection due to MRSA was found in alarming situation in the hospital patients. The high prevalence of these infections in the hospital patients shows the need of regular surveillance. Vancomycin is still found to be the most efficient drug against MRSA infection whereas chloramphenicol was found the most potent drug in overall staphylococcal infection. The study also shows the importance of evaluation of antibiotic disks prior to the study.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

I. Altogether 744 pus samples were collected from both outpatients and admitted patients. Out of 744 samples. Out of 744 samples collected, 187 (25.1%) were from admitted patients and 557 (74.8%) were from outpatients.

II. Out of 744 samples, *S. aureus* was isolated from 125 (16.8%) samples, which comprised 64.8% (n=81) from outpatients and 35.2% (n=44) from admitted patients.

III. Similarly, out of 125 *S. aureus* strains, 53.6% (n=67) were from male patients and 46.4% (n=58) were from female patients.

IV. Among various clinical specimens included in pus category, the highest number of samples received was of pus/wound swabs which comprised 73.7% (n=549) of total. Out of 549 pus/wound swab samples, *S. aureus* strains were isolated from 108 samples (19.6%).

V. In male, maximum number of sample 30.4% (n=138) was observed in age group 21-30 years and also maximum number of *S. aureus* isolates (n=24) were isolated from the same age group.

VI. Likewise, in female, highest number of sample 27.8% (n=81) was analyzed in age group 31-40 years, showing 12.3% (n=10) strains of *S. aureus*, whereas the highest number of *S. aureus* strain (n=15) was observed in age group 21-30 where total sample size was 68.

VII. Regarding antibiotic sensitivity pattern, the most effective drug for *S. aureus*, excluding vancomycin, was chloramphenicol (77.6%). Vancomycin was found to be 100% effective.

VIII. In the same way, the highest number of isolates was found to be resistant to co-trimoxazole (56%), followed by amoxycillin (50.4) and erythromycin (45.6%).

IX. Likewise, antibiogram of total *S. aureus* strains showed 28.8% (n=36) were methicillin resistant by the use of oxacillin disk test.

X. Out of 36 MRSA strains isolated, the maximum number (12/39) was isolated from the age group of 21-30 years whereas the rate of isolation was maximum (8/20) in the age group of above 60 years.

XI. Similarly, out of total 25 MRSA strains isolated from outpatients, majority (n=9) were found in age group 21-30, whereas, out of total 11 MRSA strains isolated from admitted patients, majority (n=3) were from age group 21-30 years.

7.2 Recommendations

I. The research should be extended to molecular level by using different molecular techniques like RFLP in order to reveal the epidemiology of the MRSA strains isolated.

II. MIC value for methicillin antibiotics should be re-determined as previous value didn't seem to be effective against the *S. aureus* strains isolated.

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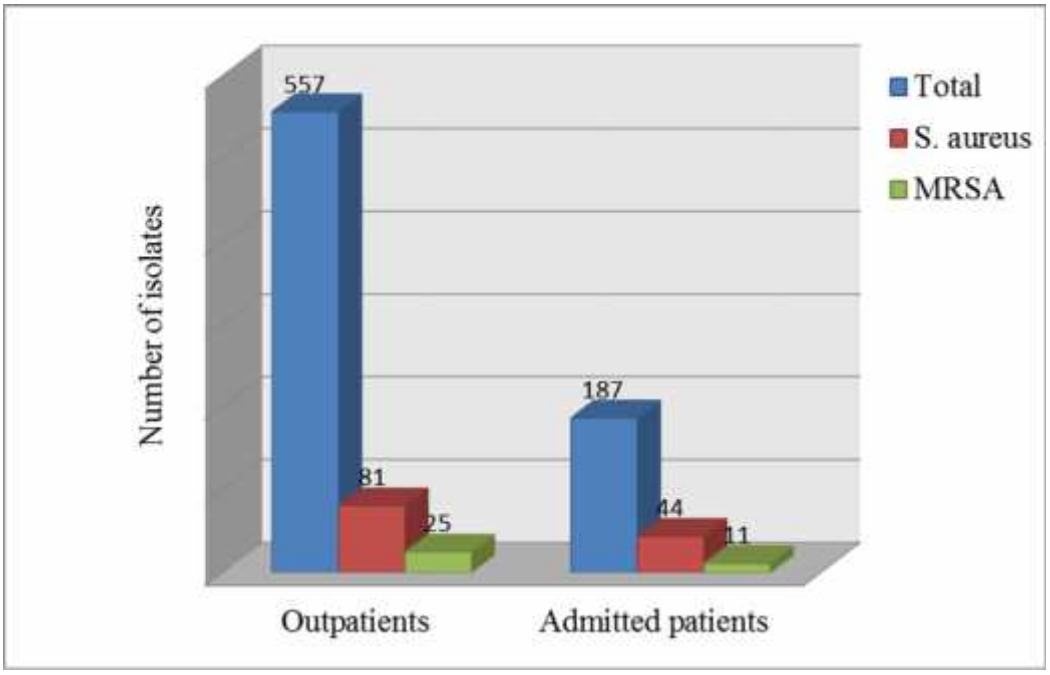

