“Evaluation of Virulence Properties of *Staphylococcus aureus* Isolated from A Tertiary Level Hospital of Dhaka City”

Thesis submitted for partial fulfillment of the requirements for the degree of Master of Philosophy (M.Phil) in Microbiology, University of Dhaka

Submitted by

Examination Roll: 01

Session: 2014-15

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Certificate

This is to certify that the thesis entitled "**Evaluation of Virulence Properties of *Staphylococcus aureus* Isolated from A Tertiary Level Hospital of Dhaka City**" submitted by Syeda Sharmin Duza, Roll No: 01, Session: 2014-15, Registration No: 207, Bangamata Sheikh Fazilatunnesa Mujib Hall, carried out her research under my supervision. This is further to certify that it is an original work and suitable for partial fulfillment of the degree of Master of Philosophy (M.Phil) in Microbiology, University of Dhaka.

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Dedicated to
My Beloved Parents

Acknowledgements

At the very beginning, I would like to thank and recall my creator the almighty Allah for His immense kindness and mercy upon me to show me the right path to learn special and specialized knowledge in the vast land of microbiology. Many people contributed and share their knowledge and provide me the time in this period of this thesis and deserve a genuine credit for completion of my study line. In this entire country there is a lot of kind soul without whom I might not be able to complete my journey.

Firstly, I am thankful to all the patients from whom I have collected samples. I am grateful to the academic selection committee of this M.Phil thesis. I would like to convey my gratefulness to my learned supervisor, Dr. Md. Majibur Rahman, Professor, Department of Microbiology, University of Dhaka, for his unconditional time and knowledge sharing in this advanced bioscience area.

I want to express my best regards to my co-supervisor Professor Dr. Sabita Rezwana Rahman, Professor and chairman, Department of Microbiology, University of Dhaka, for her support in my lab work. I would love to thank and express my gratitude to all the teachers of the department for their valuable time in my thesis work.

I am thankful to my teachers and colleagues of HFRCMCH specially, Professor Dr. M Murshed, Dr. Jannatul Ferdous and Dr. Taslima Sarwar for always boosting me up to do good in every aspects of my work . Thanks to my parents, my husband, my son and my siblings specially my younger sister for always pushing me and allowing me to act beyond my limits.

The completion of my project would not have been accomplished without the help of my classmates and junior colleagues specially Rahmat A Shakil, Tushar, Tanim, and Abu Sayem.

I would like to thank Md. Kawsar, Md. Alam, Md. Abdul Mannan, Md. Sharif and other staffs of our department who helped me during my thesis work.

Despite the fact that I cannot recall the names of all the persons who helped me, it doesn't mean that I have forgotten them. A heartwarming gratitude to them for their valuable time.

The Author June 2019

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Abstract

Staphylococcus aureus is the most frequently isolated bacterium among both community-acquired and nosocomial infections. It is the causal pathogen of a wide range of infectious diseases ranging from skin and soft tissue infections to toxin-mediated diseases and also poses antibiotic resistant. Methicillin resistant *Staphylococcus aureus* (MRSA) turns out to be fatal, because of multidrug resistance. *Staphylococcus aureus* produces many virulence factors, including toxins, immune-modulatory factors, and enzymes. In this study, investigations were done to evaluate the virulence factors. It was conducted in the laboratory of Department of Microbiology, Holy Family Red Crescent Medical College and hospital with the collaboration of the Department of Microbiology, Dhaka University. Samples were collected from both indoor and outdoor cases of HFRCMCH. In case of indoor patients, samples were taken from the ICU, NICU, dialysis units and post-operative wards. *Staphylococcus aureus* were isolated from clinical samples like blood, pus, wound swab from abscess. The organisms were identified by colonial morphology, microscopic examination and relevant biochemical test according to standard laboratory methods. Culture was done on blood agar, mannitol salt agar and nutrient agar media. After isolation and identification, virulent factors were assessed following the analysis of growth pattern and fermentation in Mannitol salt agar media; colonial pigmentation and hemolysis on blood agar plates were observed to identify the toxin haemolysin; biochemical tests like catalase, coagulase were also evaluated to identify the enzymes. 565 samples were collected from different patients. Among which 120 samples were positive for *Staphylococcus aureus*, which is **21.2%**. In this trial, it was found that 68 isolates (56.7%) were impervious to Oxacillin. The majority of the MRSA isolates were multidrug resistant (MDR). 25.8%, 38.3%, 30.8%, 50%, 15%, 95%, 24.2%, 52.5%, 85%, 20.8% of positive samples were impervious to Amoxyclav, Co-trimoxazole, Tetracyline, Ciprofloxacin, Ceftriaxone, Vancomycin, Gentamycin, Erythromycin, Azithromycin, Amikacin respectively. Whereas low degree resistance was shown towards Ceftazidime, Carbapenem**.** In determining the resistance of *Staphylococcus aureus* to methicillin PCR was done to identify *mecA* gene in the isolates. However, when amplifying a 533bp fragment, significant number of isolates were found to be *mecA* positive. Studying virulence factors could reveal a well to do pathway for the care and cure of the patients who have been suffering from nosocomial infections. Thus, the morbidity rate and sufferings of these group of people could be minimized up to a certain level by strict hygiene and preventative measures which are highly recommended to stop transmission.

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Abbreviations

BA	Blood Agar
bp	Base pair
CoNS	Coagulase Negative *Staphylococcus aureus*
EDTA	Ethyl diamine tetra-acetic acid
Kb	Kilobase
MAC	MacConkey's agar medium
MBC	Minimum bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MSA	Mannitol Salt Agar
MIIA	Mueller-Hinton Agar
Min	Minute
Ml	Milliliter
mM	Mill-molar
MRSA	Methicillin Resistance *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
NA	Nutrient Agar
PCR	Polymerase Chain Reaction
pH	Negative logarithm of hydrogen ion concentration
Rpm	Rotation per minute
SDS	Sodium Dodecyl Sulfate
Spp	Species
µ1	Micro-liter
µg	Micro-gram
v/v	Volume by volume
Vol	Volume
w/v	Weight by volume
NCCLS	National Committee for Clinical Laboratory Standards

Chapter 1: Introduction

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1 Introduction

Staphylococcus aureus is a Gram-positive bacterium which can cause high mortality and morbidity in patients leading to hospital acquired infections. It is one of the major pathogens causing diseases in man; they are commensals of the skin and mucous membranes of humans. Although they reside as a normal flora in human but they are not always pathogenic. Disease-associated strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. They can cause a wide range of illnesses, from minor skin infection such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses [1], food poisoning, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis.

Surgeon Sir Alexander Ogston was first named it *"Staphylococcus"* in 1880 in Aberdeen, United Kingdom, after isolating it from pus samples from a surgical abscess in a knee joint [2]. *Staphylococcus aureus* and coagulase–negative *Staphylococci*, are among the most leading causes of nosocomial infections. Several characteristics of the bacteria contribute to their survival strategy in the human host. They have the ability to persist intracellularly within several types of non-professional phagocytic cells[3], specifically macrophages and neutrophils. Even to combat with the antimicrobial agents sometimes they produce "Biofilm". In addition, *Staphylococcus aureus*, especially methicillin-resistant *Staphylococcus aureus* (MRSA), often causes serious problems via nosocomial infection in hospitals [4].

Staphylococcus aureus poses many virulence factors, which includes cell wall associated factors, enzymes and toxins. They are positive for Catalase and Coagulase test. Enzyme Coagulase (bound and free coagulases) which clots plasma and coats the bacterial cell thus, probably prevent phagocytosis. Hyaluronidase (also known as spreading factor) breaks down hyaluronic acid and helps in spreading of *Staphylococcus aureus*. It also produces DNAse (deoxyribonuclease) which breaks down the DNA, Lipase to digest lipids, Staphylokinase to dissolve fibrin and aid in spread, and Beta-lactamase for drug resistance[1].

Depending on the strain, *Staphylococcus aureus* is capable of secreting several toxins. Many of these toxins are associated with specific disease[5]**.** Firstly, Superantigens that induce Toxic shock syndrome toxin (TSST). This group includes the toxin TSST-1, TSST-2 which causes Toxic shock syndrome (TSS). The condition may be associated with poor menstrual hygiene, abscesses, osteomyelitis and post-surgical wound infection and tampon use in vagina. *Staphylococcus aureus* can produce enterotoxin that is the causative agent of gastroenteritis. This gastroenteritis occurs when consuming food in which *Staphylococcus aureus* has multiplied and formed the toxin. It is self-limiting, characterized by vomiting and diarrhea, one to six hours after ingestion of the toxin with recovery in eight to 24 hours. Another superantigen is exfoliative toxin which implicated in the disease Staphylococcal scalded-skin syndrome (SSSS), which occurs most commonly in infants and young children. It also may occur as epidemics in hospital nurseries. The protease activity of the exfoliative toxins causes peeling of the skin resulting in separation of epidermis of the skin from dermis and observed as SSSS. Other toxins that act on cell membranes include alpha toxin, beta toxin, delta toxin, and several bicomponent toxins. The bicomponent toxin Panton-Valentine leukocidin (PVL) acts synergistically with γ hemolysin to damage leukocytes, RBCs, macrophages resulting in severe necrotizing pneumonia in children [6,7.] In the cell wall of a virulent Staph there is protein A which binds with the Fc fragment of the IgG thus preventing phagocytosis by neutrophils[1].

For the treatment of the diseases caused by *Staphylococcus aureus,* penicillin was the first antibiotic used, but it showed resistance within 2 years of its introduction [8]. The history of very first emergence of penicillin-resistant *Staphylococcus aureus* was observed in the United Kingdom in 1961 and 80% of these isolates were penicillin resistant, and rates of penicillin resistance have remained in this range till 1970s.

The emergence of antibiotic-resistant forms of pathogenic *Staphylococcus aureus* (e.g. MRSA) is a worldwide problem in clinical medicine. Methicillin is a semi synthetic β-lactam antibiotic and a bacterium that is resistant to methicillin considered as resistant to all β-lactam antibiotics such as methicillin, oxacillin, penicillin and amoxicillin. It is also called oxacillin-resistant *Staphylococcus aureus.* This is increasingly implicated as a cause of nosocomial infections worldwide. MRSA is a major cause of hospital-acquired infections that are becoming increasingly

difficult to combat because of emerging resistance to all current antibiotics[9]. Among *Staphylococcus aureus* isolates from a U.S. network of over 300 microbiology laboratories, the number of resistances to methicillin nearly doubled between 1999 and 2006. In 2006 over 50% of *Staphylococcus aureus* strains from both in and outpatients were identified as methicillin-resistant [10].

MRSA is frequently resistant to most of the commonly used antimicrobial agents including the aminoglycosides, macrolides, chloramphenicol, tetracycline and fluoroquinolones**[9].** In addition, MRSA strains should be considered to be resistant to cephalosporin's, and other β -lactams (such as ampicillin-sulbactam, amoxicillin clavulanic acid, ticarcillin-clavulanic acid, piperacillin tazobactam and the carbapenems) regardless of the in-vitro test results obtained with those agents. The resistance results from the development of an altered penicillin binding protein (PBP2a) which interferes with the binding of β-lactam antibiotics**.** Penicillin-resistant *Staphylococcus aureus* produces β-lactamase that cleaves the lactam n-ring of the penicillin inactivate the drug activity.

There has been an upsurge in MRSA cases arising in the community in the last 5 years. The methicillin resistance gene *(mecA)* encodes an altered penicillin binding protein (PBP2a) that is absent in susceptible strains but more recent studies show that some MRSAs are very divergent, implying that *mecA* has been transferred between *S. aureus* lineages. The *mecA* gene is the structural determinant which encodes PBP2a.

Furthermore, community-acquired MRSA has recently emerged and has been reported to cause serious infectious diseases, sepsis, and pneumonia[11]. It is still one of the five most common causes of nosocomial infections and is often the cause of post-surgical wound infections. Hospitals have successfully reduced staphylococcal infections but still progress is slow. It is even more notorious in an under developed country like ours.

For the selection of antimicrobial agents' physicians face a great difficulty due to the multi drug resistance genes. Patient's morbidity and mortality rate is significantly affected by infection with multi-drug resistant strains of *Staphylococcus aureus***.** Vancomycin has been the most reliable therapeutic agent against infections caused by methicillin resistant *Staphylococcus aureus* (MRSA). However, in 1996 the first MRSA to acquire resistance to vancomycin was isolated

from Japanese patient. The patient had contracted a post-operative wound infection that was refractory to long-term vancomycin therapy[12].

The clinical importance of *Staphylococcus aureus* is attributed to notable virulence factors, surface proteins, toxins, and enzymes as well as the rapid development of drug resistance. As different enzymes and toxins varies in formation of different diseases and which are absolutely type specific so we can get the exact information regarding disease. To treat the cases associated with pathogenic *Staphylococcus aureus*, firstly it is important to maintain hygiene and take preventive measures during handling and care provided by the health care workers. *Staphylococcus aureus* pose a public health problem especially in patients with indwelling devices, immune-compromised, suffering from chronic infections, septicemia. Therefore, an effectual precaution is required for the control of growth of this notorious organism. Rapid diagnosis and prompt treatment are needed to reduce mortality and morbidity rate. Studying virulence factors could reveal a well to do pathway for the care and cure of the patients who have been suffering from nosocomial infections. The aim of this study was to identify the occurrence of virulence factors produced by *Staphylococcus aureus* strains isolated from patients of a tertiary level hospital. Thus, from the obtained knowledge it will be helpful for the admitted patients as the morbidity rate and sufferings of these group of people could be minimized up to a certain level.

1.1 Aims and objectives

1. To identify the virulence factors of *Staphylococcus aureus.*
2. To elaborate the occurrence of virulence factors produced by *Staphylococcus aureus* strains isolated from patients of a tertiary level hospital. Thus, from the obtained knowledge it will be easy to help the admitted patients.
3. To find out the rate of isolation of *Staphylococcus* from various clinical specimens.
4. To see the antibiotic sensitivities of the bacteria against the commonly used drugs.
5. To compare the drug resistance patterns between studies obtained from different hospitals.

Chapter 2: Literature Review

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2 Literature review

2.1 *Staphylococci*

The gram-positive cocci are classified into two families- *Micrococcaceae* and *Streptococcaceae*. *Micrococcaceae* are gram-positive, catalase positive cocci arranged in tetrads or clusters whereas *Streptococcaceae* are catalase negative gram-positive cocci, arranged in pairs or chains. Family *Micrococcaceae* comprises of four genera *Micrococcus, Staphylococcus, Stomatococcus, Planococcus*.

Staphylococcus aureus is a gram-positive bacteria, appear as round (cocci) or spherical and form grape like clusters under microscope as fundamentally they can divide along three definitely oriented planes that are located at right angles to each other. In Greek, *staphyle* means a bunch of grapes and *kokkos* means berry. *Staphylococci* normally found as a normal flora of the nose and skin of healthy humans [13]. Members of the genus *Staphylococcus* frequently colonize in the skin and upper respiratory tracts in different types of mammals. The bacteria can be transmitted from person to person or to fomite by direct contact. The *Staphylococcus* genus includes multiple species. Of these, nine have two subspecies, one has three subspecies, and one has four subspecies. Rosenbach described the two pigmented colony types of staphylococci and proposed the appropriate nomenclature. *Staphylococcus aureus* which is yellow in color and *Staphylococcus albus* white in color. *Staphylococcus albus* is now named *Staphylococcus epidermidis.* More than 30 species are identified among them only *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saphrophyticus* are significant in their interactions with humans [14]. *S. aureus* colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales, including the skin, oral cavity and gastrointestinal tract. They produce toxins and enzymes that are harmful to the cells. Their lipids and proteins destroy the RBC and enzymes causes the plasma clot while the leucocidins destroy the WBC. *S epidermidis* is usually nonpathogenic an inhabitant of the skin, *S. saphrophyticus* causes acute urinary tract infections specially in sexually active females. Taxonomically, the genus *Staphylococcus* is in the bacterial family *Staphylococcaceae,* which includes three lesser known genera, *Gamella, Macrococcus* and *Salinicoccus.* The best-known of its nearby phylogenetic relatives are the

members of the genus *Bacillus* in the family *Bacillaceae,* which is on the same level as the family *Staphylococcaceae.*

2.1.1 *Staphylococcus aureus*

Staphylococcus aureus is catalase positive, coagulase positive, facultative anaerobe, non-motile, non-spore forming occasionally capsulated organism. They generally stain dark violet means gram positive cocci bacterium usually forms yellow colonies on culture plates; whereas *S. epidermidis has* a relatively small white colony. On blood agar plate *Staphylococcus aureus* often appeared as hemolytic; *S. epidermidis* is non-hemolytic**.** *Staphylococci* are true facultative anaerobes that grow by aerobic respiration or by fermentation that yields lactic acid. The bacteria are catalase-positive and oxidase-negative which distinguishes them from *Streptococci*. Like other Gram-positive organisms, they have thick peptidoglycan (mucopeptide) layer with a specific pentaglycine bridges (attacked by lysostaphin) linking amino acid side chains. *Staphylococcus aureus* are relatively resistant to heat and drying and can survive for long term in fomites. They better grow at a temperature range of 15 to 45º C and at NaCI concentrations as high as 15 %. Most of the strains of *S. aureus* produce the enzyme coagulase an enzyme that citrated plasma to clot. Nearly all strains of *S. epidermidis* lack this enzyme since termed as coagulase negative staphylococci. *Staphylococcus aureus* is considered to be the most fastidious pathogen of its tribe; most strains of *S. epidermidis* are nonpathogenic and may even play a protective role in humans as normal flora. *Staphylococci* are perfectly spherical cells about 1µm (micrometer) in diameter, 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 **[14].**

2.1.2 Evolutionary History of *Staphylococcus aureus*

Staphylococcus was first observed in pus by Von Recklinghausen (1871) and by Robert Koch (1978). It was first cultured in liquid medium by Louis Pasteur (1880). It was named as *Staphylococcus* (In Greek, *staphyle* means a 'bunch of grapes' and *kokkos* means berry) by surgeon Sir Alexander Ogoston (1880) in Aberdeen, United Kingdom (Scotland), in pus from a surgical abscess in a knee joint[2]**.** At that era, *Staphylococcus aureus* infections commonly caused skin and soft tissue diseases which were painful. This name was later appended to *Staphylococcus*

aureus by Rosenbach who was credited by the official system of nomenclature at the time. The term *aureus* means golden. He named two species of staphylococcus based on pigmentation of colonies as *Staphylococcus aureus* (golden yellow) and *Staphylococcus albus* (white colonies). Later Passet (1885) named a third species as *Staphylococcus citreus* (lemon yellow colonies). In 1940s, medical treatment of *Staphylococcus aureus* infections became routine and successful after the discovery of penicillin. It is estimated that 20 to 40% of the human population are long-term carriers of the bacteria[15] which can be found as part of the normal skin flora and in mucous membrane of anterior nares of the nasal passages. *Staphylococcus aureus* is the most common species of staphylococcus to cause Staph infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies **[16]**. *Aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections**.** *Staphylococcus aureus* is not always pathogenic, it is a common cause of skin infections (e.g. boils), respiratory disease (e.g. sinusitis), and food poisoning. Disease-associated strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant forms of pathogenic *Staphylococcus aureus* (e.g. MRSA, VRSA) is a worldwide problem in clinical medicine [15].

2.1.3 Morphology of *Staphylococcus aureus*

*S*taphylococcus *aureus* is approximately 1µm in diameter, and divides to form clusters. On blood agar or nutrient agar plates, incubated at 37ºC after 18-24 hours it forms colonies. The colonies are smooth, convex, glistening, densely opaque and sometimes surrounded by a narrow zone of hemolysis on blood agar plates, depending on strains. Older colonies are translucent and sticky. Occasionally some of them are capsulated and their colonies are large, convex and glistening, becoming so slimy that they run over all the surface of the agar plate. Pigmentation is one of their bench marks it can be creamy to golden in color. Pigmentation can be enhanced by the growth of the organisms in fatty media like Tween agar. They are true facultative anaerobes. *Staphylococcus*

aureus is halo-tolerant means they can tolerate high concentration of NaCl (sodium chloride) that inhibit most other bacteria on Mannitol salt agar (MSA) plates. Hence, they grow 1mm diameter yellow colonies surrounded by yellow medium due to acid formation. On Mac Conkey's agar or CLED agars, it acquires the appropriate color of the indicator, depending on whether or not the particular strains ferments lactose. *Staphylococci* can be differentiated from *Streptococci* on the basis of shape and configuration of gram-positive cocci. *Staphylococci* are arranged in clusters whereas Streptococci are slightly oblong cells that usually grow in chains because they divide in one plane only, similar to a bacillus.

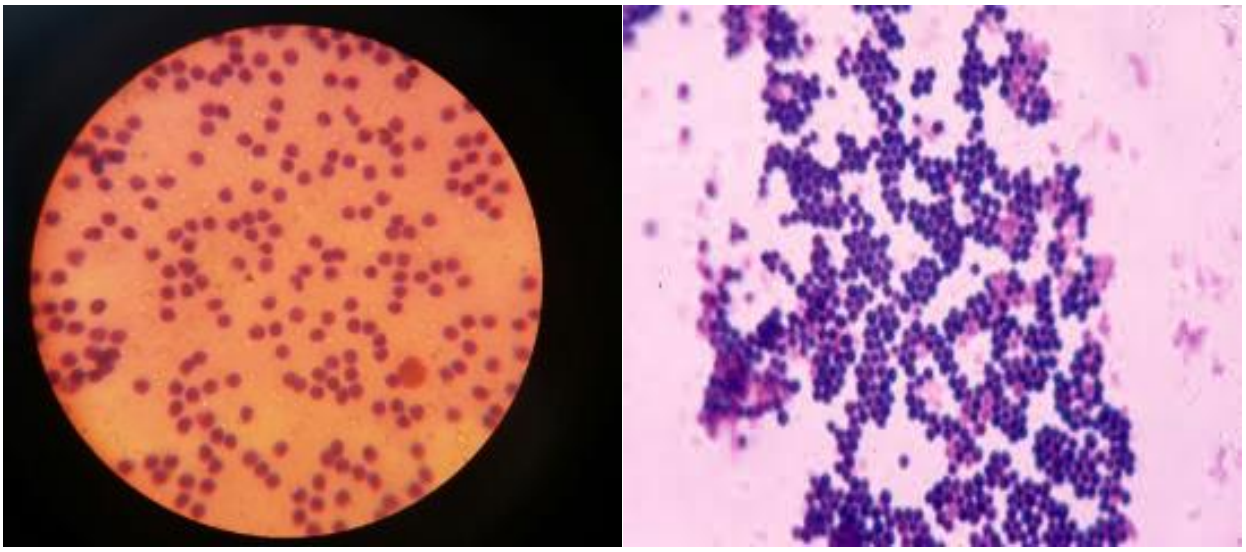

Figure 2.1 Microscopic morphology of *Staphylococcus aureus*

2.1.4 Epidemiology

Staphylococcus aureus is carried by 20 to 40 % of healthy human being and by almost everyone at some time. Most commonly they reside in skin, mucous membrane of nose especially anterior nares, vagina, perineum and oropharynx. These colonization sites act as a reservoir for future infections if the immunity of the host is reduced, resulting in opportunistic infection. They transmitted by direct contact, by fomites, or by food (enterotoxin) resulting in food poisoning. The rate of *Staphylococcal* infection is higher in insulin-dependent diabetic patients, HIV infected

patients, patients undergoing hemodialysis and individuals with skin damage. Hospital strains are often multidrug resistant, spread to patients either from hospital staff or other patients or environment or also from patient's own endogenous flora[14]. In the community, *Staphylococcus aureus* remains as an important cause of skin and soft tissue infections, respiratory infections, and infective endocarditis (among intra venous drug abusers).

2.1.5 Predisposition to Staphylococcal infection

There are some multifactorial causes resulting in accusation of Staphylococcal infections. They are as follows-

- Poor hygiene and nutrition,
- Tissue injury,
- Pre-existing primary infection,
- Diabetes,
- Immunodeficiency.

2.1.6 Nosocomial Infections or Hospital acquired infections

A Hospital-acquired infection (HAI), also known as a nosocomial infection, is an infection that is acquired in a hospital or other health care facility. It is sometimes instead called a health care–associated infection (HAI or HCAI). It is contracted because of an infection or toxin that exists in a hospital. Such an infection can be acquired in-

- Hospital,
- Nursing home,
- Rehabilitation facility,
- Outpatient department (OPD), or other clinical settings.