Fig 3: Distribution of *S. aureus* and MRSA in outpatients and admitted patients

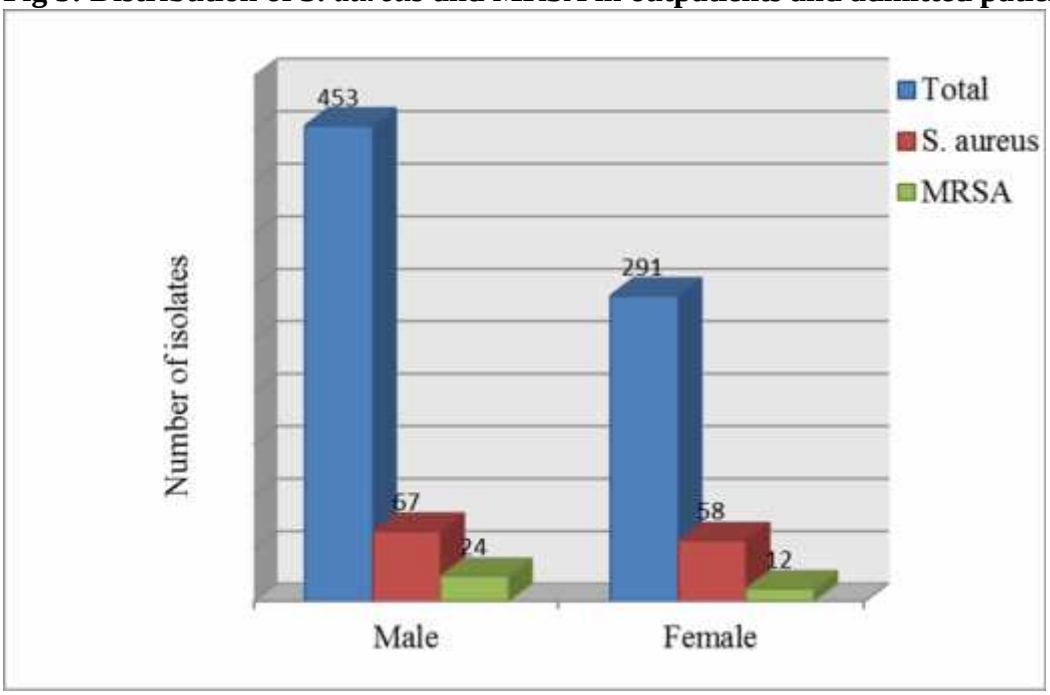

Fig 4: Distribution of *S. aureus* and MRSA in Male and Female patients

APPENDICES

Appendix I: Materials

List of Materials

Glasswares

- Beaker
- Conical flask
- Culture bottle
- Glass rod
- Glass slide
- Measuring cylinder
- Pipette
- Petri plate
- Test tube

Miscellaneous

- Bacteriological loop
- Bunsen burner
- Cotton
- Forceps
- Gloves
- Labeling sticker
- Micropipette
- Micropipette tip
- Marker
- Soap
- Spirit lamp
- Staining rack
- Sterile cotton swab
- Test tube holder

Equipments (Memmert & Olympus Company)

- Autoclave
- Compound microscope
- Hot air oven
- Incubator
- Refrigerator
- Safety cabinet
- Water bath
- Water distillation plant

Chemicals and Reagents

Blood plasma
Distilled water
Gram's Iodine
1 N Hydrochloric acid
Lysol
Nephelometer
α- Napthol solution
40% Potassium hydroxide
Crystal violet
Ethanol
Immersion oil
3% Hydrogen peroxide
Mac Farland's
Paraffin oil
Safranin

Antibiotics (HiMedia Company)

Ciprofloxacillin (5 mcg)
Cloxacillin (5 mcg)
Co-trimoxazole (25 mcg)
Erythromycin (15 mcg)
Oxacillin (1 mcg)
Oxacillin (1 mcg)*
Vancomycin (30 mcg)
Amoxycillin (30 mcg)
Tetracyclin (30 mcg)
Chloramphenicol (30 mcg)

Media (HiMedia Company)

Blood agar base
Hugh-Leifson's medium
Mac Conkey agar
Mannitol salt agar
MR/VP broth
Nutrient agar
Nutrient broth
Peptone

(* Oxoid Company)

Appendix II: Bacteriological media

Composition and preparation of different types of media

1. Nutrient Agar (NA)

Ingredients	**Gram/litre**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
pH (at 25^0 C)	*7.4±0.2*

28 gm 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.

2. Nutrient Broth (NB)

Ingredients	**Gram/litre**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
pH (at 25^0 C)	*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.

3. Mac Conkey Agar (MA)

Ingredients	**Gram/litre**
Peptone	3.0
Pancreatic digest of gelatin	17.0
Lactose	10.0
Sodium chloride	5.0
Bile salt	1.5
Neutral red	0.03

Crystal violet	0.001
Agar	13.5
pH (at 25^0 C)	*6.9-7.3*

4. Mannitol Salt Agar (MSA)

Ingredients	**Gram/litre**
Proteose 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.

5. 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.

6. Hugh-Leifson Medium

Ingredients	**Gram/litre**
Glucose	10.0
Peptone	2.0
Sodium chloride	5.0
Dipotassium phosphate	0.3
Bromothymol blue	0.05
Agar	2.0

pH (at 25^0 C)	*7.4±0.2*

19.3 grams of the medium was suspended in 1000 ml of distilled water and boiled to dissolve completely. The medium was dispensed in tubes in duplicates for aerobic and anaerobic fermentation. The media was autoclaved at 15 lbs at 121^0C for 15 minutes.

7. Methyl Red Voges-Proskauer (MR/VP) Broth

Ingredients	**Gram/litre**
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
pH (at 25^0 C)	*6.9±0.2*

1.7 grams of the medium was suspended in 100 ml distilled water and distributed in 10 ml amounts in test tubes and then autoclaved at 15 lbs at 121^0C for 15 minutes.

8. Peptone Water

Ingredients	**Gram/litre**
Peptone	10.0
Sodium chloride	5.0
pH (at 25^0 C)	*7.4±0.2*

1.5 grams of the medium was suspended in 100 ml distilled water and dissolved completely. It was then autoclaved at 15 lbs at 121^0C for 15 minutes.

Appendix III: Preparation of 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	1.0 gm
Potassium iodide	2.0 gm
Distilled water	30.0 ml

iii. Ethyl Alcohol (95%)

Absolute alcohol	95.0 ml
Distilled water	5.0 ml

iv. Safranin

Safranine (99% dye content)	10 gm
Distilled water	1000 ml

2. VP Reagent (Barrit's Reagent)

i. Reagent A

α- naphthol	5.0 gm
Absolute ethyl alcohol	100 ml

ii. Reagent B

Potassium hydroxide	40.0 gm
Distilled water	100 ml

3 volumes of reagent A and 1 volume of reagent B were mixed to prepare fresh Barrit's reagent.

3. **Catalase Reagent**

   Hydrogen peroxide 3 ml

   Distilled water 100 ml

4. **1 N Hydrochloric acid (1 mol/litre)**

   Concentrated hydrochloric acid 8.6 ml

   Distilled water 100 ml

5. **Mc Farlands' Nephelometer Standards**

Reagents	**Tube Number**										
	0.5	1	2	3	4	5	6	7	8	9	10
Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9
Approximate cell density (x 10^8 CFU/ml)	1.5	3	6	9	12	15	18	21	24	27	30

Appendix IV: Zone Size Interpretation Chart for Antibiotic Sensitivity Test

Antibiotics	Symbol	Strength	Diameter of Zone of Inhibition (mm)		
			Resistant	Intermediate	Sensitive
Amoxycillin	AM	30 mcg	≤19	-	≥20
Chloramphenicol	C	30 mcg	≤12	13-17	≥18
Ciprofloxacin	CF	5 mcg	≤15	16-20	≥21
Cloxacillin	CX	5 mcg	≤11	12-13	≥14
Cotrimoxazole	CO	25 mcg	≤10	11-15	≥16
Erythromycin	E	15 mcg	≤13	14-22	≥23
Oxacillin	OX	1 mcg	≤10	11-12	≥13
Tetracyclin	T	30 mcg	≤14	15-18	≥19
Vancomycin	VA	30 mcg	≤13	14-16	≥17

(HiMedia Laboratory Limited, India)