Infection is spread to the susceptible patient in the clinical setting by various means.

1. Health care staff can spread infection by contaminated equipment, bed linens, or air borne droplets.
2. Environment
3. Infected patients or staffs
4. In some cases, the microorganism can be a part of the normal flora, which is more frequent after surgery or other procedures that compromise the protective skin barrier. Though the patient may have contracted the infection from their own skin, the infection is still considered nosocomial since it develops in the health care setting [17].

Most common types of HAI

- Bloodstream infection,
- Pneumonia,
- Urinary tract infection (UTI),
- Surgical site infection

Studying virulence factors could reveal a well to do pathway for the care and cure of the patients.

2.2 Sterilization

Staphylococcus and other micrococci are hardiest non-spore forming bacteria who survive both in dry and moist environment and in laboratory cultures such as sealed agar slopes. Still they can be sterilized by Autoclave (121°C) and Hot air oven (160°C). It with stand moist heat at 60ºC for up to 30 min. They cannot withhold the phenol and hypochlorite disinfecting agents. Also, they cannot survive in antiseptic preparations such as hexachlorophene, chlorhexidine and povidone-iodine. Whereas there are several newer techniques to combat with the pathogenic organism in wide range industrial area like; Pulsed UV-Light.

2.3 Genome structure

The *Staphylococcus aureus* genome, which is the most common species among the Staphylococcus genome projects. It has the most completed genome sequence compared to any other microbial species. The genome of *Staphylococcus aureus* consists of a single circular chromosome (2.7-2.8 mbp) plus an assortment of extrachromosomal accessory genetic elements. The original genome map of *Staphylococcus aureus* was based on the strain NCTC 8325, it is a prototypical strain for all genetic manipulation, initiated by Peter A. Pattee and colleagues. By 2000, the entire genome of strain 8325 had been sequenced and annotated [18]. Since then, at least six other *S. aureus* strains have been completed (COL, N315, Mu50, MW2, MRSA252, MSSA476).

The *Staphylococcus aureus* strain NCTC 8325 complete circular genome map shows 2,889 open reading frames, 61 tRNA genes, 3 structural RNAs, and 5 complete ribosomal RNA operons. This strain has about 33% G+C content and an average gene length of 824 nucleotides with 85% coding sequence, similar to other *S. aureus* strains. Virulence factors are encoded by phages, plasmids, pathogenicity islands and staphylococcus cassette chromosome. Increased resistance for antibiotics is encoded by a transposon (Tn 1546) that was inserted into a conjugated plasmid that also encoded resistance to other things including disinfectants. MRSA (Methicillin-Resistant *Staphylococcus aureus),* which is resistant to the antibiotic methicillin, expresses a modified penicillin-binding protein (PBP2a) encoded by *mecA* gene. This was brought about by many evolutions thought horizontal gene transfer of *mecA* to a wide variety of methicillin susceptible *S. aureus* strains[19]. The genes for antibiotic resistance in *Staphylococcus aureus* are located on plasmids or other similar structures.

Diversification within the *Staphylococcus aureus* population is achieved through a combination of mutation, recombination and horizontal gene transfer. Evolution of this bacterium can occur through asymptomatic colonization and or during the course of the caused disease.

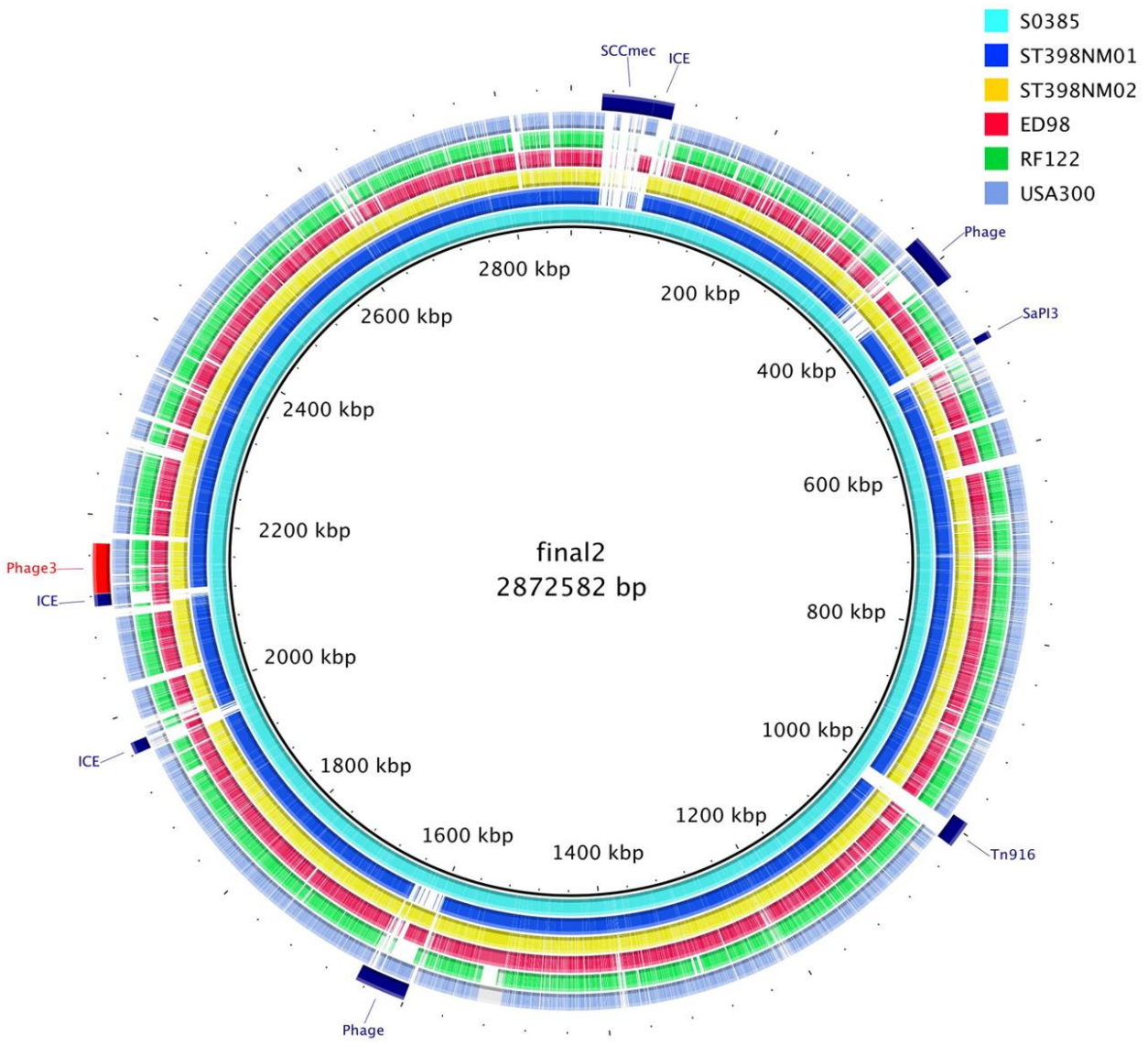

Figure 2.2 Genome structure of *Staphylococcus aureus*[20]

2.4 Evolutionary History of MRSA

Methicillin resistant *Staphylococcus aureus* (MRSA) was first identified just over 5 decades ago. This has now undergone rapid evolutionary changes. Many studies have tried to elucidate the origin of MRSA strains and significant advances have been made in recent years. In 1961, British scientists identified the very first strains of *Staphylococcus aureus* bacteria that resisted methicillin. The first reported human case of MRSA in USA came in 1968. It became a

worldwide epidemic since 1970s[21]. It occupies the methicillin resistance gene, *mecA*, which is carried by the genetic element known as *SCCmec*. The origins of *SCCmec* are unknown, and although investigations have found that these elements are widely distributed in staphylococci, including *S. aureus*, they have not been found in any other genera of bacteria[22]. *SCCmec* is integrated near the *S. aureus* origin of replication, and this location might have been critical for providing MRSA with the ability to acquire other antibiotic-resistant genes. Dependent on its intrinsic virulence or the ability of the host to contain its opportunistic behavior, *Staphylococcus aureus* can cause a range of diseases in man. The bacterium readily acquires resistance against all classes of antibiotics by one of two distinct mechanisms: mutation of an existing bacterial gene or horizontal transfer of a resistance gene from another bacterium. Several mobile genetic elements carrying exogenous antibiotic resistance genes might mediate resistance acquisition. Of all the resistance traits *Staphylococcus aureus* has acquired since the introduction of antimicrobial chemotherapy in the 1930s, methicillin resistance is clinically the most important, since a single genetic element confers resistance to the most commonly prescribed class of antimicrobials the beta-lactam antibiotics, which include penicillin, cephalosporin, and carbapenem[23.] Resistance is attributed due to **Beta-lactamase** production due to-

- ✓ **Genes** located on the extra-chromosomal plasmids.
- ✓ Presence of unusual **PBP** in the cell wall of resistant strains.

Many studies have characterized MRSA isolates from individual hospitals or countries and have identified MRSA strains. Those strains being well adopted are established in several hospitals inside a country and then have spread internationally. MRSA isolates are generally characterized by pulsed-field gel electrophoresis. According to Enright MC, *et al,* 2000, this method is insufficiently discriminatory for evolutionary studies [24]. The overall genetic characteristic of *S. aureus* isolates is unambiguously determined through multi-locus sequence typing (MLST) which requires a procedure that is highly discriminatory. Multi-locus sequence typing (MLST) characterizes isolates of bacteria by using the sequences of internal fragments of seven housekeeping genes.

2.4.1 Danger of MRSA

Staphylococcus aureus is present in 20-40% of normal healthy human nose and is also commonly found on people's skin but does not cause disease [55]. It is now threatening to be a severe Community acquired infection which is likely to be more severe and need long term Hospitalization. It's tougher to treat because it is resistant to some commonly used antibiotics. The presence of organism on the skin or in the nose or in the back of the throat without any illness is termed as colonization. However, patients having fever or inflammation associated with the presence of MRSA are considered to be infected. MRSA does not pose a risk to the health official staff, unless they are suffering from a debilitating disease or immune compromised or family members of an affected patients of their close social or work contacts. Symptoms of MRSA depends on where it is affected. From mild skin infections to wound infections sometimes it may arise a life-threatening condition. Though it is no more dangerous but some public health experts alarmed by the spread of tough strains of MRSA which is often termed as "super bug"[6].

2.5 Vancomycin resistant *Staphylococcus aureus* (VRSA)

Vancomycin, a glycopeptide antibiotics that inhibits cell wall bio-synthesis, remains a drug of choice to treat MRSA cases. Vancomycin resistant *Staphylococcus aureus* (VRSA) are resistant to vancomycin. Sometimes there are Vancomycin intermediate *Staphylococcus aureus* (VISA) who exhibits increased resistance with the drug. By the end of 1990s the relatively few multidrug-resistant and highly epidemic clones of methicillin-resistant *Staphylococcus aureus* (MRSA) had become the most frequent causative agent of diseases in both hospitals and communities[8]**.** In spite of the availability of several structurally different antibacterial agents, the therapy most frequently used for treatment of MRSA infections has remained the glycopeptide antibiotics, primarily vancomycin**.** From 1980 on, there was an abrupt and continued increase in the use of vancomycin in the United States and several countries, which seems to parallel the increasing frequency of MRSA infections in hospitals.

An MRSA isolate with decreased susceptibility to vancomycin was first reported in Japan in 1997. The isolate had only a modestly increased minimum inhibitory concentration (MIC) value

for vancomycin, in the range of 3-8 µg/ml, and become known as vancomycin intermediate-resistant *S. aureus* (VISA). VISA isolates do not carry imported foreign genetic elements; rather the increased vancomycin MIC values are related to mutations that appear in the invading pathogen during vancomycin therapy in vivo. VISA began to reported with increasing frequency among MRSA isolates identified all over the world [19]. In spite of their moderate increase in MIC value, vancomycin treatment of infections by VISA isolates often ended in treatment failure**.**

2.6 Virulence factors of *Staphylococcus aureus*

Virulence of *Staphylococcus aureus* is multi-factorial depending on a series of toxins, adhesion, immune evasion and other virulence determinants, various combination of which occur in different strains. The pathogenic capacity of a given strains is a combined effect of toxins, enzymes and cell wall associated factors together with invasive properties. As a pathogen, it is important to understand the virulence mechanisms of *S. aureus* especially the methicillin-resistant *Staphylococcus aureus* (MRSA) in order to successfully combat the pathogen. The increasing population of "super germs" and antibiotic resistant pathogens have increased pressure on researchers as well as physician to find alternative, more effective ways of fighting these "super bugs." DNA sequencing of this microbe has already isolated the source code of its resistance to antibiotics. Furthermore, higher case fatality rates have been observed for certain MRSA infections, including bacteremia **[25]** and surgical site of infections **[26].**

Table: 2.1 Virulence factors of *Staphylococcus aureus*[27]

Cell wall associated factors
1. Peptidoglycan 2. Teichoic acid 3. Cell surface adhesins- e.g. clumping factor 4. Protein A 5. Capsule 6. Cytoplasmic membrane
Toxins
1. Membrane active toxins • Hemolysins –α, β, γ, δ • Leukocidin / PVL (Panton valentine leukocidin) 2. Epidermolytic toxin (exfoliative toxin) 3. Enterotoxins 4. Toxic shock syndrome toxin
Extracellular enzymes
1. Catalase 2. Coagulase 3. Heat stable thermonuclease 4. Deoxyribonuclease 5. Staphylokinase (fibrinolysin) 6. Others- hyaluronidase, lipase, protease and Penicillinase.

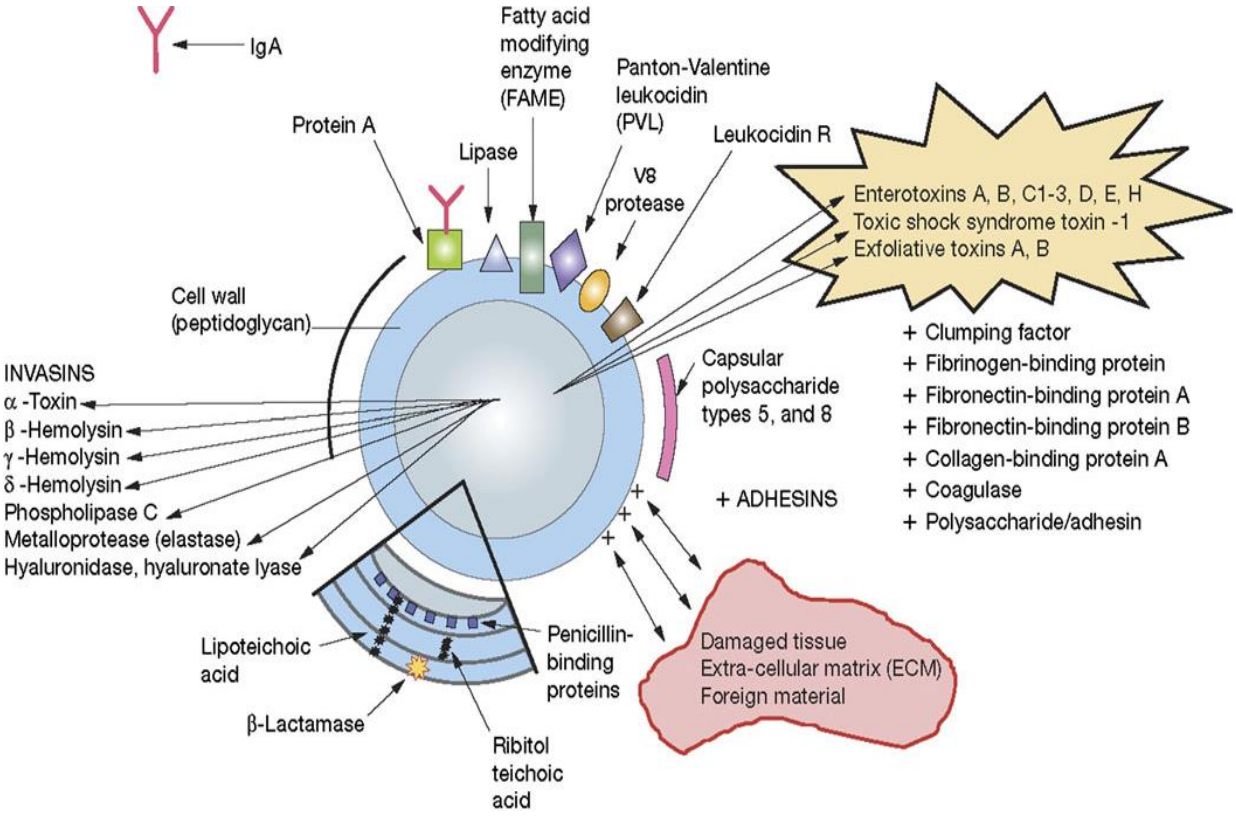

Figure 2.3 Virulence factors of *Staphylococcus aureus*[28]

2.6.1. Cell wall associated factors

Like most gram-positive bacteria, the cell wall of *staphylococcus* consists of a thick peptidoglycan layer and teichoic acid. *Staphylococcus aureus* has additional factors in the cell wall, such as Protein A and Clumping factor. Surface proteins play multiple important roles in *S. aureus* pathogenesis. They function in bacterial cell wall metabolism, serve to find to host tissue, facilitate internalization and immune evasion. They are involved in bacterial aggregation and biofilm formation[29].

- **Peptidoglycan**

  Peptidoglycan is one of the most important part of the cell wall. Similar to other gram-positive bacteria, the peptidoglycan layer of *Staphylococcus aureus* is thicker (15-80 nm, up to 100 layers thick). It confers rigidity to the cell wall and maintains the shape. It

includes inflammatory response and also has endotoxin like activity. It comprises almost half of the cell wall. It is thicker (15-80 nm, up to 100 layers thick), confers rigidity to the cell wall and maintains the shape. They are endogenous pyrogens. To combat with the host immunity, they help by activation of complement and production of Interlukin-1 from monocytes thus resulting in aggregation of polymorphonuclear cell[14].

- **Teichoic acid**

  Teichoic & lipoteichoic acid is of two types

  - Polysaccharide A in *S. aureus*
  - Polysaccharide B in *S. Epidermidis*

  It is made up of ribitol phosphate polymers, helps in adhesion of cocci to mucosal surfaces and inhibits opsonization. They are poor immunogens (except when bound to peptidoglycan).

- **Cell surface adhesins**

  Clumping factor or bound coagulase – it is a fibrinogen binding adhesion, responsible for slide coagulase reaction. There are also fibrinonectin binding adhesion and Collagen binding adhesion.

- **Protein A**

  Protein A is an antiphagocytic virulence factor, covalently incorporated into cell wall of *S.aureus.* They anchored to staphylococcal peptidoglycan pentaglycine bridges (chains of five glycine residues) by the transpeptidase sortase A [30.] Protein A in various recombinant forms has been used for decades to bind and purify a wide range of antibodies by immunoaffinity chromatography. Transpeptidases, such as the sortase responsible for anchoring factors like Protein A to the staphylococcal peptidoglycan, are being studied in hopes of developing new antibiotics to target MRSA infections [31].

  It is a 42 kDa polypeptide encoded by spa gene. It is present on 90-99% of human *S.aureus* strains (specially cowan I strains). They have many biological properties such as anti-

complementary, chemotactic, mitogenic, inhibition of opsonization and induction of platelet damage. Protein A binds with the Fc region of any IgG antibody, leaving Fab region free which binds to the corresponding antigen thus mediates co-agglutination reaction. In fact, studies involving mutation of genes coding for protein A resulted in a lowered virulence of *S. aureus* as measured by survival in blood, which has led to speculation that protein A-contributed virulence requires binding of antibody Fc regions [32].

- **Microcapsule**

  Some strains of *Staphyloccus aureus* have polysaccharide microcapsule, which inhibits phagocytosis by neutrophils. The capsular polysaccharides are zwitterionic-i.e. they have both negative and positive charges, which is a nature that is critical for abscess formation.

2.6.2 Toxins

Staphylococcus aureus produce disease both through their ability to multiply and spread widely in tissues and through their production of many extracellular substances. Some of these substances are considered to be toxins. Many *S. aureus* toxins and other virulence determinants are encoded on MGEs. Their presence varies considerably between strains. Such MGE (mobile genetic elements) encoded toxins include superantigens such as TSST (toxic shock syndrome toxin), some leukotoxins such as Panton-Valentine leukocidin, and exfoliative toxins. In contrast, α-toxin, γ-toxin, some leukotoxins and phenol-soluble modulins (PSMs) are also produced by most strains.

- **Toxic Shock Syndrome Toxin**

Majority *Staphylococcus aureus* isolated from patients with toxic shock syndrome produce a toxin called Toxic shock syndrome toxin (TSST). It is of two types TSST-1 and TSST-2. TSST-1 which is structurally similar to enterotoxin. Enterotoxin F or Pyrogenic exotoxin C is the most common type of TSST-1; rarely enterotoxin-B and C may also be associated. It is the prototypical superantigen which gets absorbed into circulation. It stimulates protein manifestation of toxic shock syndrome by simultaneously binding with MHC class II molecules and to variable portion of β

chain of helper T cell receptor of many T_h cells, thus allowing T cell stimulation. It promotes the T –cells non-specifically causing excessive production of cytokines (Cytokine Storm) which leads to potentially fatal multisystem disease. The toxin is associated with fever, shock and multi system involvement, including a desquamative skin rash [14]. Because of their similarities in molecular structure, it is sometimes referred to as Staphylococcal enterotoxin F although it does not cause food poisoning when ingested. The gene for TSST-1 is found in about 20% of *Staphylococcus aureus* isolates including MRSA. In 1970s it was recorded of having toxic shock syndrome in menstruating women using tampons in vagina. It is said to be colonized by TSST 1 strains of *Staphylococcus aureus*. Subsequently TSS has been reported from both men and non-menstruating women as a complication of staphylococcal abscesses, osteomyelitis and post-surgical wound infection. It is heat resistant. Detection of TSST can be done by latex agglutination test and enzyme immunoassay. PCR based assays are available for detection of TSST genes 1 and 2.

- **Epidermolytic / Exfoliative Toxin (ET)**

The toxin of *Staphylococcus aureus* includes at least two proteins that yield the generalized desquamation of the staphylococcal scalded skin syndrome (SSSS) in children. Specific antibodies protect against the exfoliative action of the toxin**.** This is a superantigen too. It comprises of two proteins – ET –A (chromosomal, heat stable) and ET-B (plasmid coded, heat labile). Illness may vary from localized tender blisters and bullae formation to exfoliation and separation of outer epidermal layer leaving denuded underlying skin (Nikolsky's sign). Severe form in newborn is called as Ritter's syndrome; characterized by fever, lethargy, and irritability with poor feeding. Epidermolytic strains belong to *S.aureus* bacteriophage group II[14].

- **Enterotoxin**

Enterotoxin is expressed by nearly 50% of *S.aureus* and is responsible for staphylococcal food poisoning. It is a preformed toxin secreted in food before consumption so that it can act rapidly. As a result, the incubation period is short (1-6 hours). It stimulates the vagus nerve and the vomiting center of the brain. It also stimulates the intestinal peristaltic activity. Thus, resulting in nausea, vomiting, occasionally diarrhea without fever. The symptoms usually resolve within 8-10 hours.

Most common foods which may contain the toxin are milk products, custards, potato salad or processed meat. It is a heat stable toxin who is resistant to gastric juice. Enterotoxin can be serotyped into (A-E G-I, R-T, and V). Type A mostly causes food poisoning. Type F is responsible to cause toxic shock syndrome. Serotype –J, Q and U are enterotoxin like toxins. Staphylococcal enterotoxin is also responsible for some cases of pseudomembranous colitis following use of antibiotics. Detection of enterotoxin in food can be done by latex agglutination test and enzyme immunoassay or by multiplex PCR.

- **Superantigens**

Superantigens are class of antigens that causes nonspecific activation of T cells resulting in polyclonal T call activation and massive cytokine release such as IL2, TNF –α, Interferon γ. *S.aureus* can express two different types of toxin with superantigen activity, enterotoxins, of which there are eight serotypes (A, B, C, D, E, G, H and I) and toxic shock syndrome toxin (TSST1). **Enterotoxins** cause diarrhea and vomiting when ingested and are responsible for staphylococcal food poisoning. When expressed systemically, enterotoxins F can cause toxic shock syndrome (TSS) - indeed enterotoxins B and C cause 50% of non-menstrual TSS. **TSST-1** is very weakly related to enterotoxins and does not have emetic activity[33]**.** Superantigens stimulate T cells non-specifically without normal antigenic recognition**.**

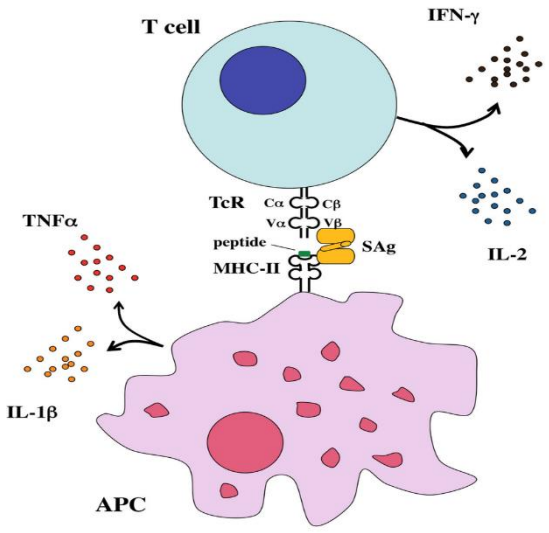

Figure 2.4 Polyclonal activation of T cell[35]

They simultaneously bind with MHC class II molecules and to variable portion of β chain of helper T cell receptor of many T_h cells. Normally for T-cell activation Up to one in five T cells may be activated, whereas only **1** in 10,000 are stimulated during antigen presentation by Super

Antigens[34]. 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 grove.

- **Membrane Active Toxins /Cytolytic Exotoxin/ Hemolysin**

  The toxins are α, β, γ and δ toxins who attack mammalian cell specially RBC membrane and causes haemolysis. They differ from one another by their action on RBCs of different animals.The toxins are dermonectrotic, leucocidal and lethal for animals on injection, cause necrosis in skin and contain soluble hemolysins, which can be separated by electrophoresis. The alpha toxin (hemolysin) is a heterogeneous protein that can lyse identically with the lethal and dermonecrotic factors of exotoxin**.**

Table:2.2 Hemolysin of *Staphylococcus aureus* and their activity[27]

Hemolysin	**Activities**
α Hemolysin	It is inactivated at 70º C but again reactivated paradoxically at 100ºC. It is dermonectrotic, leukocidal, lethal, cytotoxic and neurotoxic activities. Lyse rabbit RBCs but not sheep or human RBCs.
β Hemolysin	It is sphingomyelinase in nature. Lyse sheep RBCs but not rabbit or human RBCs. Exhibits hot-cold phenomenon, i.e. hemolysis starts at 37ºC but becomes evident only after chilling.
γ Hemolysin	It has three protein fragments which act together along with leucocidin to exhibit hemolytic activity. Lyse rabbit, sheep and human RBCs.
δ Hemolysin	It has detergent like action. Lyse rabbit, sheep, human, horse RBCs. It is dermonectrotic, leukocidal and lethal.

- **Panton-Valentine leukocidin (PVL)**

Staphylococcal toxins that act on cell membranes include alpha toxin, beta toxin, delta toxin, and several bicomponent toxins. The bicomponent toxin Panton-Valentine leukocidin (PVL) is

associated with severe necrotizing pneumonia in children[6,7]. It has two components F(fast) and S(slow) based on their migration on carboxymethyl cellulose column. Both the components act synergistically with γ hemolysin to damage leukocytes, RBCs, macrophages. It can kill WBC of humans and rabbits. PVL and γ hemolysin are called synergohymenotropic toxins as the cannot active individually but in combination they are capable of producing hemolytic and leukocidal activity. The genes encoding the components of PVL are encoded on a bacteriophage found in community-associated methicillin-resistant *S. aureus* (MRSA) strains.

2.6.3 Enzymes

- **Catalase**

This Staphylococci produce catalase which converts hydrogen peroxide (H_2O_2) into water and nascent oxygen. The nascent oxygen causes oxidative damage of host tissue. This enzyme is produced after phagocytosis or during metabolism of the bacteria. It differentiates *staphylococci* from *streprococci.* The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are vigorous catalase-producers. The test is performed by adding 3% hydrogen peroxide(H_2O_2) to a colony on an agar plate or slant or test tube. Catalase-positive cultures produce 0_2 and bubble at once.

$$2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$$

Streptococci vs. Staphylococci

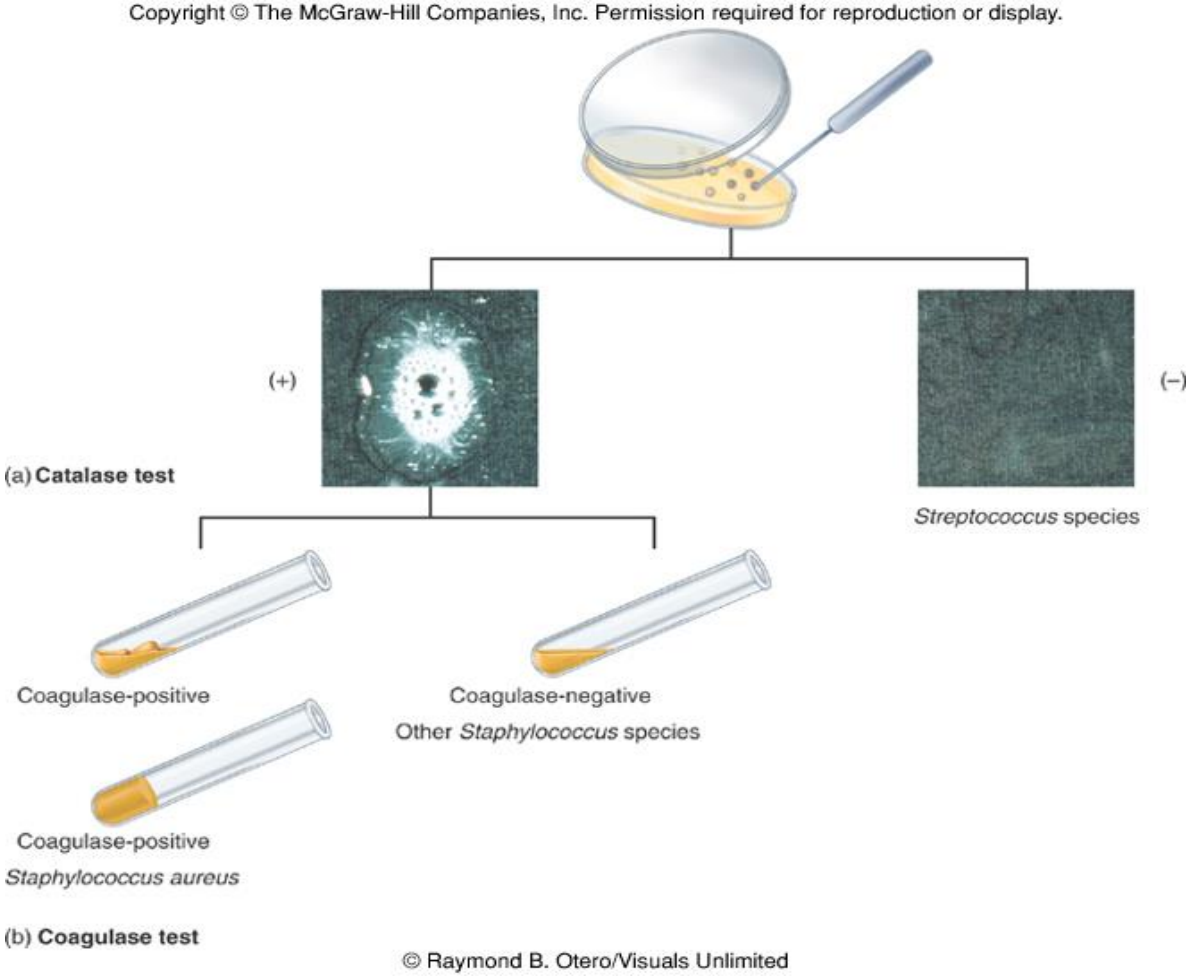

Figure 2.5 Differentiation between the gram-positive coccis' on the basis of biochemical tests

- **Coagulase and Clumping factor**

  The unique feature of *Staphylococcus aureus* is that, it secrets coagulase enzyme which brings about clotting and coagulation of plasma. Staphylococci were divided into two groups on the basis of their ability to clot blood plasma. The Coagulase positive staphylococci constitute the most pathogenic species *S aureus.* The Coagulase negative staphylococci (CoNS) are known to comprise over 30 other species. The CoNS is common commensal of skin, although some species can cause infections. Coagulase is a marker for *S aureus* in the clinical microbiology but there is no direct evidence that is a

virulence factor. Also, some natural isolates of *Staphylococcus aureus* are defective in coagulase. It is an extracellular protein which combines with plasma protein called CRF (coagulase reacting factor) and together they activate prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. This is the basis of the tube coagulase test, in which a clot is formed in plasma after incubation with the *Staphylococcus aureus* broth culture. Coagulase enzyme of 8 antigenic types (A-H). Type A is the most common type secreted by human strains of *Staphylococcus aureus.* Production of coagulase is indicative of an S. aureus strain. The enzyme acts within host tissues to convert fibrinogen to fibrin. It is theorized that the fibrin meshwork that is formed by this conversion surrounds the bacterial cells or infected tissues, protecting the organism from nonspecific host resistance mechanisms such as phagocytosis and the anti-staphylococcal activity of normal serum. In the coagulase tube test for bound and free coagulase, a suspension of the test organism in citrated plasma is prepared and the inoculated plasma is then periodically examined for fibrin formation, or coagulation. Clot formation within 4 hours is interpreted as a positive result and indicative of a virulent *Staphylococcus aureus* strain. The absence of coagulation after 24 hours of incubation is a negative result, indicative of an avirulent strain[36].

Clumping factor is a surface *Staphylococcus aureus* compound that is responsible for adherence of the organisms to fibrinogen and fibrin. When mixed with plasma, *Staphylococcus aureus* forms clumps. Clumping factor is distinct from coagulase.

- **Leukocidin**

  Staphylococcus aureus can express a toxin that specifically acts on polymorphonuclear leukocytes. Its role in pathogenesis is uncertain, because pathogenic staphylococci may not kill white blood cells and may be phagocytosed as effectively as non-pathogenic cellular multiplication.

- **Hyaluronidase or spreading factor**

  It hydrolyzes the hyaluronic acids (acidic mucopolysaccharides) present in the matrix of the connective tissues, thereby facilitating the spread of bacteria in tissue.

- **Penicillinase**

  More than 90% of *Staphylococcus aureus* produce enzyme penicillinase. The enzyme inactivates penicillin group of antibiotics, hence is responsible for widespread occurrence of penicillin-resistant staphylococci. The gene for this enzyme is acquired through plasmids.

- **Staphylokinase**

  Staphylokinase is a plasminogen activator which expressed by many strains of *Staphylococcus aureus*. The genetic determinant is associated with lysogenic bacteriophages. A complex formed between staphylokinase and plasminogen activates plasmin-like proteolytic activity which causes dissolution of fibrin clots (Fibrinolysis). The mechanism is identical to streptokinase, which is used in medicine to treat patients suffering from coronary thrombosis [37].

- **Other enzymes**

  Other enzymes include phosphatase, deoxyribonucleases, nucleases, proteases, phospholipase, proteinase and lipase and β- lactamase.

2.7 Regulation of Virulence determinants

The expression of staphylococcal virulence is regulated by three systems those are sensitive to environmental signals. They are noted in two proteins (two-component system), a sensor kinase and a response regulator. The sensors bind with extracellular ligands resulted in phosphorylation cascade leads to binding of the regulator to specific DNA sequences which ultimately leads to activation of transcription-regulating functions. In two proteins (two-component system) *agr,sae*

RS, srrAB,arlSR and lytRS are included. The accessory gene regulator (agr) is essential for quorum-sensing. The sae regulates gene expression in transcriptional level and is essential for production of α toxin, β- haemolysis, and coagulase. ssrAB is responsible for regulation of virulence factor expression that is influenced by environmental oxygen. The arlSR is responsible for control of autolysis and also decreases the activation of the agr locus. The lytRS locus is also responsible for autolysis.

2.8 Biofilm formation

The survival strategy of the bacteria is changing every single day to combat with the potential antibiotics. Biofilms are thin but robust layer of mucilage adhering to a solid surface and containing a community of bacteria. Staphylococci are known as to a good biofilm former because of the production of a series of surface molecules that promote extracellular matrix formation. Biofilm producing strains have higher tendency to exhibit antimicrobial resistance, even sometimes multidrug resistance. Biofilms provide significant protection from antibiotics and host defenses and enable the bacteria to remain attached to biotic or abiotic surfaces. Many colonizing *S. aureus* isolates were shown to be defective in the global virulence regulator**.** There is evidence to suggest that increased capacity to form biofilms and adhere to epithelial cells may have been linked for example to the spread of the so-called Brazilian MRSA clone (ST239)[38]**.** Thus, increases the chances of mortality and morbidity specially in patients with wound. Regular surveillance of biofilm formation by *Staphylococcus aureus* with their anti-biogram may lead to the early treatment of wound infections.

2.9 Available Antibiotics for the Treatment of Infections

Table 2.3 Treatment options for the management of Infection[27]

Treatment	***Staphylococcus aureus***
Since *Staphylococcus aureus* rapidly develops drug resistance antibiotics should be cautiously chosen. **Parenteral therapy for serious infections** Sensitive to penicillin **DOC:** Penicillin G Sensitive to methicillin **DOC:** Nafcillin or Oxacillin Resistant to methicillin (MRSA) **DOC:** Vancomycin (15-20mg/kg body weight) **Or Alternative Drugs for MRSA:** 1. Teicopanin 2. Daptomycin 3. Linezolid 4. Quinopristin/Dalfopristin **5.** ceftobiprole **Emperical therapy (if MRSA status not yet known)** Vancomycin with or without an aminoglycoside (vancomycin is indicated only if MRSA risk is high or condition is serious, e.g. cardiac implant) **Oral therapy for skin and soft tissue infections** Sensitive to methicillin **DOC**: Dicloxacillin, Cephalexin/Cefazolin Resistant to methicillin (MRSA) **DOC:** Clindamycin **Or Alternative Drugs for MRSA:** 1. Linezolid 2. Doxycyclin 3. Cotrimoxazole	

***DOC (Drug of choice)**

Table:2.4 Mechanism of Action of Important Antibacterial Drugs[39]

Mechanism of Action	Drugs
Inhibition of cell wall synthesis	
1. Antibacterial activity Inhibition of cross-linking (transpeptidation)of peptidoglycan	**Penicillin, Cephalosporin, Imipenem, Vancomycin**
2. Inhibition of other steps in peptidoglycan synthesis	**Cycloserine, Bacitracin**
Inhibition of Protein Synthesis	
1. Action on 50S ribosomal Subunit	**Chloramphenicol, Erythromycin, Clindamycin, Linezolid**
2. Action on 30S ribosomal Subunit	**Tetracycline, Aminoglycoside**
Inhibition of Nucleic Acid Synthesis	
1. Inhibition of Nucleotide Synthesis	**Co-trimoxazole (Sulphonamides, Trimethoprim)**
2. Inhibition of DNA Synthesis	**Quinolones e.g. Ciprofloxacin**
3. Inhibition of RNA Synthesis	**Rifampin**

2.9.1 β-lactam Antibiotics

Penicillin, Cephalosporin (3rd generation cephalosporin like Ceftazidime, Ceftriaxone), Carbapenem (Imipenem, Meropenem) are the major β-lactam drugs they are named so because of the importance of the β-lactam ring. Penicillin has a five membered ring which is substituted in only one place whereas Cephalosporin has six membered rings adjacent to β-lactam ring are substituted in two places on the 7-aminocephalosporanic acid nucleus. Carbapenem are even more different than the above two. Imipenem (N-formimidoylthienamycin) has a methyl group in the ring in place of the sulfur. The β-lactam drugs act by inhibiting transpeptidase enzyme, the enzyme catalyzes the final cross-

linking step in the synthesis of peptidoglycan. In *Staphylococcus aureus*, transpeptidation occurs between the amino group on the end of pentaglycine cross-link and the terminal carboxyl group of the D-alanine on the tetrapeptide side chain. Because the stereochemistry of penicillin is similar to dipeptide, D-alanyl-D-alanine, penicillin can bind to the active site of the transpeptidase and inhibit its activity[39]. There are two other factors involved in the action of penicillin,

a) Penicillin binding protein (PBP)

b) Autolytic enzyme: Murein hydrolases are activated in penicillin treated cells and degrade peptidoglycan. In *Staphylococcus aureus* these enzymes are not activated so they are tolerant to the action of penicillin.

The Beta-lactam drugs act on the cell resulting in influx of water into the high-osmotic-pressure interior of the cell ultimately the cell dies. Penicillin in bactericidal, it kills cells the growing cells as at that time synthesis of new peptidoglycan and transpeptidation occurs.

The intact ring is essential for the antibacterial activity. Penicillinases (β-lactamases) cleave the ring. Benzylpenicillin (penicillin G) was frequently used in the treatment of *Staphylococcus aureus*. But soon it became resistant due to the β-lactamase enzyme. The access of the enzyme to the β-lactam ring was overcome by modification in the side chain with the addition of large aromatic rings containing bulky methyl or ethyl groups (methicillin, oxacillin, nafcillin, etc.). There are some structural analogues of penicillin like clavulanic acid and sulbactam. They have very little antibacterial activity but together they bind strongly to β-lactamase enzyme and thus protect the penicillin. The drug Amoxyclav is a combination of Amoxicillin and clavulanic acid, where

Amoxicillin inhibit bacterial cell wall mucopeptide synthesis and the acid inactivates wide range of β-lactamase enzyme.

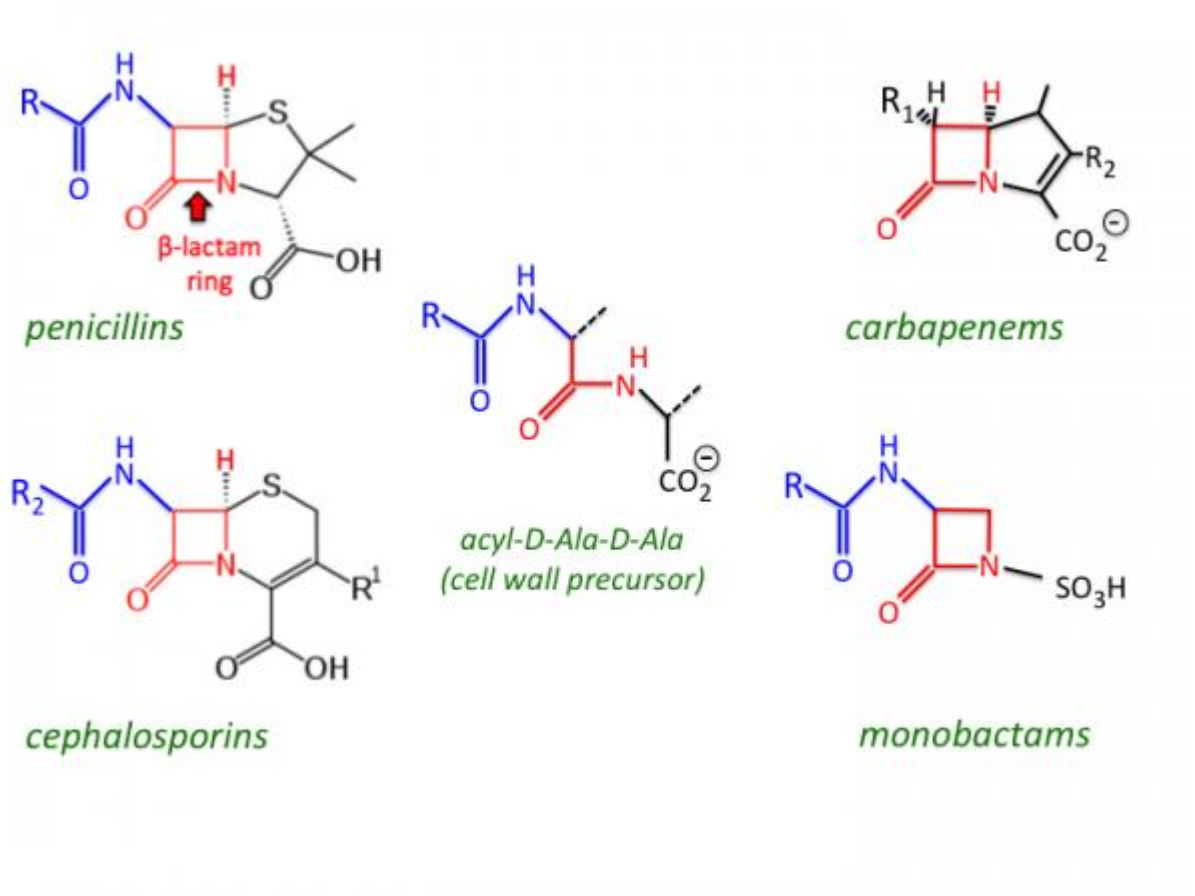

Figure 2.6 Beta-lactam drugs[40]

The major mechanism of resistance of MRSA to β-lactam antibiotics is due to the acquisition of the *mecA* gene encoding an additional penicillin-binding protein (PBP)**[41].** This can function as transpeptidase. Bacterial resistance to β-lactamase is generally driven either by the production of enzymes that inactivate them (β -lactamases), or by the modification of their targets in the cell wall (penicillin-binding proteins, PBPs), sometimes in conjunction with mechanisms leading to diminished permeability or active efflux **[42.]**

All *Staphylococcus aureus* isolates, both methicillin-sensitive and resistant strains, carry three high-molecular-weight penicillin binding proteins (PBPs), **PBP1, PBP2 and PBP3.** Most β-lactam

antibiotics bind to them. The β-lactam antibiotics generally target the transpeptidase domain of PBPs. This leads to a reduction in cell wall cross-linking and consequently a loss of cell wall integrity occurs [43]. PBP4 is the single low-molecular weight PBP. This has been shown to have a low affinity for most β-lactams. Thus, it is capable of substituting the biosynthetic functions of the normal PBPs. This is unique among low-molecular weight PBPs, it possesses both transpeptidase and carboxypeptidase activities [44].

Penicillin-binding protein (PBP2a) is coded by the *mecA* gene, which has a remarkable reduced affinity for many β-lactams[41]. β-lactamase enzyme is coded by *blaZ* which is present in >95% of MRSA strains. This confers penicillin-resistance. The genetic organization of the *mecA* locus is similar to that of the β-lactamase, which contains the structural gene *blaZ* and regulatory genes *blarRl-blal.* The mechanism involves two proteolytic steps:

i. Binding of the β-lactam antibiotic to the extracellular sensor domain of MecR1 leads to the autocatalytic cleavage of the sensor-inducer and activation of the cytoplasmatic inducer domain, which appears to be a prometalloprotease.

ii. The activated inducer domain of MecR1 either directly cleaves the promoter-bound MecI dimers por promotes the repressor cleavage, which disables the ability of the repressor protein to dimerize and bind to the *mecA* promoter, enabling the expression of the resistance gene"

2.9.2 Vancomycin

Vancomycin is a glycopeptide that inhibits cell wall synthesis by blocking transpeptidation. Other than penicillin they bind directly with the D-alanyl-D-alanine portion of the pentapeptide which blocks the transpeptidase from binding. They inhibit a second enzyme namely bacterial transglycosylase, which helps in synthesis of peptidoglycan partially. It has been regarded as the first-line drug for the treatment of MRSA. They are bactericidal work well against *Staphylococcus aureus, Staphylococcus epidermidis* and *enterococci*. In 1858, when this drug was first introduced, it was perceived that there would be no resistance to this antibiotic as resistance was very difficult to induce [19]. However, in 1997 the first strain of *S. aureus* with reduced

susceptibility to vancomycin was reported in Japan**.** According to the National Committee for Clinical Laboratory Standards (NCCLS), *Staphylococcus aureus* for which MIC of vancomycin is ≤4 µg/ml are sensitive, while isolates for which MIC of vancomycin is 8-16 µg/m1 are considered to be intermediate sensitive (Vancomycin intermediate *S. aureus).* Strains having MIC of vancomycin ≥32 µg/ml are designated resistant (vancomycin-resistant *S. aureus).* However, in Japan isolates with 8 µg/ml are considered VRSA. The resistance occured due to the replacement of alanine with the lactate in the peptidoglycan. The gene responsible for the resistance is *vanA* gene which is carried by transposons.

Compared with β-lactam therapy, vancomycin therapy has been associated with slower clinical response and longer duration of MSSA bacteremia, and it has been associated with more frequent complications in patients with endocarditis **[45].** A well-known adverse effect of the drug is "red man" syndrome. Red refers to the flushing caused by vasodilatation induced by histamine release from mast cells and basophils. Failure of vancomycin therapy may be observed in the treatment of patients with bacteremia due to strains of MRSA that have MICs of vancomycin well within the range considered susceptible. Heterogeneous vancomycin resistance, which is not readily detected by routine clinical laboratory methodology, is also associated with failure of vancomycin therapy**[46].**

2.9.3 Fluoroquinolones

Quinolones are bactericidal drugs that block bacterial DNA synthesis by inhibiting DNA gyrase (topoisomerase). Nearly all quinolone antibiotics in use are fluoroquinolones (Ciprofloxacin, Norfloxacin, Ofloxacin, etc.) which contain a fluorine atom in their chemical structure and are effective against both Gram-negative and Gram-positive bacteria. Quinolones are not recommended for Children and pregnant women as they cause damage to the growing bones[39]. DW286, a naphthyridone, is among several fluoroquinolones in development that have in vitro activity against MRSA. Active against MRSA strains that are resistant to other fluoroquinolones, it selects fluoroquinolone-resistant mutants at a lower frequency than do older agents (as may another fluoroquinolone, ABT-492)**[47].**

2.9.4 Linezolid

Linezolid is useful for the treatment of methicillin-resistant *Staphylococcus aureus*, it is bacteriostatic against the organism. They bind with the 23S ribosomal RNA in the 50S sub unit and inhibit protein synthesis, but the precise mechanism is unknown. It appears to block the early initiation step of ribosome formation. Together with vancomycin yield comparable results in hospitalized patients with MRSA infections at a variety of anatomic sites in a randomized, open-label trial [48], as well as in the treatment of skin and skin-structure infections caused by gram-positive organisms [49]. A retrospective subset analysis of two prospective randomized clinical trials found evidence suggesting that linezolid was superior to vancomycin in the treatment of hospital-acquired pneumonia due to MRSA [48].

2.9.5 Daptomycin

Daptomycin is a novel lipopeptide antibiotic with bactericidal activity against *S. aureus* that binds, in a calcium-dependent manner, to the bacterial cell membrane, disrupting membrane potential [50]. Daptomycin has received approval from the US Food and Drug Administration for the treatment of complicated skin and skin-structure infections due to susceptible gram-positive pathogens [51]. Daptomycin therapy failed in a trial involving patients with community-acquired pneumonia; daptomycin not only has limited penetration into pulmonary epithelial lining fluid, but its activity is inhibited by pulmonary surfactant [51].

2.9.6 Rifampin

Rifampin is a very potent, bactericidal and staphylococcal agent. This works with MICs of ≤ 0.05 µg/ml. It blocks mRNA synthesis by bacterial RNA polymerase without affecting the human cell RNA polymerase. They penetrate well into tissues and abscesses. The role of Rifampin in the treatment of staphylococcal infections is controversial. Rifampin is recommended as a component of nafcillin or vancomycin combination regimens for treatment of staphylococcal prosthetic valve endocarditis (Whitener, C., *et al.* 1993). In combination with other drugs they are widely used for the treatment of prosthetic-valve-endocarditis caused by *Staphylococcus epidermidis*. This combination has been used for the treatment of methicillin resistant

staphylococcal infections in patients who have not responded to vancomycin [52]. Rifampin is red in color and it is highly excreted in the urine, saliva and sweat of the patient turning their color into orange, which is absolutely harmless but disturbing for the patients. Resistance is due to chromosomal mutation in the gene coding for the b subunit of the bacterial RNA polymerase, resulting in ineffective binding of the drug.

2.9.7 Aminoglycosides

Aminoglycosides (Streptomycin, Gentamicin, Amikacin) are named for the amino sugar component of the molecule, which is connected by a glycosidic linkage to other sugar derivatives. They are bactericidal and work by inhibition of the initiation complex and misreading of messenger RNA (mRNA). The initiation complex is composed of a streptomycin treated 30S subunit, a 50S subunit, and mRNA will not function- like no peptide bonds are formed, no polysomes are made resulting in formation of "streptomycin monosome". The misreading of messenger RNA resulted in insertion of wrong amino acid into the protein. Ultimately the membrane of the bacteria is damaged a resulted in cellular death. Aminoglycosides have certain limitations in their use like renal toxixicity and autotoxicity (auditory and vestibular portion of eighth cranial nerve); poor gastrointestinal absorption; ineffectiveness against anaerobes as transport inside the cell requires oxygen.

Resistance to the drug occurs due to-

a) Modification of the drug by plasmid-encoded phosphorylating adenylylating and acetylating enzymes
b) Chromosomal mutation
c) Decrease drug permeability of the bacteria.

2.9.8 Tetracyclines

Tetracycline means four cyclic rings with different substituents at the three R group. The various tetracyclines (doxycycline, minocycline, oxytetracycline) have similar antimicrobial activity but differ in pharmacology. They are bacteriostatic but not selectively toxic for the human cell, because tetracycline in vitro will inhibit protein synthesis equally well in purified ribosomes in both

bacteria and human cells. The mode of action is inhibition of protein synthesis by binding to the 30S ribosomal subunit and by blocking the aminoacyl transfer RNA (tRNA) from entering the acceptor site on the ribosome.

Generally, they have low toxicity but may be presented with some side effects like-

a) Suppression of normal flora of the intestine lead to diarrhea and overgrowth of drug resistant bacteria;
b) Suppression of *Lactobacillus* in vaginal normal flora resulting in increased pH leads to over growth of *Candida albicans* , vaginitis.
c) Brown staining of the teeth of fetus and young children.
d) Photosensitivity

Tigecycline is the first clinically available member of the glycylcycline group of antibiotics, who shares almost same mode of action as well as similar adverse effects like tetracycline. They are used for the treatment of skin and skin structures infections caused by Methicillin sensitive as well as resistant *Staphylococcus aureus*[39].

Resistance to the drug occurs due to the failure of the drug to reach inhibitory concentration inside the bacteria. This occurs as the plasmid-encoded processes may reduce the uptake of the drug or enhance its transport out of the cell. In vitro susceptibility results involving tetracycline derivatives must be interpreted with caution, because *S. aureus* isolates that are tetracycline-resistant but that have relatively low MICs of doxycycline and or minocycline may, in fact, harbor inducible efflux genes [53].

2.9.9 Glycylcyclines

The minocycline derivative tigecycline has bacteriostatic activity against both MSSA and MSRA, including tetracycline-resistant strains [53]. In a randomized dose-comparison study, clinical cure rates were 67% and 74% in patients with skin and skin-structure infections who received 25 mg and 50 mg daily, respectively[10].

2.9.10 Macrolides

Macrolides (Azithromycin, Erythromycin and Clarithromycin) are bacteriostatic drugs. The name refers to their large (13-16 carbon) ring structure. They inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit and preventing the release of the uncharged tRNA after it has transferred its amino acid to the growing peptide chain. Thus, the donor site is occupied, a new tRNA cannot bind and protein synthesis seize. Erythromycin has shorter half-life so frequent administration needed. Resistance to the drug is primarily plasmid-encoded enzyme that methylates the 23S rRNA, thereby blocking the drug binding. An efflux pump may reduce the intracellular concentration of the drug resulting in low-degree resistance[59].

2.9.11 Clindamycin

Clindamycin has been used successfully in the treatment of invasive CA-MRSA infections in children. They are bacteriostatic drug. The mode of action comprises bind to the 50S subunit and blocks peptide bond formation by an undetermined mechanism. Its specificity for bacteria arises from its inability to bind to the 60S ribosomal subunit of human cell. Inducible resistance to clindamycin, however, is not detected by routine susceptibility testing, but requires the use of other methods (e.g. a double-disk diffusion test). Flattening of the zone in the area between the disks to resemble the letter indicates the presence of inducible resistance [54.]

2.9.12 Dalfopristin, Quinupristin

This combination is active in vitro against MSSA and MRSA. It is bactericidal against *S. aureus*, although in the presence of constitutive expression of macrolide-lincosamide-streptogramin resistance, it is only bacteriostatic. In a randomized trial, patients with nosocomial MRSA pneumonia who received quinupristin or dalfopristin had a clinical response rate of 19.4%, compared with 40% in vancomycin recipients [55.]

2.9.13 Co-trimoxazole

In combination with Trimethoprim the sulfonamides have wide range of antibacterial activities. The sulfonamides are bacteriostatic drugs that are produced by chemical synthesis. In 1935, the parent compound Sulfanilamide became the first clinically effective antimicrobial agent. They are the structural analogues of *p*-aminobenzoic acid (PABA). The mode of action of the drug is to block the synthesis of tetrahydrofolic acid, which is required as a methyl donor in the nucleic acid precursor adenine, guanine, and thymine. PABA condenses with a pteridine compound to form dihydropteroic acid, a precursor of tetrahydrofolic acid. Sulfonamides compete with PABA for the active site of the enzyme dihydropteroate synthetase. Whereas trimethoprim inhibits the production of tetrahydrofolic acid by inhibiting the enzyme dihydrofolate reductase. They are selectively toxic, as many bacteria synthesize their folic acid from PABA-containing precursors, whereas human cells require preformed folic acid as an exogenous nutrient because they lack the enzyme for synthesis. Human cells thus bypass the step. These drugs are cheap however the side effects include fever, rash, photosensitivity and bone marrow suppression. Sometimes they are responsible for erythema multiforme, Stevens Johnson Syndrome. The advantages of combined drug therapy are that they act synergistically thus bacterial mutants resistant to one drug will be inhibited by other[39].

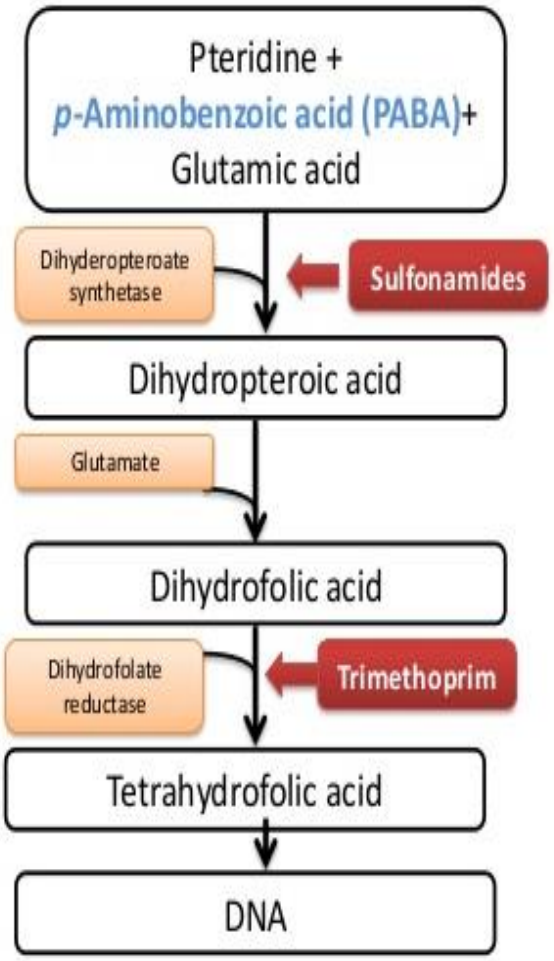

Figure 2.7 Site of action of Sulfonamide and Trimethoprim Drugs

Resistance to sulfonamides is due to plasmid- encoded transport system that actively exports the drug out of the cell and chromosomal mutation in the gene coding for the target enzyme thus reduces the binding affinity of the drug. Whereas Trimethoprim resistance occurs due to chromosomal mutation in the gene coding for the target enzyme

2.9.14 Novel β-lactams

A series of β-lactamase stable cephalosporins with high affinity for PBP2a are in clinical development. The PBP2a affinity of BMS-247243 is 100-fold greater than that of methicillin or cefotaxime, and the drug is bactericidal against MRSA at twice the rate of vancomycin. Other drugs of this class in development include the zwitterionic cephem RWJ-54428, CB-181963, BAL5788, a prodrug of BAL9141, and S-3578. ME1036 (formerly CP5609) is a C2-modified carbapenem with high affinity for PBP2a and with an MIC of 2.0μg/mL -against MRSA. SM-197436, SM-232721, and SM-232724 are novel methyl-carbapenems that are also active in vitro against MRSA[56].

2.10 Different Antibiotics Resistance Mechanism

2.10.1 Mechanism of β-lactam resistance

Penicillin resistance in the bacteria is increasingly recognized since 1945. Nearly 80% or more strains of *Staphylococcus aureus* became resistant to penicillin. There are three known mechanisms for which S. aureus becomes resistant to penicillin.

- **Hyper production of β-lactamase or plasmid mediated resistance**

  It occurs due to production of enzyme beta-lactamase which is plasmid mediated. The production of β-lactamase that cleave the β-lactam rings, thus inactivates penicillin. *Staphylococcus* produce four types of penicillinases (A, B, C, D) anchored on the cytoplasmic membrane that participate in the cross linking of the peptidoglycan of the bacterial cell wall. Normally PBP's activity similar to that of serine proteases and have high affinity for β-lactam agents. When this binding occurs, the PBPs are not able to function in the assembly

of the cell wall causing bacterial death. Penicillinase plasmids are transmitted to the *Staphylococci* by both transduction and conjugation. The plasmids also carry markers of resistance to heavy metals, such as arsenic, cadmium, mercury, lead and bismuth as well as to other antibiotics, such as erythromycin and fusidic acid. It is difficult to treat staphylococcal infection caused by β-lactamase producing strains. However, *S. aureus* strains resistant to these agents [57].

- **Chromosomal mediated resistant:** This type of resistance occurs due to modification of normal penicillin-binding proteins (PBPs). Thus, reduction in the affinity of the PBPs to the β lactam drug also contributes to the resistance of the bacteria to penicillin and other β lactam antibiotics.
- **Tolerance to Penicillin**: The presence of an acquired penicillin-binding protein **[19]** play role. Staphylococcus developing tolerance to penicillin are only inhibited but not killed. Penicillin resistant strains can be treated with beta-lactamase resistant penicillin e.g. oxacillin, flucloxacillin, cloxacillin, methicillin, or vancomycin.

Borderline oxacillin resistant *Staphylococcus aureus* (BORSA)

Occasionally a non *mec A* gene mediated low level resistance to oxacillin is observed in some strains of *Staphylococcus aureus* this might be due to hyper production of β lactamase.

2.10.2 Mechanisms of Methicillin Resistance

Methicillin resistant *Staphylococcus aureus* (MRSA) denotes resistance of *Staphylococcus aureus* to penicillin, as well as to all other β-lactam antibiotics including the third generation Cephalosporins and Carbapenems. Resistance to penicillin is due to the production of novel PBPs, designated as PBP2a. PBPs are target structure of β-lactam antibiotics. Infections caused by penicillin-resistant *S. aureus* were initially limited to hospitalized patients and were only later detected in the community, where they eventually became common[21] after 1980**.** In an historical reprise, the identification of methicillin-resistant *S. aureus* (MRSA) was reported within 1 year after the 1960 introduction of this semisynthetic medicine. This resistance -

- Is Chromosome mediated
- Can be transferred between *Staphylococcus aureus* strains by transduction
- Is produced by >90% strains of *Staphylococcus aureus*.

PBP is an essential protein needed for cell wall synthesis of bacteria. The β lactam drugs bind and inhibit this protein there by inhibit the cell wall synthesis. Normally PBPs activity similar to that of serine proteases and have high affinity for β-lactam agents. When this binding occurs, the PBPs are not able to function in the assembly of the cell wall causing bacterial death. On the other hand, PBP2a is not part of the intrinsic set of PBPs of *S. aureus* has a unique protein that has a molecular weight of approx.76kDa. This is produced only by methicillin-resistant staphylococci. Isolates containing the PBP2a resistance mechanism are clinically resistant to all available β-lactam, including penicillin, cephalosporin, β-lactam or β-lactamase inhibitor combinations**.**

PBP2a is mediated by a chromosomally coded gene called the *mec A* gene, which alters PBP present on the *Staphylococcus aureus* cell membrane to PBP2a. The altered PBP2a of MRSA strains has less affinity for β lactam antibiotics. Hence MRSA strains are resistant to all β-lactam drugs. There is an increasing trend of MRSA rate over last few decades. Though it varies from place to place, overall about 30-40 % strains of *Staphylococcus aureus* are MRSA. The spread of MRSA from the hospital to the community was a predictable event. The emergence in the past decade of novel strains of MRSA in the community that are genetically distinct from MRSA strains originating in the hospital was perhaps less anticipated. MRSA usually colonizes the broken skin and can cause a wide range of local and systemic infections. Their association with health care may, however, have been indirect; household contacts of individuals with hospital-acquired MRSA (HA-MRSA) are at significantly increased risk for MRSA colonization. These strains can cause a wide range of infections including bacteremia, endocarditis, and pneumonia. More increasingly they have been recognized in hospitalized patients undergoing prosthetic heart valve surgery.

At a Houston pediatric hospital, 74% of community-acquired S *aureus* strains isolated since 2001 have been resistant to methicillin **[58].** Clusters and outbreaks in adolescents and adults have been reported to occur in Native Americans, homeless youth, military recruits, children in child care centers, and competitive athletes. Although most infections have involved skin and skin structures, potentially lethal invasive infections have also occurred.

MRSA strains can be treated with glycopeptide antibiotics such as Vancomycin and Teicoplanin in serious systemic infections bacteremia, endocarditis, and pneumonia. MRSA is sensitive to one or more of the second line drugs, which include Erythromycin, Clindamycin, Quinolones, Fusidic acid, Trimethoprim, Chloramphenicol, Tetracycline, Rifampin. However, Ciprofloxacin, Rifampin and Fusidic acid are not used regularly because of possibility of emergence of resistance.

Tab. 2.5 Types of MRSA[27]

Community Associated MRSA(CA-MRSA)	**Hospital Associated MRSA(HA-MRSA)**
1. These strains express *mec*A gene subtype IV, V, VI.	1. These strains express *mec*A gene subtype I, II, III.
2. They are usually more virulent and express several toxins such as PVL.	2. They are multidrug resistant (but their virulence is relatively low)
3. They cause invasive skin and soft tissue infection such as necrotizing fasciitiss.	3. They cause perioperative wound infections in hospitals and nosocomial outbreaks (hospital staffs are the major carriers)

2.10.3 Mechanisms of Macrolide Resistance

The first mechanism of macrolide resistance in clinical isolates of *Staphylococci* due to post-transcriptional modifications of the 23S rRNA by the adenine-N-6-methyltransferase, that leads to methylation of adenosine 2058 (A2058) of 23S rRNA within the large ribosomal subunit **[60].** These ribosomal methylases are encoded by *erm* genes. In some cases, ABC transporters encoded by plasmid borne *msrA* gene cause active efflux of 14-membrane-ring (Erythromycin, Clarithromycin and Dirithromycin) or 15-membrane-ring (Azithromycin) macrolides. Rarely staphylococci

strain has been reported to produce a macrolide phosphotransferase which inactivate some to these antimicrobials.

2.10.4 Mechanisms of Cephalosporin resistance

The most important bacterial mechanism of resistance to the Cephalosporin is the production of β-lactamases. These enzymes are present in virtually all gram-negative and gram-positive bacteria and act to hydrolyze the cyclic amide bond of the β-lactam ring, rendering it inactivate. Decreased binding affinity of PBPs for antibiotics is another mechanism of bacterial resistance and is the reason that cephalosporins are ineffective against methicillin-resistant *Staphylococcus aureus*.

2.10.5 Mechanisms of Vancomycin Resistance

Staphylococcus aureus infections causes mainly due to strains that had become resistant to methicillin [so-called Methicillin Resistant Staphylococcus aureus (MRSA)]. However, in July 2002, things changed when the Centers for Disease Control (CDC) in the USA published the first documented report of *Staphylococcus aureus* that was resistant to vancomycin as well as being resistant to methicillin. Erroneous and overuse of vancomycin has leads to the emergence of resistant to vancomycin. It may be of low-grade resistance to vancomycin known as VISA (vancomycin intermediate *Staphylococcus aureus*) or high-grade resistance, known as VRSA (Vancomycin resistant *Staphylococcus aureus*). Approximately 5 years earlier, the Japanese had reported the first strain of S. aureus with reduced (or intermediate) susceptibility to vancomycin followed by 2 additional cases from the USA. These earlier isolates were termed Vancomycin Intermediate Staphylococcus aureus (VISA).

The mechanisms of resistance that have been identified for VISA and VRSA strains are quite different and are not fully understood. For VISA strains, the proposed mechanism resulting in reduced susceptibility to Vancomycin is believed to be a thickening of the bacterial cell wall such that vancomycin is trapped within the bacterial cell wall and is thus unable to reach its target on the surface of the bacterial cytoplasmic membrane. The VRSA strain that was isolated from the patient in Michigan as well as a second isolate from a patient in Pennsylvania contained the *vanA* gene

which codes for an altered target such that the binding of vancomycin to the target is significantly reduced and thus it cannot carry out its normal function of inhibiting bacterial cell wall synthesis. The source of the *vanA* gene isolated in VRSA appears to have come from co-infection with vancomycin resistant *Enterococcus faecalis* (VRE) by horizontal conjugal transfer. The treatment of VRSA should be based on antimicrobial susceptibility report. Linezolid, telavancin, daptomycin and quinupristin/ dalfopristin are effective drugs.

The future of VISA and VRSA strains is not clear and much research is needed to help further understand all aspects of these organisms including their epidemiology, microbiology, clinical and infection control implications and optimal treatment. As well, in addition to VISA and VRSA strains, there appear to be strains of *S. aureus* that are referred to as hetero-resistant.

2.10.6 Mechanisms of Quinolones Resistance

Fluoroquinolone antibiotics exert their antibacterial effects by inhibition of certain bacterial topoisomerase enzymes, namely, DNA gyrase (bacterial topoisomerase II) and topoisomerase IV. These essential bacterial enzymes alter the topology of double-stranded DNA (dsDNA) within the cell **[61].** DNA gyrase and topoisomerase IV are heterodimeric proteins composed of two subunits, designated A and B. The genes encoding A and B subunits are referred to as *gyrA* and *gyrB* (DNA gyrase)[62]. Mechanisms of bacterial resistance to fluoroquinolones fall into two principal categories, alterations in drug target enzymes and alterations that limit the permeation of drug to the target. The target enzymes are most commonly altered in domains near the enzyme active sites, and in some cases reduced drug binding affinity has been demonstrated. Alterations of target enzymes appear to be the most dominant factors in expression of resistance to quinolones.

Staphylococcus aureus show resistance to quinolones by an efflux system that expels quinolones and prevents antibiotics from reaching enough concentration in the cytoplasm to exert their antimicrobial activity. The efflux activity is not enough to explain the full range resistance to quinolones it is suggested that fluoroquinolone efflux must occur together with other resistance mechanism**.**

Chapter 3: Methods and Materials

University of Dhaka
Department of Microbiology

3 Methods and Materials

3.1 Methodology

This study was conducted in the laboratory of Department of Microbiology, Holy Family Red Crescent Medical College and hospital with the collaboration of the Department of Microbiology, Dhaka University. Samples were collected from both indoor and outdoor cases of HFRCMCH. In case of indoor patients, samples were taken from the ICU, NICU, dialysis units and post- operative wards where mostly foreign bio-materials inserted frequently. *Staphylococcus aureus* were isolated from clinical samples like blood, pus, wound swab from abscess. The patients comprised of both sexes and all age groups. The various clinical specimens were identified by colonial morphology, microscopic examination and relevant biochemical test according to standard laboratory methods. Culture was done on blood agar, mannitol salt agar, nutrient agar media. All specimens were inoculated onto the media and incubated at 37°C. The identification of *Staphylococcus aureus* was performed by subsequent gram staining. After isolation and identification virulence factors were assessed following the analysis of growth pattern and fermentation on mannitol salt agar media, colonial pigmentation and hemolysis pattern on blood agar media. Hemolysis on blood agar plates was observed for the analysis of haemolysin[20]; catalase, coagulase biochemical tests were also evaluated to identify the enzymes. PCR based methods was done to determine the *mecA* gene in *Staphylococcus aureus.*

3.2 Over view of Method and Materials

- **Sample Collection**

⬇

- **Swab**

⬇

- **Mannitol Salt Agar** ➡ **Gram staining**

⬇

- **Blood Agar**

⬇

- **Nutrient Agar**
  - ✓ **Stock Preparation**
  - ✓ **Biochemical tests**
  - ✓ **Genomic DNA Extraction**
- **Antibiotic Susceptibility testing by Disc diffusion method (modified Kirby Bauer method)**

3.3 Sample collection

Staphylococcus aureus is hardy and is usually easy to recover from swabs, pus, tissue and blood culture specimens. Under aseptic precaution pus was collected by swab from the patients with abscess, wound infection, burnt area, anterior nares infection. Swabs were in wooden applicator sticks which were sterilized earlier in tubes in the autoclave. Care was taken to avoid contact of the cotton swab with another source. After collection, the tubes were properly capped, labeled and transferred to the laboratory. All the clinical samples transported immediately within half an hour to the laboratory to avoid drying.

3.4 Culture media used for isolation of the bacteria

The following media were used for isolation of bacteria:

1. **Mannitol salt agar (MSA)**

   MSA was used for selective isolation of *Staphylococcus aureus*. They are salt tolerant. According to mannitol fermentation they can be differentiated. After incubation at 37°C Coagulase positive *Staphylococci* produce yellow halo surrounding the colonies, whereas coagulase negative *Staphylococci* produce small pink or red colonies with no color change to the medium[63].

2. **Nutrient agar**

   This media was used for sub-culturing purpose and for studying morphological properties of bacterial colonies.

3. **Blood agar**

   Media was used for the isolation of all possible bacterial species. The collected samples were streaked on the media for the detection of hemolytic properties of the pathogens. Then incubated at 37°C overnight for the growth. Blood agar is a enrich media. Most strains of *Staphylococcus aureus* are β-hemolytic (i.e. produce complete lysis of red blood cells resulting in a zone of clearing). Colonies grown on blood agar are small, shiny, golden-yellow pigmented and surrounded by a zone of clearing[64].

3.5 Inoculation of specimens

Cotton swabs on wooden applicator sticks were prepared. They were sterilized in tubes in the hot air oven earlier. The cotton-swab stick containing specimen was smoothly rolled on the Mannitol salt agar (MSA) media. A sterile inoculating wire loop was used to streak the specimen loaded in the corner of the culture plate. The inoculated plates were incubated at 37°C. Following 24 hours growth the morphological and cultural characteristics were observed.

3.6 Identification of *Staphylococcus aureus*

3.6.1 Isolation of the organism

After collecting the samples, immediately inoculated in MSA medium. *Staphylococcus* grows easily on MSA plates. Which is selective for *Staphylococcus* as it contains 7.5 % NaCl (Halo-tolerant). They gave small, round, smooth, white, creamy colonies on them after 18 to 24 hours of incubation at 37℃.

Colony characteristics

- Size : 1-3mm
- Pigmentation : Yellow
- Form : Circular
- Margin : Entire
- Elevation : Raised

3.6.2 Bacterial pure Culture

Following incubation, growth on bacteriological media was observed carefully under proper illumination. Once colonies found on MSA plates the morphological discrete colonies were isolated, inoculated and grown on Nutrient agar (NA) and in Blood (Sheep) agar medium for pure culture[63]. When incubated for extended time on blood agar plates *Staphylococcus* produce hemolytic (mainly β) zones around the colonies and sometimes yellow pigmentations. The colonies on both the plates were observed for the characteristics mentioned below:

- **Abundance of growth:** The amount of growth was defined as none, slight, scanty, moderate or large.
- **Hemolytic properties:** β (complete hemolysis)
- **Shape of the colonies:** The organisms were circular having unbroken peripheral edge, irregular having indented peripheral edge and rhizoid having root-like spreading growth.
- **Size of the colonies:** The colonies were pinpoint, small, moderate, or large.
- **Margin:** The appearance of the outer edge of the colony was described as entire that is sharply defined and even, lobate with marked indentation,

undulate with wavy indentation, serrate with tooth like appearance and filamentous having thread like spreading edge.

- **Surface texture:** Depending on the species, the colony surface may be smooth shiny glistening.
- **Elevation:** The degree to which colony growth was raised on the surface of the agar plate was cited as flat with elevation which was not discernible, raised with slight elevation, convex having dome-shaped elevation and elevated convex central region.

3.6.3 Microscopic observation after gram staining

The size, shape, arrangements, staining, and properties of the selected strains were determined through the microscopic examination. The total procedure of gram staining is described below according to the following steps[65,66].

3.6.3.1 Preparation of smear

A small colony was picked up from blood agar media with a sterile inoculating wire loop, smeared on separate glass slide with a drop of sterile normal saline and fixed by allowing it to dry in the air [64].

3.6.3.2 Fixation of smear

Each smear was fixed by heating the under surface of the slide over a gas burner. Then the slide was allowed to cool before staining. The fixed slide was stained by following Gram staining method.

3.6.3.3 Gram Staining

The portion of the slide containing smear was covered with crystal violet (primary stain) for one minute. Crystal violet was washed rapidly with distilled water and smear was covered with Gram's iodine (mordant) for one minute. Then the smear was decolorized rapidly with 95% ethyl alcohol (decolorization). The alcohol was rinsed off immediately with distilled water. Finally, the slide was counter stained with safranin (counter stain). Waited for another 30 sec and rinse well with distilled water and allowed to air dry. The stained slide was examined microscopically. The 100x oil immersion object was checked to look for the bacteria[67].

3.6.3.4 Microscopic characteristics

Organisms in stained slides were examined microscopically to observe their arrangements. The isolates revealed Gram-positive, cocci shaped organisms, arranged singly or in pairs, and in irregular, grape like clusters.

3.7 Identification of isolate by standard biochemical tests

All suspected *Staphylococcus aureus* colonies were inoculated firstly on nutrient agar and allow them to grow overnight at 37°C. On the following day the several biochemical tests including catalase, coagulase tests were performed to identify *Staphylococcus aureus*[36].

3.7.1 Controls for tests

The sterility of each batch of test medium was confirmed by incubating one uninoculated tubes of the batch along with the inoculated ones. Controls were used to confirm that the test media have been made up correctly and they were used and observed under the proper conditions. There was no growth on the uninoculated tubes thus confirmed the validity of the test.

3.7.2 Catalase test

The enzyme catalase is capable of decomposing hydrogen peroxide into water and molecular oxygen. 5ml of 3% hydrogen peroxide was taken on a test tube and a loopful of fresh test culture was added. Production of bubbles indicated the positive results.

3.7.3 Indole test

Freshly grown isolated colony of *Staphylococcus aureus* was inoculated into peptone water medium and incubate at 37℃ for 24hrs. After incubation 10 drops of kovac's reagent was added and gently agitated. Absence of red coloration in reagent indicated that indole was not produced.

3.7.4 Citrate utilization test

Simmon's citrate agar medium was streaked with a loopful of fresh *Staphylococcus aureus* culture and incubated at 37℃ for 24hrs. There was no change in color from green to deep Prussian blue indicates negative citrate utilization.

3.7.5 Oxidase test

For this test, a freshly grown isolated colony on nutrient agar was taken with a sterile loop and rubbed on a strip of a filter impregnated with freshly prepared solution of 1% tetramethyl-p-phenylenediamine dihydrochloride. Dark blue color indicates positive result.

3.7.6 Methyl red (MR) and Voges Proskauer (VP) test

A freshly grown isolated colony of *Staphylococcus aureus* was inoculated into MR-VP broth and incubated at 37℃ for 24hrs. Then the culture split into two part one for MR test, 5-6 drops of methyl red solution was added into medium and shaken well for change in color (red) which indicate the presence of *Staphylococcus aureus*. While yellow or orange color indicated a negative result. Other portion for VP test, 5 drops of alcoholic α- napthol solution was added into medium followed by 5 drops of potassium hydroxide (KOH) and shaken vigorously for 1-2 minutes. Appearance of crimson ruby color indicated the production of acetyl methyl carbinol means positive result.

3.7.7 Kligler's Iron Agar (KIA) test

Kligler's Iron Agar was used for the differentiation of microorganisms on the basis of dextrose and lactose fermentation and hydrogen sulfide production. A needle was touched to an isolated colony and tabbed into the soft agar then streaking of colony into the slant. The tubes were incubated at 37°C for 24 hours.

3.7.8 Motility Indole Urea (MIU) Test

The test was carried out in motility indole urea semisolid media. Suspected isolated colony was touched with a sterile needle into agar very carefully down the tube without touching the bottom. The tube was incubated at 37°C for 24 hours.

3.8 Serological Test

3.8.1 Slide coagulase test

The slide coagulase test detects the bound coagulase or the clumping factor. It was performed by making a heavy mixture to a homogeneous composition, adding 1 drop of plasma with normal saline. Clumping within 10 second indicated a positive result for coagulase. This test is done for differentiation of *Staphylococcus aureus* from the coagulase negative *Staphylococcus* species.

3.8.2 Latex agglutination test

The latex agglutination test is a rapid diagnostic slide test for Staphylococcus aureus. The HiStaph^{TM} latex test kit (HIMEDIA) uses protein-coated latex particles that are able to detect the clumping factor (bound coagulase and protein A) that causes the *Staphylococcus aureus* to adhere to the black latex particles, producing a visible agglutination. This is a confirmatory test for the identification of presumptive Staphylococcus aureus colonies from primary plate culture. After labeling slides, one drop of *Staphylococcus* latex reagent in the center of the circle was added. With an applicator stick spreading of colony of each organism in the reagent was done. By rotating the slide in a circular motion for 60 seconds all slides were observed for the presence or absence of agglutination[36].

3.9 Methods of Antibiotic Sensitivity Test

The methods for susceptibility testing of *Staphylococci* most commonly used in clinical laboratories are disc diffusion, broth dilution, agar screen methods. Sensitivity in detection of resistance can be strain dependent, which probably is due to differences in sizes of the resistant sub-population within strains. The specificity of these systems is high however and strains testing resistant are unlikely to be susceptible by other methods. Standard methods of sensitivity testing by disc Diffusion are adequate for *Staphylococcus.* There will be zone of inhibition.

3.9.1 Determination of Antimicrobial susceptibility by disc diffusion method (modified Kirby Bauer method)

To prepare the inoculums from the primary culture plate, 3-5 colonies of appearance of the test organism were collected with a sterile loop and transferred to a tube of sterile saline. The inoculum had to be made from a pure culture, loopful of the confluent growth was similarly suspended in saline. The tube was compared with the 0.5 McFarland standard and the density of the test suspension was adjusted to that of the standard by adding more bacteria or more saline. Proper adjustment of the turbidity of the inoculums was essential to ensure that the resulting lawn of growth was confluent or almost confluent. The plates were inoculated by dipping a sterile swab into the inoculums. Excess inoculums ware removed by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. The swab was streaked all over the surface of the medium three times, rotating the plate through an angle 60 º after each application. Finally, the swab was passed round the edge of the agar surface. The inoculums were left to dry for a few minutes at room temperature with the lid closed. The antibiotic discs were placed on the inoculated plates using a sterile needle tip. It was convenient to use a template to place the disc uniformly. A several discs were placed on 90mm plate. The plates were placed in an incubator at 35°C within 30 minutes of preparation. A temperature above 35°C might be invalid for oxacillin or methicillin. After overnight incubation, the diameter of each zone (including the diameter of the disc) was measured with a ruler on the under surface of the plate without opening the lid and recorded in mm[68]. The MSA plates were incubated at 33-35^0 C which is favorable for the growth of (MRSA).

Table 3.1 Concentrations and diffusion zone breakpoints for resistance for antimicrobial agents tested in this study

Antibiotics	Abbreviation form	Disc drug concentration (µg)	Diffusion zone breakpoint (mm)		
			Resistant	Intermediate	Sensitive
I.Oxacillin	OX	1	≤10	15-16	≥17
II.Amoxyclav	AMC	20	≤13	14-17	≥18
III.Co-trimoxazole (trimethoprim/Sulfamethoxazole)	COT	1.25/23.75	≤10	11-15	≥16
IV.Tetracycline	TET	30	≤14	15-18	≥19
V.Ciprofloxacin	CIP	5	≤15	16-20	≥21
VI.Ceftriaxone	CTR	30	≤13	14-20	≥21
VII.Ceftazidime	CAZ	30	≤14	15-17	≥18
VIII.Vancomycin	VAN	30	≤9	10-14	≥15
IX.Gentamycin	GN	10	≤12	13-14	≥15
X. Erythromycin	E	15	≤13	14-22	≥23
XI.Azithromycin	AZ	15	≤15	15-16	≥17
XII. Amikacin	AK	30	≤14	15-16	≥17
XIII.Imipenem	IMI	10	≤15	16-18	≥19
XIV. Meropenem	MEM	10	≤15	16-18	≥19

Reference [119,120]

3.10 Detection of MRSA

Antimicrobial susceptibility test by disc diffusion method. Test can be done by using Cefoxitin or oxacillin discs[69,70]. Cefoxitin is the recommended disc to be used. If oxacillin disc is used, then certain conditions to be maintained such as- using media containing 2-4% NaCl, incubation at 35ºC and full 24 hours incubation. A colony of *Staphylococcus aureus* was picked and a suspension of 0.5 McFarland standard was made. Inoculation of this suspension was done in Muller Hinton Agar media. An oxacillin disc containing 1 microgram of oxacillin was placed over the inoculated media and incubated at 35°C for 24 hours. Zone diameter of < 10mm was taken as oxacillin resistance and this was regarded as MRSA. According to the Clinical and Laboratory Standards Institute (CLSI) S. aureus strains exhibiting growth on MHA supplemented with 2% NaCl and ≥ 4 μg/ml Oxacillin are considered methicillin-resistant while S. aureus strains only exhibiting growth on MHA supplemented with 2% NaCl when Oxacillin-levels are ≤ 2 μg/ml are considered methicillin-susceptible[63].

3.11 Stock preparation

(Preservation of Bacterial Isolates / Sub- culturing of Isolates.)

Isolate those produced yellow colonies on MSA media were sub-cultured in Nutrient agar plates for pure colony. After streaking, the plates were incubated overnight at 37ºC. After that Glycerol Stock preparation was done. Duplicate stock samples were prepared from each of the isolates in 20 % glycerol. Isolates were grown overnight on Nutrient broth at 37ºC. After 16-18 hours of growth, 0.80 ml of culture was transferred to sterile microcentrifuge tube containing 0.20ml of sterile glycerol. Content of centrifuge tube mixed well and kept primarily at -20ºC for future use.

3.12 Molecular Characterization

3.12.1 Chromosomal DNA extraction by DNA boiling method

Drug resistance to methicillin and oxacillin is independent of β-lactamase production. The *mecA* gene for the drug resistance resides on the chromosome, and the gene encodes a low affinity penicillin binding protein (PBP2 or PBP2a)[65]. The resistance is linked to the acquisition of a mobile genetic element

(staphylococcal cassette chromosome) harboring the *mecA* gene, (SCCmec) (Ubukata et al., 1989). DNA extraction of all isolates with reference strain was performed by following procedure-

For total DNA extraction, an isolated colony of each strain was inoculated into 5 ml of LB (Luria Bertani broth) media and incubated overnight at 37^0C with shaking at 200rpm in orbital shaker. 1 ml of culture was taken into an autoclaved Eppendorf tube and centrifuged for 5 min at 10000 rpm. Supernatant was discarded and the pellet was dissolved in 200µl PCR grade water into the pellet by pipetting up and down vigorously. Then the bacterial suspension was boiled at 100℃ for 10 min. After boiling, Eppendorf's containing boiled culture were placed in ice for 10 min. 100-150µl of supernatant was collected into another Eppendorf tube and stored at -20℃. Concentration of DNA was measured by Nanodrop 2000 spectrophotometer (Thermo SCIENTIFIC, USA). It was kept at -20^0C until used for PCR reaction.

3.12.2 PCR amplification

The PCR amplification was performed in a thermal cycler (Primus 25) by using a recombinant Taq DNA polymerase. The reaction mixture consisted of 50µl of lysate, 10µl of 10X PCR amplification buffer (200 mM Tris-HC1 [pH 8.3], 500 mM KCI, 15 mM $MgCl_2$, 0.1% [w/v] gelatin, 0.5% Tween 20), 2.0 µl of each primer (20 µM stock solution), 10 µl of the deoxynucleoside triphosphates (1 mM each in stock solution), 0.4 µl of AmpliTaq (5 U/µ1 of stock solution), and 25.6 µl of nuclease free water. Mineral oil (50 µ1) was added to the mixtures to inhibit evaporation. A total of 30 PCR cycles were run under the following conditions:

- DNA denaturation was done at 94°C for 1 min,
- Primer annealing at 55 ° C for 0.5 min, and
- DNA extension at 72°C for 1.5 min.

After the final cycle, the reaction was terminated by keeping it at 72^0 C for 3.5 min. The PCR products were stored in the cycler at 4^0 C until they were collected.

3.13.3 Detection of *mecA* gene by PCR

Sets of primer specific for the detection of methicillin resistance determinant of *Staphylococcus aureus* species are used (Table 3.4) to confirm the methicillin resistance determinant. Isolated DNA was amplified [71,72]. The following oligonucleotide primers were used:

Table: 3.2 Primer used in multiplex PCR for the detection of methicillin resistant determinants

Target sequence	Name of the primer	Primer sequence	Product size (bp)
mecA	A-1(F) A-2(R)	5′-AAAATCGATGGTAAAGGTTGGC 5′-AGTTCTGCAGTACCGGATTTGC	533

3.13.4 Visualization and Interpretation of results

After staining with ethidium bromide(0.5µg/ml) and de-staining, gel was observed under UV Transilluminator (Gel Doc, Major science, Taiwan) and DNA bands were identified according to their molecular size by comparing with 1000bp DNA ladder. Samples showing the presence of specific DNA band corresponding to 533bp were considered positive for presence of *mecA* gene.

Chapter 4: Results

University of Dhaka
Department of Microbiology

4.Results

4.1 Prevalence of Staphylococcus aureus

In this study a total of **565 samples** were collected from different patients (both indoor and outdoor cases) of Holy Family Red Crescent Medical College and Hospital. Among them 283 (50.05%) samples were positive for *Staphylococcus.* Out of 283 *Staphylococcus* **120 samples (21.2%)** were coagulase positive for *Staphylococcus aureus*, whereas 163 samples (28.85%) were coagulase negative *Staphylococcus*. That means among **565 clinical samples 21.2%** cases were ***Staphylococcus aureus***. (Table 4.1)

Table 4.1 Isolation rate of *Staphylococcus aureus* from different clinical samples (n=565)

Staphylococcus	**Number of isolates (%)**
Staphylococcus aureus	**120 (21.2%)**
Coagulase negative Staphylococcus	163 (28.85%)
Total	283 (50.05%)

4.2 Phenotypic Characterization

Mannitol salt agar (MSA) is a selective medium for *Staphylococcus aureus*. High salt concentration act as a selective agent that allows only the growth of *Staphylococcus spp.* The phenol red used as an indicator that differentiate *Staphylococcus aureus* from other *Staphylococcus sp.* by fermenting phenol, thus changes the color of MSA plate from red to yellow due to production of acid.

Colony characteristics of *Staphylococcus aureus*

- Size : 1-3mm
- Pigmentation : Yellow
- Form : Circular
- Margin : Entire
- Elevation : Raised

4.2.1 Microscopic characteristics of *Staphylococcus aureus* collected from different clinical samples

Organisms in stained slides were examined microscopically to observe their arrangements. The isolates revealed Gram-positive; cocci shaped organisms; arranged singly or in pairs, and in irregular, grape like clusters.

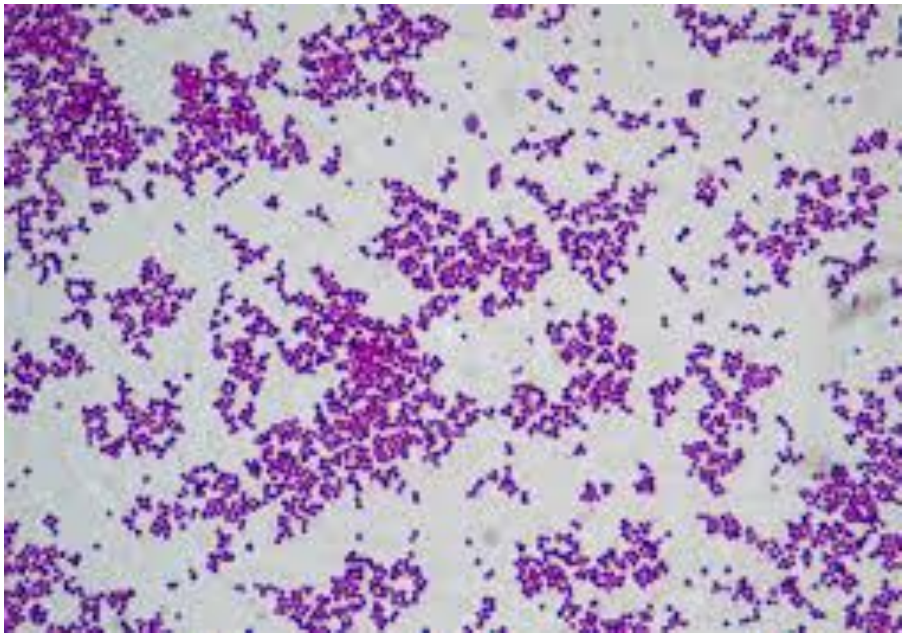

Figure 4.1 Microscopic examination of *Staphylococcus aureus* (100x magnification by oil immersion lens)

4.2.2 Growth characteristics of *Staphylococcus aureus* collected from different clinical samples

Once colonies found on MSA plates the morphological discrete colonies were isolated, inoculated and grown on Nutrient agar (NA) and in Blood (Sheep) agar medium for pure culture. When incubated for extended time on blood agar plates 81 (67.5%) samples of *Staphylococcus* produce hemolytic (mainly β) zones around the colonies and sometimes golden yellow pigmentations (40%). (Table 4.2)

Table:4.2 Growth Characteristics in Blood agar media of isolated *Staphylococcus aureus* from different clinical samples

Media: Blood Agar

Growth Characteristics

Colony color	**Number of isolates**	**Percentages (%)**
Golden yellow	48	40
Light yellow	41	34.2
Creamy yellow	18	15
Yellow	13 **Total= 120**	10.8

Growth Pattern

Colony size	**Number of isolates**	**Percentages (%)**
Medium(~3mm)	18	15
Small(>2mm)	39	32.5
Pin head(≤2mm)	30	25
Pin point(~1mm)	33 **Total= 120**	27.5

Hemolytic property (β)

Present	81	67.5%
Absent	39 **Total= 120**	32.5%

4.2.3 Biochemical characteristics of *Staphylococcus aureus* collected from different clinical samples

Table 4.3 Biochemical characterization of isolated *Staphylococcus aureus* from clinical samples

Biochemical tests	Positive isolates ID	Negative isolates ID
Catalase test	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26, 27,28,29,30,31,32,33,34,35,36,37, 38,39,40,41,42,43,44,45,46,47,48, 49,50,51,52,53,54,55,56,57,58,59, 60,61,62,63,64,65,66,67,68,69,70, 71,72,73,74,75,76,77,78,79,80,81, 82,83,84,85,86,87,88,89,90,91,92, 93,94,95,96,97,98,99,100,101,102,103,104,105,106,107,108,109,110,111,112,113,114,115,116,117,118,119,120.	**X**
Coagulase test	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16, 17,18,19,20,21,22,23,24,25,26, 27,28,29,30,31,32,33,34,35,36,37, 38,39,40,41,42,43,44,45,46,47,48, 49,50,51,52,53,54,55,56,57,58,59, 60,61,62,63,64,65,66,67,68,69,70, 71,72,73,74,75,76,77,78,79,80,81, 82,83,84,85,86,87,88,89,90,91,92, 93,94,95,96,97,98,99,100,101,102,103, 104,105,106,107,108,109,110,111,112, 113,114,115,116,117,118,119,120.	**X**

Biochemical tests	Positive isolates ID	Negative isolates ID
Oxidase test	**X**	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15, 16,17,18,19,20,21,22,23,24,25,26, 27,28,29,30,31,32,33,34,35,36,37, 38,39,40,41,42,43,44,45,46,47,48, 49,50,51,52,53,54,55,56,57,58,59, 60,61,62,63,64,65,66,67,68,69,70, 71,72,73,74,75,76,77,78,79,80,81, 82,83,84,85,86,87,88,89,90,91,92, 93,94,95,96,97,98,99,100,101,102, 103,104,105,106,107,108,109,110, 111,112,113,114,115,116,117,118, 119,120.
MIU (Motility Indole Urea)	**X**	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15, 16,17,18,19,20,21,22,23,24,25,26, 27,28,29,30,31,32,33,34,35,36,37, 38,39,40,41,42,43,44,45,46,47,48, 49,50,51,52,53,54,55,56,57,58,59, 60,61,62,63,64,65,66,67,68,69,70, 71,72,73,74,75,76,77,78,79,80,81, 82,83,84,85,86,87,88,89,90,91,92, 93,94,95,96,97,98,99,100,101,102, 103,104,105,106,107,108,109,110, 111,112,113,114,115,116,117,118, 119,120.

KIA (Kligler's Iron Agar)	Slant	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100,101,102,103,104,105,106,107,108,109,110,111,112,113,114,115,116,117,118,119,120.		**X**
	Butt	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100,101,102,103,104,105,106,107,108,109,110,111,112,113,114,115,116,117,118,119,120.		
Mannitol Fermentation	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100,101,102,103,104,105,106,107,108,109,110,111,112,113,114,115,116,117,118,119,120.			

Biochemical tests	Positive isolates ID	Negative isolates ID
Simmons Citrate Agar	**X**	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15, 16,17,18,19,20,21,22,23,24,25,26, 27,28,29,30,31,32,33,34,35,36,37, 38,39,40,41,42,43,44,45,46,47,48, 49,50,51,52,53,54,55,56,57,58,59, 60,61,62,63,64,65,66,67,68,69,70, 71,72,73,74,75,76,77,78,79,80,81, 82,83,84,85,86,87,88,89,90,91,92, 93,94,95,96,97,98,99,100,101,102, 103,104,105,106,107,108,109,110, 111,112,113,114,115,116,117,118, 119,120.
Methyl Red (MR)	1,3,4,7,9,10,13,15,16,19,21,22,25,27, 28,31,33,34,36,38,39,42,44,45,48,50, 52,53,56,58,59,62,64,66,67,70,72,73, 76,78,80,81,84,86,87,90,92,94,95,98, 100,101,104,106,108,109,112,114,115, 118,120.	1,2,5,6,8,11,12,14,17,18,20,23,24, 26,29,30,32,35,37,40,41,43,46,47, 49,51,54,55,57,60,61,63,65,68,69, 71,74,75,77,79,82,83,85,88,89,91, 93,96,97,99,102,103,105,107,110,1 11,113,116,117,119.
Voges Proskauer (VP)	1,2,5,6,8,11,12,14,17,18,20,23,24, 26,29,30,32,35,37,40,41,43,46,47, 49,51,54,55,57,60,61,63,65,68,69, 71,74,75,77,79,82,83,85,88,89,91, 93,96,97,99,102,103,105,107,110,111, 113,116,117,119.	1,3,4,7,9,10,13,15,16,19,21,22,25, 27,28,31,33,34,36,38,39,42,44,45, 48,50,52,53,56,58,59,62,64,66,67, 70,72,73,76,78,80,81,84,86,87,90,9 2,94,95,98,100,101,104,106,108,10 9,112,114,115,118,120.

Table: 4.4 Identifying Features of *Staphylococcus aureus*

1. *Staphylococcus aureus are* **Gram-positive cocci** arranged in irregular grape-like clusters.
2. On **Nutrient Agar**, *Staphylococcus aureus* colonies produced characteristic golden-yellow colonies.
3. On **Blood Agar**, *Staphylococcus aureus* produced clear zone of hemolysis (β-hemolysis)
4. On **Mannitol salt agar,** *Staphylococcus aureus* produced yellow colonies.
5. *Staphylococcus aureus* were Catalase positive, Coagulase positive.
6. They are Oxidase negative, Indole negative, they show negative reactions in Simmons Citrate Agar.

Figure 4.2 Growth of *Staphylococcus aureus* on MSA plate

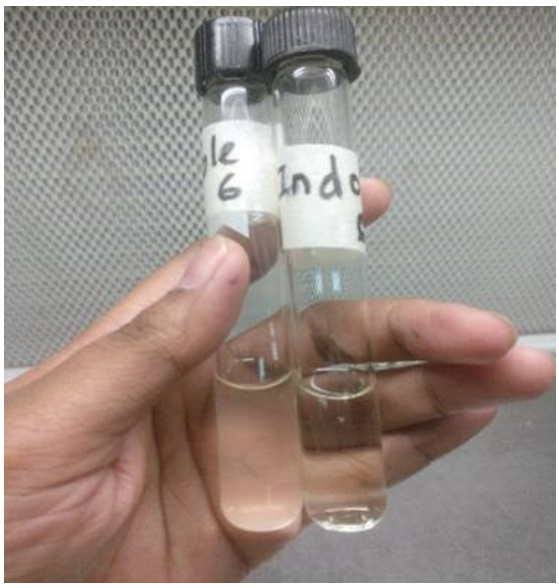

Figure 4.3 Finding of *Staphylococcus aureus* in indole test

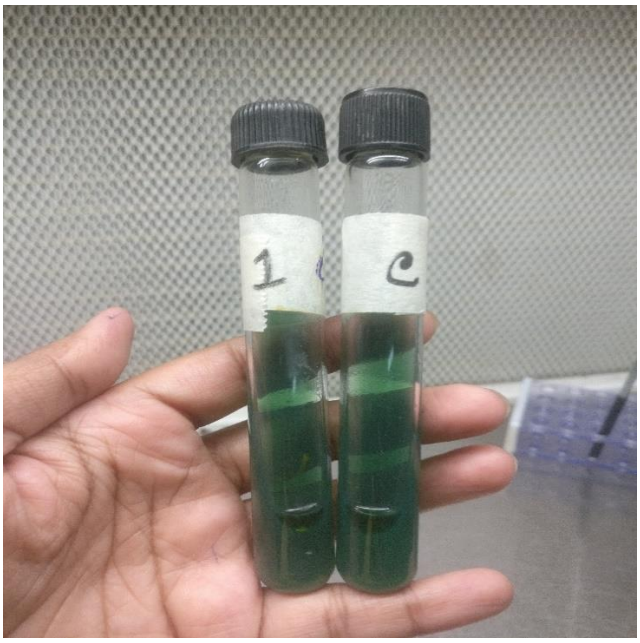

Figure 4.4 *Staphylococcus aureus* in Citrate utilization test

Figure 4.5 *Staphylococcus aureus* in Slide coagulase test

Figure 4.6 Growth of *Staphylococcus aureus* on Blood agar plates

Figure 4.7 *Staphylococcus aureus* in Methyl red test

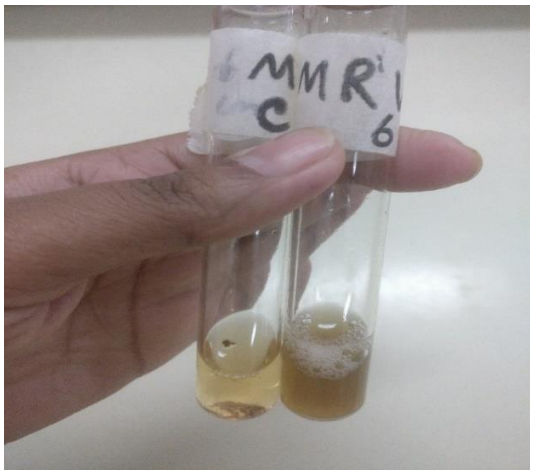

Figure 4.8 *Staphylococcus aureus* in Voges Proskauer test

Figure 4.9 *Staphylococcus aureus* in Kligler's Iron Agar test

Out of 120 clinical samples of *Staphylococcus aureus*, 68 (56.7%) strains were detected as MRSA and 52 (43.3%) strains were detected as MSSA by phenotypic method and by detection of *mecA* gene by PCR (Table 4.5)

Table 4.5 Isolation rate of MRSA and MSSA among *Staphylococcus aureus* (n =120)

Staphylococcus aureus	**Number of isolates (%)**
MRSA	68 (56.7%)
MSSA	52 (43.3%)
Total	120 (100%)

Here the prevalence of the organism in indoor admitted patients were 92.5% and outdoor or day cases were 7.5 %. The indoor cases were admitted in ICU (25%), NICU (17.5%), Dialysis unit (16.66%), General wards (25.8%) and Children wards (7.5%) of HFRCMCH. (Table 4.6)

Table 4.6 Prevalence of *Staphylococcus aureus* in samples collected from different wards of HFRCMCH (n=120)

Places in the hospital	Number of isolates	Percentages of isolates among positive samples
ICU	30	25%
NICU	21	17.5%
Dialysis Unit	20	16.66%
General Wards /Cabins	31	25.8%
Children Wards	9	7.5%
OPD	9	7.5%
	Total =120	

Clinical samples which were positive for *Staphylococcus aureus* were collected from the patients of all age group. Among which Blood samples were 53 (44.2%), Pus samples were 47 (39.2%) and Wound swab were 20 (16.66%) (Table 4.7) *Staphylococcal* species identified from different positive clinical samples were plotted in a pie diagram showed below (Figure 4.10).

Table 4.7 Staphylococcus aureus identified from different clinical samples

Name of the specimen	Number of positive isolates	Percentages (%)
Blood	**53**	**44.2**
Pus	**47**	**39.2**
Wound Swab	**20**	**16.6**
	Total =120	

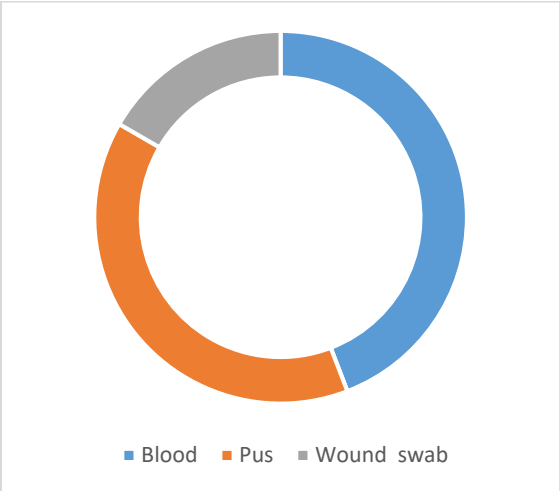

Figure 4.10 *Staphylococcus aureus* identified from different positive clinical samples

Near about 55% of the positive cases were female population. It might be because of a large number of samples were collected from the patients with infected wound in their incisional area of Caesarian section (Table 4.8)

Table 4.8 Sex Distribution of patients suffered from diseases caused by *Staphylococcus aureus* (n=120)

Sex	Number of Isolates	Percentages
Female	67	55.8%
Male	53	44.2%
	Total= 120	

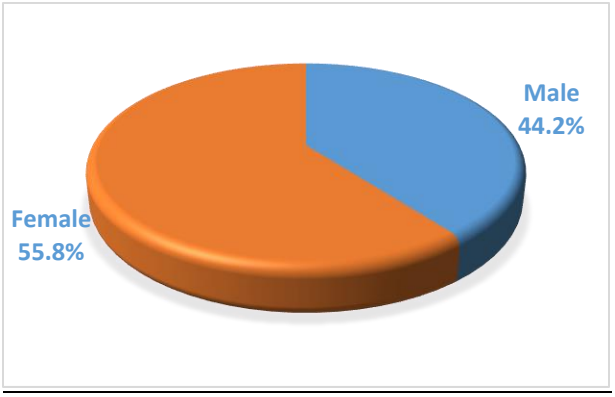

Figure 4.11 Sex Distribution of patients suffered from diseases caused by *Staphylococcus aureus*

All the samples were received from the patients age limit 0 to 87 years who came to Holy Family Red Crescent Medical College and hospital either as an indoor or outdoor patient. Children below 18 were affected by the organism and manifested clinical symptoms including fever, abscesses. Alarming finding was that very young people specifically age limit between 0-8yrs are affected more (Figure 4.13). This may be due to cases were NICU (17.5%) admitted patients and mostly neonates. 57% of the total sample were adult (Table 4.9).

Table 4.9 Prevalence of Age in patients suffered from diseases caused by *Staphylococcus aureus*

Age group of the patients	Number of isolates	Percentages
Adult (18+)	68	56.7%
Child (below 18)	52	43.3%

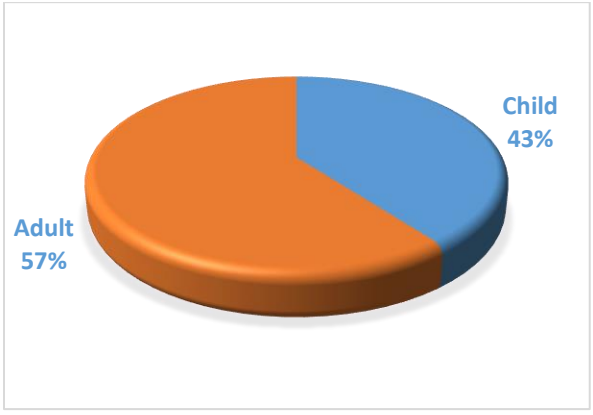

Figure 4.12 Prevalence of Age in patients suffered from diseases caused by *Staphylococcus aureus*

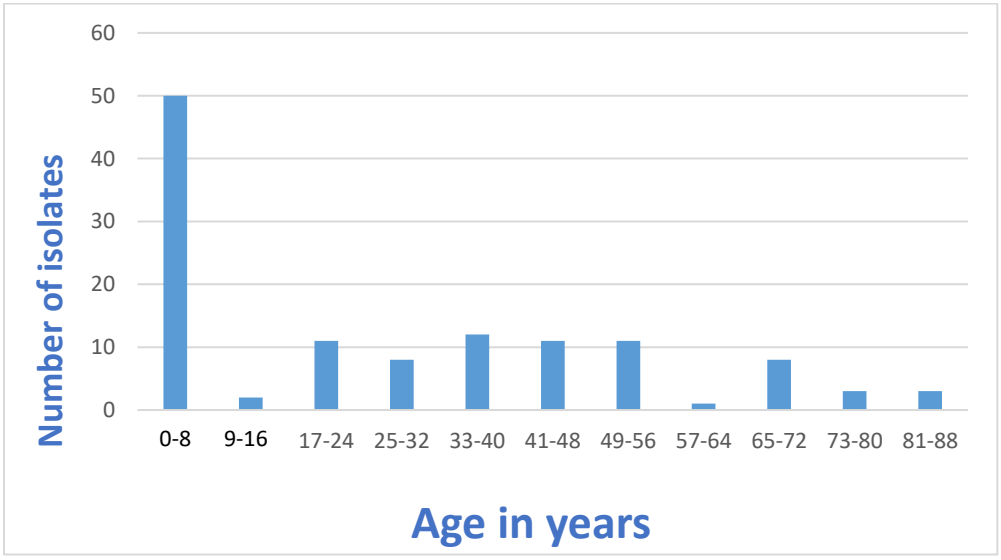

Figure 4.13 Prevalence of Age in patients suffered from diseases caused by *Staphylococcus aureus*

4.3 Anti-Biogram

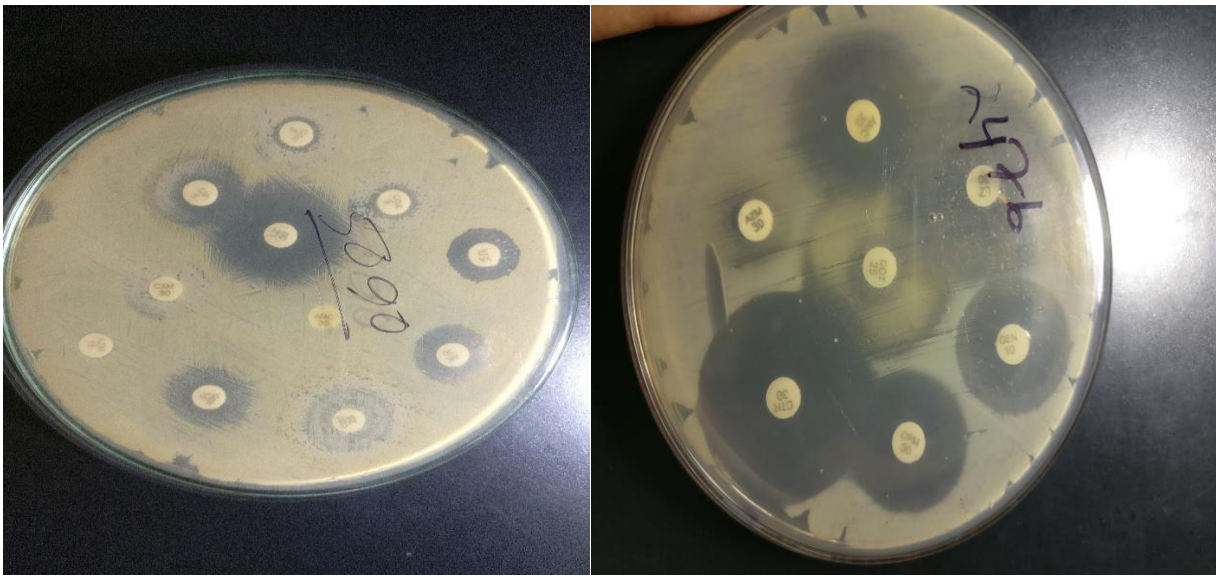

Figure 4.14 Antimicrobial susceptibility by disc diffusion method (modified Kirby Bauer method)

4.3.1 Antibiotic Susceptibility Test

The Antibiotic susceptibility test was performed to determine the susceptibility pattern of the 120 isolates against fourteen different antibiotics. The sensitivity pattern of fourteen different antibiotics were determined for these isolates. Kirby-Bauer method was chosen for the susceptibility test of the isolates (Bauer et al.) and susceptibility status was defined as outlined in National committee for clinical laboratory standards (NCCLS) guideline. The antibiotic susceptibility pattern of *Staphylococcus aureus* reveals that 56.66% of the isolates were resistant to Oxacillin, 25.8% of the isolates were resistant to Amoxyclav, 38.3% of the isolates were resistant to Co-trimoxazole, 30.8% of the isolates were resistant to Tetracyline, 50% of the isolates were resistant to Ciprofloxacin, 15% of the isolates were resistant to Ceftriaxone, 95% of the isolates were resistant to Vancomycin, 24.2% of the isolates were resistant to Gentamycin, 52.5% of the isolates were resistant to Erythromycin, 85% of the isolates were resistant to Azithromycin, 20.8% of the isolates were resistant to Amikacin. Apparently, Ceftazidime, Imipenem, Meropenem were good choice as they were more sensitive (Table 4.11). All isolates showed Multi Drug Resistance Pattern (MDR). Oxacillin Resistant Isolates (n=68) showed resistance to at least three up to six other drugs out of fourteen antibiotics. On the basis of structural classification antibiotics, 100%

of the isolates were resistant to β-lactam drugs, 60% of the isolates were resistant to Fluoroquinolone, 20.6% of the isolates were resistant to Aminoglycosides and 80.8% of the isolates were resistant to Macrolides (Table 4.12)

Table 4.10 Quick view of the antibiotic susceptibility pattern

Over all Findings:
▪ Oral drugs like **Amoxyclave, Ciprofloxacin, Azithromycin** are becoming ineffective. ▪ 3rd generation of **Cephalosporin** is still a good choice. ▪ **Amikacin** is more effective than **Gentamycin**. ▪ Low degree resistance is shown towards **Aminoglycoside**, **Carbapenem.**

4.3.2 Drug resistance pattern of *Staphylococcus aureus* isolated from different clinical samples from the patients of HFRCMCH.

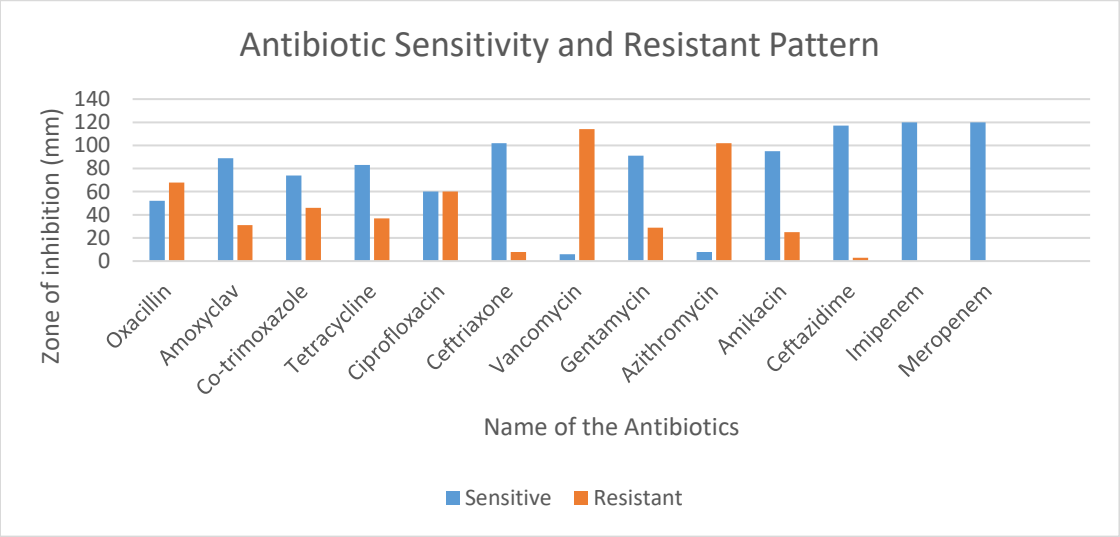

Figure:4.15 Susceptibility pattern of *Staphylococcus Aureus* isolated from different clinical samples in HFRCMCH

Table 4.11 Antimicrobial sensitivity pattern of *Staphylococcus aureus* isolated from different clinical samples (n=120)

Name of the Antimicrobial agents	Number of Isolates	
	Susceptible Organisms [%]	**Resistant Organisms (%)**
I. **Oxacillin** (n=120)	52 (≥17mm) [43.3%]	68 (56.7%)
II. **Amoxyclav** (n=120)	89 (≥18mm) [74.2%]	31 (25.8%)
III. **Co-trimoxazole** (n=120)	74 (≥16mm) [61.7%]	46 (38.3%)
IV. **Tetracyline** (n=120)	83 (≥19mm) [69.2%]	37 (30.8%)
V. **Ciprofloxacin** (n=120)	60 (≥21mm) [50%]	60 (50%)
VI. **Ceftriaxone** (n=120)	102 (≥21mm) [85%]	18 (15%)
VII. **Ceftazidime** (n=120)	117(≥18mm)[97.5%]	3 (2.5%)
VIII. **Vancomycin** (n=120)	6 (≥15mm) [5%]	114 (95%)
IX. **Gentamycin** (n=120)	91(≥15mm) [75.8%]	29 (24.2%)
X. **Erythromycin** (n=120)	57(≥23mm) [47.5%]	63 (52.5%)
XI. **Azithromycin** (n=120)	18 (≥17mm) [15%]	102 (85%)
XII. **Amikacin** (n=120)	95 (≥17mm) [79.2%]	25 (20.8%)
XIII. **Imipenem** (n=120)	120 (≥19mm)[100%]	0 (0%)
XIV. **Meropenem** (n=120)	120(≥19mm) [100%]	0(0%)

Table 4.12 Multi drug Resistant (MDR) pattern of MRSA on the basis of structural classification antibiotics (n=68)

Structural classification of antibiotics	Resistant number of Isolates (%)
β-lactam drugs	68 (100%)
Fluoroquinolone	41(60%)
Aminoglycosides	14 (20.6 %)
Macrolides	55 (80.8%)

4.4 Molecular Characterization

4.4.1 Detection of *mecA* gene by PCR

In determining the frequency of the resistance of *Staphylococcus aureus* to methicillin the present study aimed to identify the isolates using PCR and to correlate the presence of the organism with conventional method. In this regard, 120 *Staphylococcus aureus* isolates were collected from patients with different diseases attending different wards of HFRCMCH of Dhaka. Phenotypic Kirby-Bauer method confirmed the presence of methicillin resistant *Staphylococcus aureus* in 56.7% (Table 4.5) of the subjected isolates. However, when amplifying a 533bp fragment of the *mecA* gene by PCR, significant number of isolates were found to be *mecA* positive.

A total of 30 PCR cycles were run under the following conditions. DNA denaturation was done at 94°C for 1 min, primer annealing at 55° C for 0.5 min, and DNA extension at 72°C for 0.5 min. After the final cycle, the reaction was terminated by keeping it at 72^0C for 5 min. The PCR products were stored in the cycler at 4^0 C until they were collected.

M NC C BS PS WS

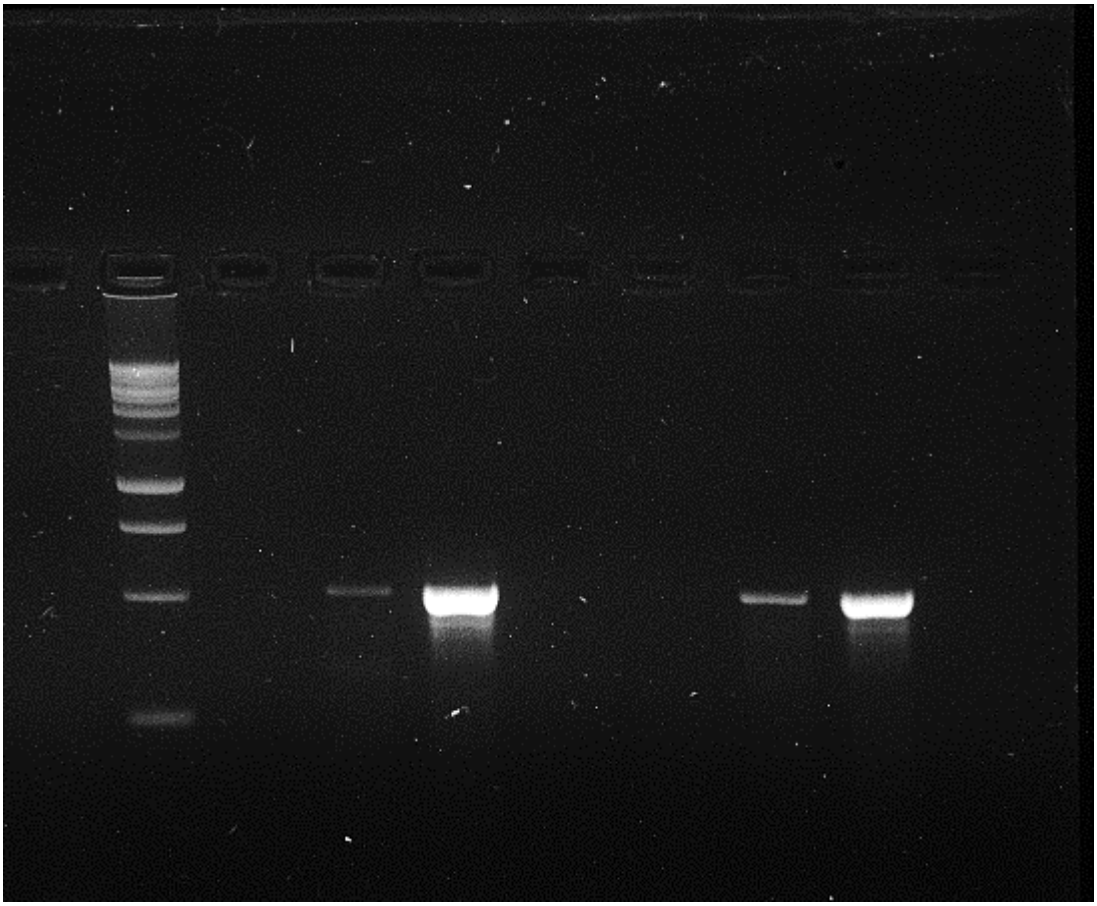

Figure 4.16 PCR products for detection of *S. aureus* specific *mecA* gene resolute on 2% agarose gel. Lane M indicate marker, lane C indicate positive control, NC indicate negative control and lane BS, PS, WS indicate sample IDs. The product size of *mecA* is 533 bp. Here, 1kb ladder was used.

Chapter 5: Discussion

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5. Discussion:

Staphylococcus aureus is the usual commensal of skin, mucous membrane, nose and vagina of healthy susceptible individual. Almost one third of healthy adults harbor the bacteria. That is actually one of the significant causes for the patients to become very much prone to acquire hospital-acquired infections (HAI) once their immune status is depressed. In fact, at the same time the bacteria are becoming increasingly difficult to combat because of emerging resistance to all current antibiotics. As well as the multi-drug resistant (MDR) gene is circulating in a significant amount in the community. In a developing and highly populated country like Bangladesh picture is even more blur and dark. The level of microbial contamination, exposure to different diseases, frequent and irrational practice of prescribing antibiotics play major role to increase the morbidity as well as mortality rate. Now a days the pathogenic microbial contamination becomes the major concern because of their higher contamination level and transmission of disease. Hospitals have successfully reduced the number of *Staphylococcal* infections but still the rate of progress is very slow.

Every single day the survival strategy of the bacteria is modifying and they are adopting newer methods for protection. The drugs usually used for the treatment of the diseases are becoming invalid so the reserve drugs are in need. Every time we get a good antibiotic that works against it, the bacteria gets even more smarter and develop resistance against that antibiotic as well. MRSA is a common strain that is resistant to common antibiotics that is usually used to treat *Staphylococcal* infections. The survival of nosocomial pathogens, including MRSA in the environment is a major concern and of great interest to the infection control professionals. MRSA often causes serious problems via nosocomial infection in hospitals. They continue to evolve and become even more potent to be resistant. The main focus of the study was to isolate and identify *Staphylococcus aureus* from different samples and to correlate the individual data in different categories. Virulent factors of the organism are surely responsible for the pathogenesis of the bacteria and vital for my study line. Finally, antibiotic resistant factor of the *Staphylococcus aureus* was revealed. The overall objective of the study was to find out the rate of isolation of *Staphylococcus* from various clinical specimens submitted in the HFRCMCH's microbiology lab, to see the antibiotic sensitivities of the bacteria against the commonly used drugs, to compare the

changing patterns of drug resistance with the nearby hospitals. After obtaining adequate knowledge from the study it was easy to evaluate of virulence properties of *Staphylococcus aureus*.

Hospital acquired infections are those which circulate because of an infection or toxin that exists in a hospital. They can be transmitted to a healthy individual through a direct contact (inanimate object) from the contaminated equipment, bed linens, or air borne droplets from the environment, infected patients or health care staffs. In some cases, the microorganism which is a part of the normal flora, can flair up after surgery or other procedures that compromise the protective skin barrier. Though the patient may have contracted the infection from their own skin, the infection is still considered nosocomial since it develops in the health care settings. There are chances of cross infections too. Most commonly bloodstream infection, pneumonia, surgical site infection, wound infection, skin and soft tissue infections are promptly manifested. Studying virulence factors could reveal a well to do pathway for the care and cure of the patients thus reduce their sufferings. The person usually suffers if he has poor hygiene and nutrition, un-attended tissue injury, pre-existing primary infection, intra-venous medical devices, drug abusers, diabetes, or any sort of immunodeficiency disorder, direct or indirect contact with infected person.

Methicillin Resistant *Staphylococcus aureus* (MRSA) is emerging enormously in the community. Infection with MRSA is likely to be more severe and need long term hospitalization. It is tougher to treat and the treatment cost is increasing too. Resistance of the strain is attributed to β-lactamase production due to presence of unusual PBP in the cell wall of the resistant strains. The major mechanism of resistance of MRSA to β-lactam antibiotics is due to the acquisition of the *mecA* gene encoding an additional penicillin-binding protein (PBP). From mild skin infections to wound infections sometimes MRSA may arise a life-threatening condition. So, it is threatening to be a "Super bug". MRSA does not pose a risk to the health official staff unless they are suffering from a debilitating disease or immune compromised or in direct contacts.

Staphylococcus aureus after attachment and persistence at first colonize in axilla, perineum and nose then they got entry inside the body. The invading process is performed by using enzymes. Thus, they evade the host immunity and evasion of defense mechanisms occurs, or tissue invasion and penetration, as well as enabling the production of toxins that mediate more aggressive disease and spread the organism to various organs of the body.

Staphylococcus aureus is a gram-positive coccus, arranged in cluster which showed creamy to golden color pigmentation in blood agar media. They are mainly aerobic but sometimes yield facultatively anaerobic. The *Staphylococcus* can be classified further on the basis of coagulase test and on pathogenicity. *Staphylococcus epidermidis and Staphylococcus saprophyticus* are two other pathogens of the same species.

The identification of *Staphylococcus aureus* was performed by subsequent gram staining. After isolation and identification virulence factors were assessed. The virulence of the organism is multifactorial. It comprises of basically three sub-type. Firstly, the cell wall associated factors were identified. Clumping factor or bound coagulase was identified by coagulase test, Protein A was identified by latex agglutination test. Secondly, in the toxin segments analysis of Hemolysin was done by observing the pattern of hemolysis on the blood agar. Lastly the virulence properties of the enzymes were evaluated by performing catalase, coagulase test and evaluation of the resistance pattern of β- lactamase drugs by observing the oxacillin resistant pattern by disc diffusion method (modified Kirby Bauer method). PCR based methods was done to determine the *mecA* gene in *Staphylococcus aureus.*

Mannitol salt agar (MSA) is a selective medium for *Staphylococcus aureus*. High salt concentration act as a selective agent that allows only the growth of *Staphylococcus spp.* The phenol red used as an indicator that differentiate *Staphylococcus aureus* from other *Staphylococcus spp.* by fermenting mannitol, thus changes in the color of MSA plate from red to yellow due to production of acid. Organisms in gram stained slides, were examined microscopically to observe their arrangements. The isolates revealed Gram-positive; cocci shaped organisms; arranged singly or in pairs, and in irregular, grape like clusters (Figure 4.1). Once colonies found on MSA plates the morphological discrete colonies were isolated, inoculated and grown on Nutrient agar (NA) and in Blood (Sheep) agar medium for pure culture. When incubated for extended time on blood agar plates 81 (67.5%) samples of *Staphylococcus* produce hemolytic (mainly β) zones around the colonies and sometimes golden yellow pigmentations (40%). (Table 4.2).

Biochemical tests were performed to confirm the samples for *Staphylococcus aureus*. The organisms were catalase positive, coagulase positive while they are oxidase negative, Indole negative. They show negative reactions in Simmons Citrate Agar (Table 4.3).

All the samples were received from the patients age limit 0 to 87 years who came to Holy Family Red Crescent Medical College and hospital either as an indoor or outdoor case. Here, samples were collected from the newborn children to adults. Samples included blood, wound swab and pus. All laboratory works were performed in the department of microbiology, HFRCMCH with the collaboration of the department of microbiology of Dhaka university. In this study a total of **565 samples** were collected from different patients (both indoor and outdoor cases) of Holy Family Red Crescent Medical College and Hospital. Among which 283 (50.05%) samples were positive for *Staphylococcus.* Out of 283 *Staphylococcus* **120 samples (21.2%)** were positive for *Staphylococcus aureus*, whereas 163 samples (28.85%) were coagulase negative *Staphylococcus*. That means among **565 clinical samples, 21.2%** cases were ***Staphylococcus aureus*** (Table 4.1). In another study conducted in Dhaka medical college during January 2010 to December 2011 showed, 112 (22.4%) *Staphylococcus aureus* isolates after screening 500 nasal swabs[73]. A study conducted in ICU in 2014 of HFRCMCH shows only 16 (5.3%) of *Staphylococcus aureus* isolates among 1282 samples[74]. The number or percentages of *Staphylococcus aureus* have markedly increased in these years. Another study in Dhaka city revealed 162 (22.2%) of *Staphylococcus aureus* isolates among 729 aerobic bacteria[75]. In 2005 a study was conducted to find out the methicillin resistant *Staphylococcus aureus* in Bangladesh. In that multicenter study they found the rate of isolation of the bacteria was 510 (14.1%) among 3611 clinical specimens[81].

Out of 120 *Staphylococcus aureus,* 68 (56.7%) isolates were detected as MRSA and 52 (43.3%) strains were detected as MSSA by phenotypic method and by detection of *mecA* gene by PCR (Table 4.5). In another study, in Dhaka out of 112 *Staphylococcus aureus* 38 (33.93%) strains were detected as MRSA. They have also identified *mecA* gene in their isolates[73]. Resistance pattern was also discouraging in another study, they found 43% MRSA strains and 77% were resistant to ampicillin and 93% to penicillin [75]. In India resistance is apparently lower in southern India which significantly varies from Assam, India[76]. The pattern has resemblance with my study. A study in Germany based on databases were searched (studies published between 1976 and 2007) to identify the role of antibiotics as a risk factor for MRSA isolation in adult patients reveals 25.9% of *Staphylococcus aureus* strains isolated from outpatients were methicillin resistant, most of these strains were recovered from individuals who were likely to have acquired them in the health care environment**[77].** Prevalence of MRSA has increased substantially over last few years in hospital

patients in Bangladesh. In a multicenter study, high incidence of MRSA in large hospitals in four different regions of Bangladesh was observed in 2005[81]. Prevalence of MRSA ranged from 23.6 % in Australia to over 61 % in Taiwan and Singapore, and more than 70 % in Japan and Hong Kong[86]. Resistant strains are circulating and increasing every year, so it is high time in antibiotic selection which may be a key factor causing the dissemination of predominant MRSA clones in hospitals.

In this study, clinical samples which were positive for *Staphylococcus aureus* were collected from the patients of all age group. It was found that among the positive samples, 43% isolates harbor in the young population and 57% of the total sample were adult (Table 4.9). Some of them were even neonates who are very much prone to any sort of illness. Children below 18 were affected by the organism and manifested clinical symptoms including fever, abscesses. Alarming finding was that very young people specifically age limit between 0-8 years were affected more (Figure 4.13). This may be due to cases were NICU (17.5%) admitted patients and mostly neonates. In another study held in Iran from January 2008 to November 2012 revealed that, the bacteria isolation percentage were slightly higher (9.9 % – 16.3 %) in the age group less than 19 years. They have collected specimens from different infected body sites from 358 patients during the period[82].

Staphylococcus aureus was identified from the clinical samples which includes blood 53 (44.2%), pus 47 (39.2%) and wound swabs 20 (16.66%) (Table 4.7). *Staphylococcal* species identified from different positive clinical samples were plotted in a pie diagram (Figure 4.10). In another study, *Staphylococcus aureus* was isolated from 10 body sites and were predominant in wound (29.3%), abscess (25.7%) and blood (25.7%) specimens. The MRSA and MDR strains were mainly present in wound specimens[82]. In another study, in Armed Forces Hospital, Al-Kharj Saudi Arabia they have collected 689 clinical specimens over a period of three years from November 2004 to October 2007. The isolation of the bacteria in pus (22.2%) and wound swabs (23.8%) was remarkable[83]. In a study in Dhaka, clinical samples were collected during the months from August to November, 2015. Among those 65 samples 18 (27.69%) pus samples were positive for *Staphylococcus aureus*[84]. A study from India showed, the isolation of 450 cultures of *Staphylococcus aureus* from different samples. Amongst which, 234 isolates were from pus (52%), 164 isolates were from blood (36.4%)[85]. Of 1360 clinical specimens from a study in Ethiopia, *Staphylococcus aureus* was

recovered from the clinical specimens was the highest in pus (118, 55.4 %). The studies vary from mine may be due to collection of a lot of specimen from patients admitted to an intensive care unit (ICU:25%, NICU: 17.5%). Blood samples are quite common for patients with septicemia.

For the indoor cases samples were collected from patients admitted in ICU, NICU, dialysis unit, children and general wards while OPD (Out Patient Department) cases were also included for isolation of the bacteria. Here the organism isolated from indoor admitted patients were 92.5% and outdoor or day cases was 7.5 %. The indoor patients were admitted in ICU (25%), NICU (17.5%), Dialysis unit (16.66%), General wards (25.8%) and Children wards (7.5%) of HFRCMCH. (Table 4.6). Maximum isolates were from the ICU, almost 25% of the total isolates. While the NICU cases were also remarkable. The immune status in young patients are vulnerable due to less nutrition, poor hygiene during and after delivery, leading a large number of children were presented with the *Staphylococcal* infection.

Near about 55% of the positive cases were female (Table 4.8). It might be because of a large number of samples were collected from the patients with infected wound in their incisional area of caesarian section. After examining the patients, inflamed wound area with abscess or pus was found. In Dhaka city, a large number of anti-natal cases are available where patient party and even sometimes physicians prefer to go for a caesarian section rather than conventional Normal vaginal delivery (NVD) now. Caesarian section is a major invasive surgery which need maximum aseptic precautions. But unfortunately, it is not maintained well in all the cases. Even maintaining hygiene is important during ante-natal period. In other study from Nigeria reported that, mounting evidence appears to support increasing *Staphylococcus aureus* colonization and infection among pregnant (6.9%)[80] women. The organism is sometimes even responsible for infertility in female due to asymptomatic cervical infection, while the majority of the patients remain undiagnosed. A study from Iran intended to assess the frequency of *Staphylococcus aureus* isolated from infertile women's endocervix. The study revealed that 26% women's urogenital tracts were colonized by the bacteria[79]. In an Iranian study, 57% of *Staphylococcus aureus* were isolated from males and 43 % from females. The percentage of MRSA and MDR strains were similar in both genders[82]. The percentages vary due to different sample size, sample collecting site from the patients and different part of the hospital. Poor hygiene after operation, lack of nutrition must be allowing the

organism to grow from the normal flora in a massive amount after colonization in the incisional site, thus they resulted in wound infection.

The antibiotic susceptibility test was performed to determine the susceptibility pattern of the 120 isolates against fourteen different antibiotics. The sensitivity pattern of fourteen different antibiotics were determined for these isolates. Kirby- Bauer method was chosen for the susceptibility test of the isolates (Bauer *et al*.) and susceptibility status was defined as outlined in National committee for clinical laboratory standards (NCCLS) guideline. The antibiotic susceptibility pattern of *Staphylococcus aureus* revealed that 56.66% of the isolates were resistant to Oxacillin, 25.8% of the isolates were resistant to Amoxyclav, 38.3% of the isolates were resistant to Co-trimoxazole, 30.8% of the isolates were resistant to Tetracyline, 50% of the isolates were resistant to Ciprofloxacin, 15% of the isolates were resistant to Ceftriaxone, 95% of the isolates were resistant to Vancomycin, 24.2% of the isolates were resistant to Gentamycin, 52.5% of the isolates were resistant to Erythromycin, 85% of the isolates were resistant to Azithromycin, 20.8% of the isolates were resistant to Amikacin. Apparently, Ceftazidime, Imipenem, Meropenem were good choice as they were more sensitive (Table 4.11). All isolates showed Multi Drug Resistance Pattern (MDR). Oxacillin Resistant Isolates (n=68) showed resistance to at least three up to six another drugs out of fourteen antibiotics .On the basis of structural classification antibiotics, 100% of the isolates were resistant to β-lactam drugs, 60% of the isolates were resistant to Fluoroquinolone, 20.6% of the isolates were resistant to Aminoglycosides and 80.8% of the isolates were resistant to Macrolides (Table 4.12). In another study conducted in Dhaka reveled that, both health care related and community-associated- MRSA strains were resistant to β-lactam antibiotics (Oxacillin, Cefoxitin, Ceftriaxone). They found high resistant to ciprofloxacin (nearly 90%), macrolids (erythromycin 88.23%) as well[73]. The studies were nearly similar and was in agreement with the study of Neelaet al (2008) form Malaysia[78]. So, in short it can be mentioned that oral drugs like Amoxyclave, Ciprofloxacin, Azithromycin are becoming ineffective; third generation of Cephalosporin is still a good choice; Amikacin is more effective than Gentamycin; low degree resistance is shown towards Aminoglycosides and Carbapenems. Resistance pattern of *Staphylococcus aureus* against various antimicrobials are considerably different among countries, centers and even among different wards of the same hospital; therefore, such type of local surveillance studies are important in deciding most adequate therapy for combating infection.

In determining the frequency of the resistance of *Staphylococcus aureus* to methicillin the present study aimed to identify the isolates using PCR and to correlate its presence with conventional method. After Phenotypic Kirby-Bauer method confirmed the presence of methicillin resistant *Staphylococcus aureus* in 56.7% (Table 4.5) of the subjected cases. However, when amplifying a 533bp fragment of the *mecA* gene by PCR, significant number of isolates were found to be *mecA* positive. In a study from Iran, all isolates were positive for *mecA* genes, while they were resistant to the gentamicin, ciprofloxacin and cefoxitin[79].

The study emphasizes on isolation and identification of the virulence factors of *Staphylococcus aureus* by modern methods from patients of a tertiary level hospital. Studying virulence factors could reveal a well to do pathway for the care and cure of the patients who have been suffering from nosocomial infections. Thus, from the obtained knowledge it will be helpful for the admitted patients as the morbidity rate and sufferings of these group of people could be minimized up to a certain level.

5.1 Prevention and Control measure

Staphylococcus aureus is a Biosafety Level 2 (BSL-2) pathogen. Prevention of spread of *Staphylococcus aureus* infections in hospitals involves:

- **Proper hand washing and hand hygiene**: It is the most efficient way to prevent hospital spread of *Staphylococcus aureus*. Scrupulous-hand hygiene of the physicians by alcohol-based rub in between patient visit can reduce direct contamination.
- **Safe disposal of hospital wastes.**
- **Isolation of the infected cases.**
- **Screening of MRSA carriers:** Screening should be done among hospital staff when there is an outbreak.
- **Treatment of carriers:** For major betterment and reduce the severity of the disease it must be done at least by the use of topical mupirocin (for nasal carriers) and chlorhexidine (for skin carriers) promptly.

- **Stoppage of antibiotic misuse and execute proper antibiotic guideline in hospitals.** Antibiotics should be OTCs and rationally prescribed.
- **Un ethical practice and use of medical instruments must be reduced:** Performing medical interventions such as the insertion of Intravenous catheters in a sequence of prescribed steps **c**an reduce the rates of nosocomial infections related to such procedures.

5.2 Conclusion

Study of the bacterial pathogenesis and virulence determinants are mandatory in order to design effective prevention and control measures. Thus, we can rapidly diagnose the cases and promptly treat to reduce the mortality and morbidity rate. Finally, we can help the patients' in-need and reduce their pain and treatment cost. Maintaining hygiene and to take preventive measures during handling and care of the patients by the physicians are mandatory. The MDR strains are circulating in a tertiary Hospital. The emergence of resistant strains of *Staphylococcus aureus* lead to prolong hospitalization, increased medical expenses and mortality. Injudicious use of the antibiotics, cross infection, poor hygiene and nutrition, tissue injury, pre-existing primary infection, immunodeficiency (e.g. diabetes) are potential risk factors for the development of the disease. So, it is high time to follow Antibiotic guideline and stop the misuse. It seems that the infection control strategies may help to control the evolving problem of *Staphylococcus aureus* infections and prevent nosocomial life-threatening infection.

5.3 Ethical approval

The study was conducted after it was ethically reviewed and approved by the Department of Microbiology, HFRCMCH. Ethical clearance was also obtained from the hospital. Informed written consent was obtained from participants before data collection. All the information obtained from the study subjects were coded to maintain confidentially. When the participants were found to be positive for *Staphylococcus aureus*, they were informed by the hospital clinician and received proper treatment.

References

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References

1. Franklin DL. Aug 20,1998. Staphylococcus aureus Infections, Review article. N Engl J Med 1998; 339:520-532. DOI: 10.1056/NEJM199812313392716. Available at: https://www.nejm.org/doi/full/10.1056/NEJM199808203390806
2. Staphylococcus aureus. (n.d.). Available at: http://eastafricaschoolserver.org/Wikipedia/wp/s/Staphylococcus_aureus.htm. Citated on: 29th May, 2016.
3. Shinji H, Yosizawa Y, Tajima A, Iwase T, *et. al.* 2011. Role of Fibronectin-Binding Proteins A and B in In Vitro Cellular Infections and In Vivo Septic Infections by Staphylococcus aureus. Infection and Immunity, 79(6), 2215-2223. doi:10.1128/iai.00133-11
4. Schaberg DR, Culver DH, & Gaynes RP. 1991. Major trends in the microbial etiology of nosocomial infection. *The American Journal of Medicine*, *91*(3), S72-S75. doi:10.1016/0002-9343(91)90346-y
5. Dinges MM, Orwin PM, & Schlievert PM. 2000. Exotoxins of Staphylococcus aureus. *Clinical Microbiology Reviews*, *13*(1), 16-34. doi:10.1128/cmr.13.1.16
6. Lina G, Piémont Y, Godail GF et al. November 1999. Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. Clin. Infect. Dis. 29 (5): 1128–32. doi:10.1086/313461.PMID 10524952
7. Gillet Y, Issartel B, Vanhems P et al. (March 2002). "Association betweenStaphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients". Lancet 359 (9308): 753–9. doi:10.1016/S0140-6736(02)07877-7. PMID 11888586
8. Henry FC, Frank RD. 1st Sep 2009. Waves of Resistance: Staphylococcus aureus in the Antibiotic Era. Nat Rev Microbiol. 7(9): 629-641. Doi: 10.1038/nrmicro2200. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2871281/
9. Romero VJ, Rubio M, Fernandez C, & Picazo JJ. (1995). Mortality Associated with Nosocomial Bacteremia Due to Methicillin-Resistant Staphylococcus aureus. Clinical Infectious Diseases, 21(6), 1417-1423. doi:10.1093/clinids/21.6.1417
10. Postier RG, Green SL. 2004. Results of a multicenter,randomized,open-label efficacy and safety of two doses of tigcycline for complicated skin and skin- structure infections in hospitalized patients. Clin Ther.;26.5:704-714.
11. Berman DS, Eisner W, Kreiswirth B. 1993. Community-acquired methicillin-resistant *Staphylococcus aureus* infection. N. Engl. J. Med. 329:1896. DOI: 10.1056/NEJM199312163292517.
12. Keiichi Hiramatsu. October, 2001. Vancomycin-resistant Staphylococcus aureus: a new model of antibiotic resistance; Lancet Infect Dis.1(3):147-55. DOI: https://doi.org/10.1016/S1473-3099(01)00091-3

13. Staphylococcus Bacteria. (2015, November 3). Available at: https://www.moldbacteria.com/types-of-bacteria/staphylococcus-bacteria.html
14. Whitman WB. 2011.Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes. 2nd ed. Georgia: Springer Science & Business Media. pp 392-433.
15. Kluytmans J, Belkum A van, Verbrugh H. 1997. Nasal carriage of Staphylococcus aureus:epidemiology,underlying mechanisms,and associated risks. Clin Microbiol.10(3):505-520.
16. Cole AM,Tahk S,Oren AMI,*et al*. 2001. Determinants of Staphylococcus aureus Nasal Carriage. Clin Diagn Lab Immunol. 8(6):1064-1069. doi:10.1128/CDLI.8.6.1064.
17. Hospital-acquired infection. 2004, August 3. Available at: https://en.wikipedia.org/wiki/Hospital-acquired_infection
18. Genetic Variation in the Staphylococcus aureus 8325 Strain Lineage Revealed by Whole-Genome Sequencing. (2013, September 30). Available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0077122
19. Mehul J, Soham S, Kelsey H , *et. al*. 2017 Dec. Deciphering pathogenicity and antibiotic resistance islands in methicillin-resistant Staphylococcus aureus genomes. Open Biol. 7(12): 170094. doi:10.1098/rsob.170094
20. Donald EL, Editor. Identification of a Highly Transmissible Animal-Independent Staphylococcus aureus ST398 Clone with Distinct Genomic and Cell Adhesion Properties. doi: 10.1128/mBio.00027-12. Available at: https://mbio.asm.org/content/3/2/e00027-12/figures-only?related-urls=yes&legid=mbio%3b3/2/e00027-12
21. Keiichi H, *et. al*. 2013 Jun 2013. Genomic Basis for Methicillin Resistance in Staphylococcus aureus. Infect Chemother. 45(2): 117–136. doi: 10.3947/ic.2013.45.2.117.
22. Karinne SC, Elsa MM, Paulo PGF. Salvador Jan./Feb 2010. Methicillin/Oxacillin-resistant Staphylococcus aureus as a hospital and public health threat in Brazil. Braz J Infect Dis vol.14 no.1
23. Grundmann H, Aires-de-SM, Boyce J, Tiemersma E. 2006 Sep 2. Emergence and resurgence of meticillin-resistant Staphylococcus aureus as a public-health threat. Lancet.368(9538):874-85.
24. Enright MC, Day NPJ, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. J Clin Microbiol. 2000;38(3):1008-1015.
25. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. 2003;2001(April 2001). Comparison of Mortality Associated with Methicillin-Resistant and Methicillin- Susceptible Staphylococcus aureus Bacteremia : A Meta-analysis. Clin Infect Dis. 53¬59. doi:10.1086/345476.
26. Engemann JJ, Carmeli Y, Cosgrove SE, et al. 2003. Adverse Clinical and Economic Outcomes Attributable to Methicillin Resistance among Patients with Staphylococcus aureus Surgical Site Infection. Clin Infect Dis. 36(5):592-598. doi:10.1086/367653.

27. Apurba SS, Sandhya BK.2016. Essentials of medical Microbiology; section 3: Systemic Bacteriology, Chapter 21, Staphylococcus. Jaypee Brothers Medical Publishers. Pp 211-220.
28. Robert S. Daum, Brad J. Spellberg. 2012. Progress toward a Staphylococcus aureus vaccine. Clinical infectious diseases : an official..DOI:10.1093/cid/cir828
29. Nathan KA, Mark JM, J William C, Jeff GL, *et.al.* 2011 Sep-Oct. Staphylococcus aureus biofilms Properties, regulation and roles in human disease. Virulence. 2(5): 445–459. doi: 10.4161/viru.2.5.17724.
30. Schneewind O1, Fowler A, Faull KF. 1995 Apr 7. Structure of the cell wall anchor of surface proteins in Staphylococcus aureus. Science.;268(5207):103-6.
31. hu J, Lu C, Standland M, et al. Single Mutation on the Surface of Staphylococcus aureus Sortase A Can Disrupt Its. Biochemistry. 2008:1667-1674. doi:10.1021/bi7014597.
32. Patel AH, Nowlan P, Weavers ED, Foster T. Virulence of Protein A-Deficient and Alpha¬Toxin-Deficient Mutants of Staphylococcus aureus Isolated by Allele Replacement. Infect Immun. 1987;55(12):3103-3110.
33. Diekema, J D. Antimicrobial Resistance Trends and Outbreak Frequency in United States Hospitals. Clin Infect Dis. 2004;38(1):78-85.
34. Superantigen. (2004, October 14). Retrieved from https://en.wikipedia.org/wiki/Superantigen
35. Thomas P, John DF. February 2007.Streptococcal superantigens. Chemical immunology and allergy 93(1):1-23. DOI: 10.1159/0000100851
36. JG Cappuccino, N Sherman. 2014. Microbiology a Laoratory Manual,10th edition, pp 452-459. Pearson Education, Inc.
37. Kenneth Todar, PhD. Todar's online textbook of Bacteriology. Staphylococcus aureus. Pp 3. Available at: http://textbookofbacteriology.net/staph_3.html
38. Amaral MM, Coelho LR, Flores RP, et al. 2005. The Predominant Variant of the Brazilian Epidemic Clonal Complex of Methicillin-Resistant Staphylococcus aureus Has an Enhanced Ability to Produce Biofilm and to Adhere to and Invade Airway Epithelial Cells. J Infect Dis. 801-810. doi:10.1086/432515.
39. Warren L. 2010. Review of Medical Microbiology and Immunology.11th Edition. Chapter-10,pp:60-74.The Mc Graw Hill Companies, Inc.
40. betalactam_pharm [TUSOM | Pharmwiki]. (2016, November 9). Retrieved May 30, 2019, from http://tmedweb.tulane.edu/pharmwiki/doku.php/betalactam_pharm
41. Hartman BJ, Tomasz A. 1984. Low-Affinity Penicillin-Binding Protein Associated with r-Lactam Resistance in Staphylococcus aureus. J Bacteriol. 158(2):513-516.
42. Poole K. Cellular and Molecular Life Sciences Resistance to b -lactam antibiotics. 1984. Cell Mol Life Sci.61:2200-2223. doi:10.1007/s00018-004-4060-9.
43. Mallorqu G, Marrero A, Garc S, Garc R, Gomis-r FX. 2004. Staphylococcal methicillin resistance : fine focus on folds and functions. FEMS Microbiol Lett. 235:1-8. doi:10.1016/j.femsle.2004.04.035.

44. Georgopapadakou NH, Smith SA, Bonner DP. 1982. Penicillin-Binding Proteins in a Staphylococcus aureus Strain Resistant to Specific-Lactam Antibiotics. Antimicrob Agents Chemother. 22(1):172-175. doi:10.1128/AAC.22.1.172.
45. Chan KE, Warren HS, Thadhani RI, Steele DJ, Hymes JL, Maddux FW, & Hakim RM. (2012). Prevalence and Outcomes of Antimicrobial Treatment for Staphylococcus aureus Bacteremia in Outpatients with ESRD. Journal of the American Society of Nephrology, 23(9), 1551-1559. doi:10.1681/asn.2012010050
46. Loomba P, Taneja J, & Mishra B. (2010). Methicillin and vancomycin resistant S. aureus in hospitalized patients. Journal of Global Infectious Diseases, 2(3), 275. doi:10.4103/0974-777x.68535
47. Yun, H. (2003). Prevalence and mechanisms of low- and high-level mupirocin resistance in staphylococci isolated from a Korean hospital. Journal of Antimicrobial Chemotherapy, 51(3), 619-623. doi:10.1093/jac/dkg140
48. Stevens DL, Herr D, Lampiris H, Hunt JL, Batts DH, & Hafkin B. (2002). Linezolid versus Vancomycin for the Treatment of Methicillin-Resistant Staphylococcus aureus Infections. Clinical Infectious Diseases, 34(11), 1481-1490. doi:10.1086/340353.
49. Croos-Dabrera RV, Wunderink RG, Rello J, Cammarata SK, & Kollef MH. (2004). Clinical cure and survival in Gram-positive ventilator-associated pneumonia: retrospective analysis of two double-blind studies comparing linezolid with vancomycin. Intensive Care Medicine, 30(3), 388-394. doi:10.1007/s00134-003-2088-1
50. Silverman JA, Perlmutter NG, & Shapiro HM. (2003). Correlation of Daptomycin Bactericidal Activity and Membrane Depolarization in Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 47(8), 2538-2544. doi:10.1128/aac.47.8.2538-2544.2003
51. Arbeit RD, Maki D, Tally FP, Campanaro E, & Eisenstein BI. (2004). The Safety and Efficacy of Daptomycin for the Treatment of Complicated Skin and Skin-Structure Infections. Clinical Infectious Diseases, 38(12), 1673-1681. doi:10.1086/420818
52. Faville RJ. (1978). Staphylococcus aureus endocarditis. Combined therapy with vancomycin and rifampin. JAMA: The Journal of the American Medical Association, 240(18), 1963-1965. doi:10.1001/jama.240.18.1963
53. Fritsche TR, & Jones RN. (2004). Antimicrobial activity of tigecycline (GAR-936) tested against 3498 recent isolates of Staphylococcus aureus recovered from nosocomial and community-acquired infections. International Journal of Antimicrobial Agents, 24(6), 567-571. doi:10.1016/j.ijantimicag.2004.07.007
54. Schmitz F. (2001). Resistance to tetracycline and distribution of tetracycline resistance genes in European Staphylococcus aureus isolates. Journal of Antimicrobial Chemotherapy, 47(2), 239-240. doi:10.1093/jac/47.2.239
55. Fagon J, Patrick H, Haas DW, *et. al*. Treatment of Gram-positive Nosocomial Pneumonia Prospective Randomized Comparison of Quinupristin / Dalfopristin versus Vancomycin. Am J Respir Crit Care Med. 2000;(6).

56. Deresinski S. Methicillin-Resistant Staphylococcus aureus: An Evolutionary Epidemiologic, and Therapeutic Odyssey. Clin Infect Dis. 2005;40(January). doi :10.1086/427703.
57. SB Al-Masaudil, MJ Day and AD Russell. 1991, A Review Antimicrobial resistance and gene transfer in Staphylococcus aureus. Journal of Applied Bacteriology. 70, 279-290
58. Bocchini CE1, Mason EO, Hulten KG, Hammerman WA, Kaplan SL. 2013 Nov. Recurrent community-associated Staphylococcus aureus infections in children presenting to Texas Children's Hospital in Houston, Texas. Pediatr Infect Dis J.;32(11):1189-93. doi: 10.1097/INF.0b013e3182a5c30d.
59. Warren L. 2010. Review of Medical Microbiology and Immunology.11th Edition. Chapter-11,pp:75-82.The Mc Graw Hill Companies, Inc.
60. Birte V, Katherine S.2009. Antibiotic Resistance in Bacteria Caused by Modified Nucleosides in 23S Ribosomal RNA - Madame Curie Bioscience Database - NCBI Bookshelf.
61. Ruiz J1.2003 May. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother. 51(5):1109-17. Epub 2003 Apr 14
62. Hooper DC. Emerging Mechanisms of Fluoroquinolone Resistance. Emerg Infect Dis. 2001;7(2):337-341.
63. Nicholas PV, Anthony RR. 2013 February. Laboratory Maintenance of Methicillin-Resistant Staphylococcus aureus (MRSA). Curr Protoc Microbiol.Unit 9(C.2).
64. JG Cappuccino, N Sherman. 2014. Microbiology a Laboratory Manual,10th edition, pp 59-62. Pearson Education, Inc.
65. Geo FB, Karen CC, Janet SB, Stephen AM. 2007. Jawetz, Melnick & Adelberg's Medical Microbiology, 24th edition, pp 224-232. The McGraw-Hill Companies,Inc.
66. Braide W. Microbiological status of processed fruit juice sold in the commercial city of Onitsha. Sci J Biol Sci. 2012; 1(December):25-30.
67. JG Cappuccino, N Sherman. 2014. Microbiology a Laboratory Manual,10th edition, pp75-80. Pearson Education, Inc.
68. Cooper RA, Wigley P, Burton NF. Susceptibility of multiresistant strains of Burkholderia cepacia to honey. Lett Appl Microbiol. 2000;31(1):20-24.
69. Bauer, Kirby. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4):493-496.
70. Ferraro, Jane M. Performance standards for antimicrobial disk susceptibility tests. Natl Comm Clin Lab Stand. 2015;(January).
71. RahimiF, BouzariM, MalekiZ, Rahimi F. Antibiotic susceptibility pattern among Staphylococcus spp. With emphasis on detection of mecAgene in methicillin resistant Staph aureus isolates. Iranian J Clin Infect Dis2009, 4:143-150.

72. Siripornmongcolchai T, Chomvarin C, Chaichumpar K. Evaluation of different primers for detecting Mec -A gene by PCR in comparison with phenotypic methods for discrimination of Methicillin-resistant Staph aureus. Southeast Asian J Trop Med Public Health2002 ;33 :758-63.
73. S Khanam, M Jobayer, SM Shamsuzzaman, JA Haq, MM Rahman, KZ Mamun. 2014. Nasal colonization of Methicillin resistant Staphylococcus aureus among patients during hospital admission-emergence of community-associated MRSA strains. Bangladesh J Med Microbial:08(02): 08-13
74. Taslima A, Mohammad M, Taslima B, Kamrun Nahar, Syeda S D, Sabeena S. 2014. Antimicrobial resistance Pattern of Bacterial isolates from ICU of a tertiary care hospital in Bangladesh. Bangladesh Journal of Medical Microbiology.08(01). 07-11,
75. Md.SA Jilani, M Murshed, L sultana, Z Hasan. July2008. Common Clinically important aerobic bacteria and their drug resistance pattern at 17 different zones of Dhaka city and its vicinity. Bangladesh Medical College Journal. 13(02). 66-71.
76. Majumdar D, Bordoloi JS, Phunak AC, Mahanta J. 2001. Antimicrobial susceptibility pattern among methicillin resistant staphylococcus isolated in Assam. Indian J Med Microbiol. 19:138-40.
77. Tacconelli E, Angelis G De, Cataldo MA, Pozzi E, Cauda R. 2008 (November 2007). Does antibiotic exposure increase the risk of methicillin-resistant Staphylococcus aureus (MRSA) isolation? A systematic review and meta-analysis. J Antimicrob Chemother. 26-38. doi :10.1093/j ac/dkm416.
78. Neela V, Sasikumar M, Ghaznavi GR, Zamberi S,Mariana S. 2008. In vitro activities of 28 antimicrobial agents against methicillin-resistant Staph aureus from a clinical setting in Malaysia. Faculty Med Health Sci. 39:885-92.
79. Mohammad TA, Aylin E, Elmira NN. 2017 Apr-Jun. The Frequency of Staphylococcus aureus Isolated from Endocervix of Infertile Women in Northwest Iran. Int J Fertil Steril. 11(1): 28-32
80. Stanley CN, Ugboma HAA, Ibezim EC, Attama AA. December 25, 2013. Prevalence and Antibiotic Susceptibility of Staphylococcus Aureus and Other Staphylococcal Infections in Pregnant Women Attending Antenatal Clinic in a Tertiary Hospital in Port Harcourt, Nigeria. Journal of Infectious Diseases & Therapy. 2:1 DOI:10.4172/2332-0877.1000125
81. Jalaluddin AH, Mohammad MR, Zahurul HA, *et.al*. April 2005. Methicillin-resistant Staphylococcus aureus in Bangladesh - A multicentre study. International Journal of Antimicrobial Agents. 25(3):272-277. DOI: 10.1016/j.ijantimicag.2005.01.004. PDF in research gate
82. Mazhar S Al-Z, Ibrahim A Al-T, Emad H, Alla Al J, and Salih K. 2015 Oct. Antimicrobial susceptibility pattern of Staphylococcus aureus isolated from clinical specimens in Northern area of Jordan. Iran J Microbiol. 7(5): 265–272.
83. Shamweel A, Faris QA, Nami F AJ, and Shad A. 2009. Prevalence and antibiotic susceptibility pattern of methicillin resistant Staphylococcus aureus at Armed Forces Hospital in Saudi Arabia. DOI: 10.3329/bmrcb.v35i1.1983

84. Parvez, M. A., Ferdous, R. N., Rahman, M. S., & Islam, S. (2018). Healthcare-associated (HA) and community-associated (CA) methicillin resistant Staphylococcus aureus (MRSA) in Bangladesh – Source, diagnosis and treatment. Journal of Genetic Engineering and Biotechnology, 16(2), 473-478. doi:10.1016/j.jgeb.2018.05.004. Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6353753/
85. Bina PG, Shruti S. 2017. Prevalence of Methicillin Resistant Staphylococcus aureus in Clinical Samples of Teerthankar Mahaveer Medical College Hospital and Research Centre (TMMCH & RC), Moradabad (UP), India. International Journal of Medical Research & Health Sciences. 6(6): 17-20.
86. Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, et al. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clin Infect Dis. 2001;32(2):114–32. doi: 10.1086/320184. [PubMed] [CrossRef] [Google Scholar]

Appendix

University of Dhaka
Department of Microbiology

APPENDIX I

Mannitol Salt Agar **Media Composition**

Protease peptone	1.0%
Beef extract	0.1%
D-Mannitol	1%
NaC1	7.5%
Phenol red	0.25%
Agar	1.5%
Distilled water	1000 ml
pH	6.9±0.2

Sterilized at 121° C under 15 lbs/sq.in pressure for 15 minutes

Blood Agar Media

Tryptone	14.0g
Peptone	4.5g
Yeast extract	4.5g
NaC1	5.0g
Agar	15g
Distilled water	1000m1
pH	7.3±0.2

For enriched hemolytic properties, 7% sterile sheep blood was added to pre sterilized medium. Following autoclave, the media was cooled down to 50° C for 30 minute and then aseptically drawn sheep blood was added to medium using a sterile 10 pipette and pipette filter. The media content was mixed homogenously by gentle rolling of conical flask on the bench top.

Muller- Hinton Agar

Beef infusion	2.0g
Bacto casamino acid	17.5g
Starch	1.5g
Agar	15g
Distilled water	1000 ml
pH	7.3±.01

Sterilized at 121° C under 15 lbs/sq.in pressure for 15 minutes

Oxacillin Agar Media

Peptone	11.80 g
Mannitol	10 g
Lithium Chloride.	5 g
Yeast extract	9 g
Sodium chloride	55 g
Aniline Blue	0.2g
Bacteriological agar	12.5 g
Distilled water	1000 ml
PH	7.2±.02

Nutrient agar

Peptone	0.5g
Beef extract	.03g
NaC1	.05g
Agar	1.5g
Distilled water	1000m1
p^H	7.2

Sterilized at 121° C under 15 lbs/sq.in pressure for 15 minutes

Kligler's Iron Agar

Peptone	2%
Dextrose	0.1%
Sodium chloride	0.5 %
Ferric ammonium citrate	0.02%
Sodium thiosulphate	.03%
Phenol red	0.25%
Agar	2%
Distilled water	1000 ml
pH	7.4

Sterilized at 121° C under 15 lbs/sq.in pressure for 15 minutes

MR-VP Broth

Peptone	7%
Dextrose	0.5%
Potassium phosphate	0.5%
Distilled water	100m1
pH	6.9±0.2

Motility Indole Urease Agar (MIU)

Peptone	3.0%
NaCl	0.5%
Urea	2%
Mono Potassium Phosphate	0.2%
Phenol red	0.0005%
Agar	0.4%
Distilled water	100m1
pH	7

Simmons's Citrate Agar

Magnesium sulphate	0.02%
NaC1	0.5%
Sodium Citrate	0.2%
Di-Potassium Phosphate	0.1%
Mono-potassium Phosphate	0.1%
Bromothymol Blue	0.008%
Agar	2%
Distilled water	100m1
pH	7

APPENDIX II

Composition of the chemical and reagent

Barret's reagent

Solution A

α-napthol: 5.0g

α-napthol ethanol(absolute): 9995 ml was dissolved in ethanol with constant stirring.

Solution B

KOH	40g
Creatine	0.3g
Distilled water	1000m1

KOH was dissolved in 75ml of distilled water. The solution as it a warm was allowed to cool to room temperature. Creatine was added and stirred to dissolve. Water was added and reagent was stored at room temperature.

Crystal Violate Strain

Solution A	
Crystal violate	1 g
Distilled water	100 ml
Solution B	
Sodium Bi-carbonate	5 g
Distilled water	100 ml

Acetic acid

5% Glacial acetic acid

Acid alcohol

This is a 3% (v/v) HC1 solution in 70% (v/v) ethanol

Gram's iodine

Iodine (re-sublimed): 5.0 g
Potassium iodide: 10 g
Distilled water: 100 ml

Hydrogen per-oxide

3% aqueous solution of H_20_2 was prepared from the absolute solution.

Kovac's reagent

para-di methyl aminobenzaldehyde (DMAB): 5.0 g

isoamyl-alcohol: 75.0 g

HC1: 25.0 g

Methyl red solution

Methyl red: 0.04 g
Ethanol: 40 ml
Distilled water: 100 ml
McFarland 0.5 standard:
BaC12 (0.048 M): 1.75 % (w/v)
H_2SO4 (0.36N): 1.0% (w/v)

α -napthol reagent

α-napthol: 50.0 g

Ethanol (45%): 95 ml

Oxidase reagent

Tetramethyl paraphenylance- diamine dihydrachloride: 1.0 g

Distilled water: 100 ml

Safranin O (Certified): 2.5g

Ethanol (95%): 10 ml

Distilled water: 100 ml

Safranin 0 was dissolved in the ethanol and water was then added.

APPENDIX- III

Tris-EDTA buffer (TE buffer)

One ml of 1M Tris-HCl (pH8.0) and 0.2ml of 0.5M EDTA (pH8.0) were added to 98.8ml distilled water to make 1000ml TE buffer solution.

O.5M EDTA

18.612g of EDTA was dissolved in 70ml distilled water and then pH was adjusted at 8.0 with 10N NaOH. Further distilled water was added to make 100ml. The solution was then autoclaved and stored at room temperature.

1 M Tris- HCI

12.1g of Tris base was·dissolved in 80ml distilled water and pH was adjusted to 8.0 with concentrated HCL. Distilled water was then added to make 100ml solution. The solution was then autoclaved and stored at 4°C temperature.

10X Tris-Borate EDTA (TBE) stock electrophoresis buffer

Tris base (108g), 40ml 0.5 EDTA (pH8.0) and 55g boric acid were dissolved in 700ml distilled water. Distilled water was further added to make 1L solution. The pH was adjusted to 8.3.

Loading buffer

Bromophenol blue: 0.15%(w/v)
SDS:0.5(w/v)

EDTA: 0.15M

Glycerol: 50%(v/v)